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• *REVIEW* •

Enzyme inhibition assay for pyruvate dehydrogenase complex: Clinical utility for the diagnosis of primary biliary cirrhosis

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Abstract
Primary biliary cirrhosis (PBC) is usually diagnosed by the presence of characteristic histopathological features of the liver and/or antimitochondrial antibodies (AMA) in the serum traditionally detected by immunofluorescence. Recently, new and more accurate serological assays for the detection of AMA, such as enzyme-linked immunosorbent assay (ELISA), immunoblotting, and enzyme inhibition assay, have been developed. Of these, the enzyme inhibition assay for the detection of anti-pyruvate dehydrogenase complex (PDC) antibodies offers certain advantages such as objectivity, rapidity, simplicity, and low cost. Since this assay has almost 100% specificity, it may have particular applicability in screening the at-risk segment of the population in developing countries. Moreover, this assay could be also used for monitoring the disease course in PBC. Almost all sera of PBC-suspected patients can be confirmed for PBC or non-PBC by the combination results of immunoblotting and enzyme inhibition assay without histopathological examination. For the development of a "complete" or "gold standard" diagnostic assay for PBC, similar assays of the enzyme inhibition for anti-2-oxoglutarate dehydrogenase complex (OGDC) and anti-branched chain oxo-acid dehydrogenase complex (BCOADC) antibodies will be needed in future.

Key words: Primary biliary cirrhosis; Enzyme inhibition assay; Antimitochondrial antibody; 2-oxo-acid dehydrogenase complex

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INTRODUCTION
Primary biliary cirrhosis (PBC) is a chronic autoimmune cholestatic liver disease characterized by the destruction of small and medium-sized bile ducts and the presence of antimitochondrial antibodies (AMA) in the serum traditionally detected by immunofluorescence[1,2]. The "gold standard" procedure for the diagnosis of PBC is histopathological examination of liver tissue. However, the characteristic histopathological changes of PBC are not always evident in biopsy specimens. Therefore, serological examination such as AMA is useful for the diagnosis of PBC because this is non-invasive and therefore can be repeated throughout the course of the disease. The major mitochondrial autoantigens recognized in the sera of PBC patients are members of 2-oxo-acid dehydrogenase complex (2-OADC) family, including E2 subunit of pyruvate dehydrogenase complex (PDC-E2), E2 subunit of branched chain oxo-acid dehydrogenase complex (BCOADC-E2), and E2 subunit of 2-oxoglutarate dehydrogenase complex (OGDC-E2)[1,3]. Unfortunately, however, there is so far no "gold standard" assay (i.e., with 100% sensitivity and 100% specificity) for the detection of AMA in PBC.

PBC is present among various ethnic and racial populations, but its incidence and prevalence varies quite widely, from the highest among Northern European populations to vanishingly low in certain parts of Asia[3]. This difference may be due, at least in part, to the diagnostic awareness of physicians for asymptomatic cases. Therefore, reliable and easy-to-use tool for screening PBC in general population is needed.

Serological assays for the detection of AMA
AMA is one of the most diagnostically useful of all autoimmune markers, since both the sensitivity and specificity for the diagnosis of PBC are acceptably high[1]. Indirect immunofluorescence assay using either Hep-2 cells or mouse kidney/stomach sections as the substrate and enzyme-linked immunosorbent assay (ELISA) using semipurified PDC as the antigen source are now widely used in clinical laboratories. Traditional indirect immunofluorescence assay has high sensitivity, and can detect reactivity to all 2-OADC enzymes. However, this assay is non-automated and labor-intensive, and the "readout" is subjective. The reactivity of serum with mitochondrial antigens other than PBC specific 2-OADC enzymes and nonspecific staining or high background could influence its specificity and sensitivity[3]. The

sensitivity, specificity, positive predictive value, negative predictive value, and accuracy determined in our previous study were 89%, 99%, 98%, 94%, and 95%, respectively[4].

Recently, new and more accurate serological assays for the detection of anti-2-OADC, such as ELISA, immunoblotting, and enzyme inhibition assay, has been developed. ELISA can detect more precisely the reactivity to a single 2-OADC enzyme in each run, and is non-subjective readout. Recently, more sensitive ELISAs using PDC-E2, BCOADC-E2 and OGDC-E2 as coating antigens have been developed[5-8]. In ELISA using commercially available MESACUP-2 Test Mitochondria M2 kit (Medical & Biological Laboratories Co., Nagoya, Japan), the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy are 90%, 98%, 95%, 96%, and 94%, respectively[9]. Immunoblotting has been reported to have almost 100% sensitivity, and can detect individual reactivity to 2-OADC enzymes[10,11]. In our immunoblotting assay condition, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 99%, 86%, 89%, 99%, and 93%, respectively[11]. However, this assay is labor intensive, and can only be performed in specialized laboratories. Moreover, its specificity has not been well established[12]. The enzyme inhibition assay, which measures the capacity of PBC sera to inhibit the catalytic activity of PDC, is non-subjective compared to immunofluorescence, is more rapid and technically simpler than immunoblotting and ELISA. This assay has almost 100% specificity[6,13-16], but the sensitivity has been reported to be around 80%[6,15,16]. This lower sensitivity can be explained by the fact that this assay does not detect the inhibitory activity of sera to 2-OADC enzymes other than PDC, such as BCOADC or OGDC.

Enzyme inhibition assay

A striking property of AMA in PBC sera is their capacity to rapidly inactivate the catalytic function of 2-OADC *in vitro*[17]. Enzyme inhibition assay has been utilized to demonstrate a population of autoantibodies in PBC sera that inhibit enzyme function, and a miniaturized semiautomated enzyme inhibition assay was developed for the detection of anti-PDC antibodies in PBC in 1991[18] (Figure 1). Several subsequent studies have assessed its objectivity, rapidity, simplicity, and costeffectiveness, by comparing these parameters to other assays such as immunofluorescence, ELISA, and immunoblotting[13,15]. Immunoblotting might be the most expensive, and enzyme inhibition assay might have most cost-effectiveness in many countries. Recently, an automated enzyme inhibition assay kit, TRACE enzymatic mitochondrial Antibody (M2) assay (EMA) kit (Thermo Trace, Victoria, australia), became available commercially. In principle, the "Substrate reagent" (250 μL) containing sodium pyruvate, magnesium acetate, cocarboxylase, coenzyme A, and nicotinamide adenine dinucleotide (NAD), is placed in flat-bottomed microtiter wells. Thereafter, 4 μL of undiluted test serum is added to each well and incubated for 1 min at 37 °C before adding 50 μL of the "Enzyme reagent" containing

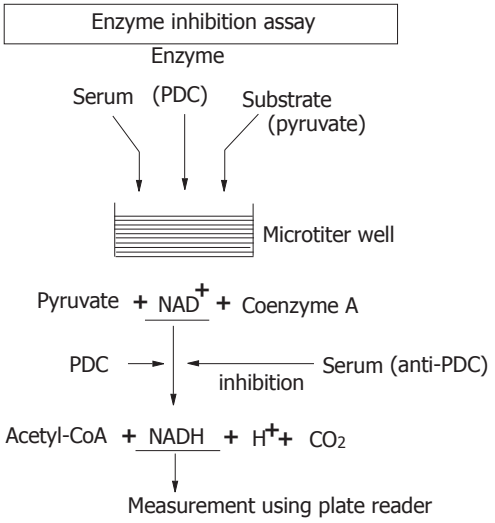

Figure 1 Principle of enzyme inhibition assay.

pyruvate dehydrogenase and dithiothreitol. After 30 s of lag time, the rate of reaction is monitored by measuring the rate of increase in absorbance at 340 nm in a microplate reader. The reaction rate (RR) is calculated using the absorbance values at 0 and 2 min based on the following formula: (final absorbance - initial absorbance)/time. The units of activity (%) are derived from the formula: (test RR/standard RR) x 100. The standard RR is derived from the "Calibrator" wells that contain anti-PDC antibody-free serum (100% activity). The unit of PDC activity of less than 70% is considered as anti-PDC positive with sensitivity and specificity of 82% and 100%, respectively, based on the information provided by the manufacturer[16] (Figure 2). By using this kit, Schmit *et al.*[14] tested the enzyme inhibition assay for 23 sera from patients with AMA-positive PBC and 92 sera from non-PBC including healthy controls, and compared the results to those of immunofluorescence and in-house ELISA. They reported that the sensitivity and specificity of EMA were quite sufficient compared to other assays. Our previous

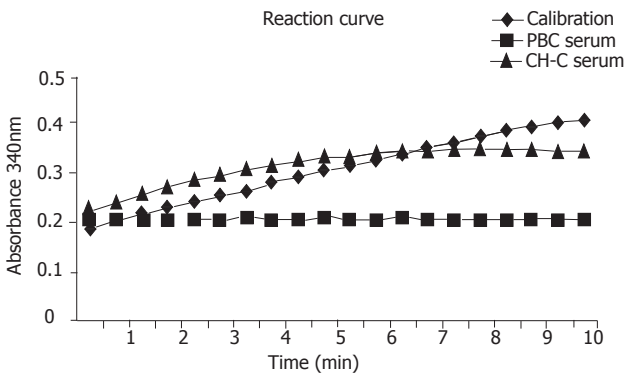

Figure 2 Representative reaction curve of automated enzyme inhibition assay using TRACE enzymatic mitochondrial antibody (M2) assay (EMA) kit. PBC; primary biliary cirrhosis, CH-C; chronic hepatitis C.

Table 1 Detection of enzymatic inhibitory antibody to PDC by enzyme inhibition assay in non-PBC s

First author	Year	Case studied	Antigen source	Serum amount (μL)	Serum dilution	Positivity rate (%)[1]
Teoh	1991	Normal subject, AIH, ALD, RA, SLE	Commercial PDC	100[2]	1:500	0 / 62 (<1.7)
Teoh	1994	Healthy subject, immunopathic diseases	Commercial PDC	100[2]	1:500	0 / 42 (<2.4)
Omagari	1996	Adult blood donors, healthy women	Commercial PDC	2	Undiluted	0 / 186 (<0.6)
Schmit	1999	Healthy controls, viral hepatitis, AIH	TRACE EMA kit	4	Undiluted	0 / 92 (<1.1)
Jois	2000	Normal subject, AIC, AIH, ALD, RA, SLE, etc.	Commercial PDC	2	Undilute	4 / 1055 (0.4)
Hazama	2000	Healthy subject, ALD, viral hepatitis, fatty liver	TRACE EMA kit	4	Undiluted	0 / 50 (<2.0)
Jensen	2000	AIH, abnormal LFT patients, Normal blood donors, etc.	TRACE EMA kit	4	Undiluted	0 / 250 (<0.4)
Masuda	2002	Healthy subject, ALD, viral hepatitis, fatty liver, etc.	TRACE EMA kit	4	Undiluted	0 / 130 (<0.8)
Hazama	2002	Healthy subject, ALD, viral hepatitis, fatty liver, etc.	TRACE EMA kit	4	Undiluted	0 / 97 (<1.1)

[1]When the numerator is zero, the percentage is calculated as a nominal value of <1.
[2]100 μL of doubling dilutions of serum from 1:500 in phosphate-buffered saline solution. PDC, pyruvate dehydrogenase complex; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; EMA kit,Enzymatic mitochondrial Antibody (M2) Assay kit; AIC, autoimmune cholangitis; LFT; liver function tests.

study of EMA indicated sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 72%, 100%, 100%, 87%, and 90%, respectively. We also concluded that EMA is useful for the diagnosis of AMA-positive PBC and could be used to monitor the disease course of PBC, particularly due to the small amount of serum requested, objective read-out, and rapid turnaround time[16,19]. Jensen *et al.*[6] also reported that the sensitivity and specificity of EMA were 83% and 100%, respectively, and the EMA compared favorably against commercial ELISA methods.

Clinical utility of enzyme inhibition assay in PBC

Enzyme inhibition assay including commercially available EMA kit has the advantages of few procedural steps, small amount of test serum requested (only 4 μL of undiluted test serum), rapid turnaround time (approximately 6 min for 10 serum samples), and non-subjective readout[14,19]. Moreover, this assay has almost 100% specificity (Table 1). This means that if the serum is positive for enzymatic inhibitory antibody to PDC by enzyme inhibition assay, the diagnosis of PBC is almost confirmed. The result from our laboratory is in line with this finding because none of 245 non-PBC sera was positive for enzymatic inhibitory antibody to PDC by enzyme inhibition assay, whereas 96 (76%) of 127 PBC sera were positive (detailed data are not shown). Therefore, enzyme inhibition assay may have particular applicability in screening the at-risk segment of the population, middle-aged to elderly females[15]. This assay is also applicable in developing countries due to its objectivity, rapidity, simplicity, and low cost. However, this assay may not be suitable for screening in a particular country or area such as Japan. This assay has relatively low sensitivity compared with that of immunofluorescence and immunoblotting due to the lower frequency of autoantibodies to PDC-E2 among the Japanese compared with Caucasian patients with PBC, and a correspondingly higher frequency of antibodies to E2 subunits of the other 2-OADC enzymes[20].

This assay could also be used for monitoring the disease course in PBC. In our previous study, we determined the serial changes in enzymatic inhibitory antibody to PDC by enzyme inhibition assay using EMA kit in Japanese patients with PBC[19]. The units of PDC activity by EMA correlated significantly and inversely with AMA titers by immunofluorescence, and serum reactivity to PDC-E2 by immunoblotting, respectively. Indeed, in three patients who showed a decrease in AMA titers by immunofluorescence, AMA titers correlated more with EMA results than immunoblotting. Moreover, in a patient with fluctuating AMA titers by immunofluorescence, the units of PDC activity by EMA paralleled AMA titers[19] (Figure 3). These data suggested that PBC disease course might influence the EMA results.

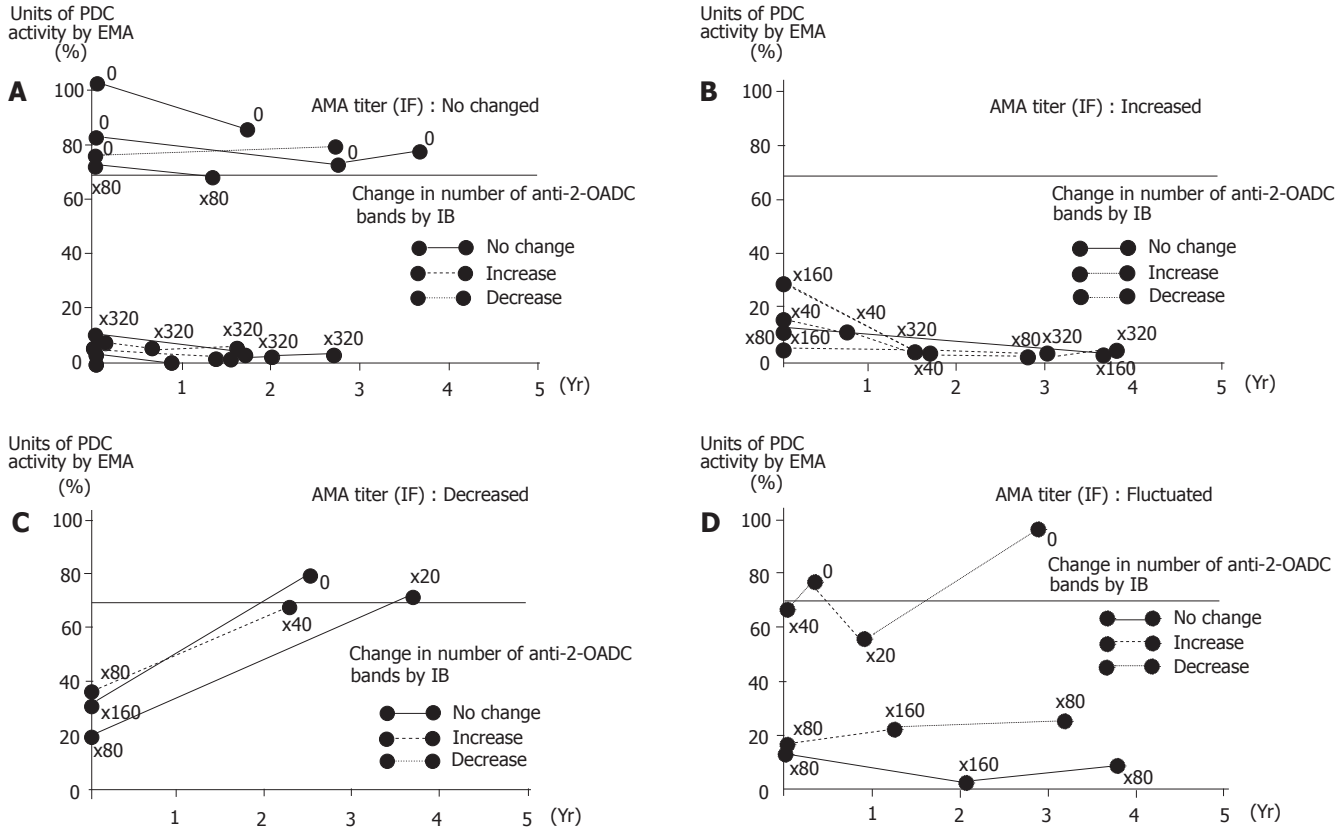

Figure 3 The units of pyruvate dehydrogenase complex (PDC) activity by enzymatic mitochondrial antibody (M2) assay (EMA) did not significantly vary in all the 9 patients in whom the titer of antimitochondrial antibodies (AMA) by immunofluorescence (IF) did not change during the course of follow-up. However, the number of anti-2-oxo-acid dehydrogenase complex (anti-2-OADC) bands by immunoblotting (IB) changed in 4 of these patients (2 increased and 2 decreased) (**A**). The units of PDC activity by EMA decreased in all 4 patients whose AMA titers by IF had increased, but the number of anti-2-OADC bands by IB did not change in 1 patient (**B**). The units of PDC activity by EMA increased in all 3 patients in whom AMA titers by IF had decreased, but the number of anti-2-OADC bands by IB in these patients did not decrease (**C**). Of the 3 patients in whom AMA titers by IF showed some fluctuation, one showed fluctuation in the units of PDC activity by EMA and two showed fluctuation in the number of anti-2-OADC bands by IB (**D**).

Table 2 Interpretation of detection of anti-2-OADC by immunoblotting and enzyme inhibition assay

Immunoblotting (IgG/IgM/IgA)	Enzyme inhibition assay	Interpretation	Estimated percentage (%)
Positive	Positive	PBC	72 - 83
Positive	Negative	Non-anti-PDC positive PBC	10 - 25
		Immunoblotting false positive?	10 - 15
Negative	Positive	Enzyme inhibition assay false positive?	Very rare
Negative	Negative	Non-PBC	Very rare

These data are based on our results that the sensitivity of immunoblotting for PBC is almost 100%, and the specificity of enzyme inhibition assay for PBC is nearly 100%, i.e., when the result by immunoblotting is negative, the serum is not from PBC, and when the result of enzyme inhibition assay is positive, the serum should be from the patient with PBC. 2-OADC, 2-oxo-acid dehydrogenase complex; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex.

Interpretation of detection of anti-2-OADC by immunoblotting and enzyme inhibition assay in PBC

Clinically, the serological diagnosis of PBC is in most instances based on the detection of AMA by indirect immunofluorescence and/or ELISA. As mentioned above, however, these two assays are not yet the "gold standard" (i.e., with 100% sensitivity and 100% specificity) for the detection of AMA in PBC, although both the sensitivity and specificity of these two assays are acceptably high. Based on the fact that immunoblotting has almost 100% sensitivity, and enzyme inhibition assay has almost 100% specificity, interpretation of anti-2-OADC results by combination of these two assays in PBC sera can be established. For example, since the negative predictive value of immunoblotting is 99%, a negative result by immunoblotting means that the serum is not from a patient with PBC. Since a positive predictive value of enzyme inhibition assay is 100%, a positive result by enzyme inhibition assay means that the serum should be from a patient with PBC. When the serum sample is positive for anti-2-OADC by immunoblotting but negative for anti-PDC by enzyme inhibition assay, it can be from non-anti-PDC positive PBC or the result of immunoblotting may be false positive. Conversely, when the serum is negative for anti-2-OADC by immunoblotting but positive for anti-PDC by enzyme inhibition assay, the result of enzyme inhibition assay may be false positive (Table 2). Thus, almost all sera from PBC-suspected patients can be confirmed for PBC or non-PBC by the combination results of immunoblotting and enzyme

inhibition assay without histopathological examination. For the development of a "complete" or "gold standard" (100% sensitivity and 100% specificity) diagnostic assay for PBC, similar assays of the enzyme inhibition for anti-OGDC and anti-BCOADC antibodies (or one-step assay for anti-PDC, OGDC, and BCOADC antibodies) will be needed in the future.

Conclusions

For the diagnosis of PBC, enzyme inhibition assay may have particular applicability in screening the at-risk segment of the population since this assay has almost 100% specificity. This assay is also applicable in developing countries due to its objectivity, rapidity, simplicity, and low cost.

REFERENCES

1 **Kaplan MM**. Primary biliary cirrhosis. *N Engl J Med* 1996; **335**: 1570-1580

2 **Talwalkar JA**, Lindor KD. Primary biliary cirrhosis. *Lancet* 2003; **362**: 53-61

3 **Gershwin ME**, Mackay I, Coppel R, Nakanuma Y. Clinical, immunologic and molecular features of primary biliary cirrhosis. *Semin Clin Immunol* 1994; **7**: 5-16

4 **Kadokawa Y**, Omagari K, Ohba K, Masuda J, Hazama H,Kinoshita H, Ohnita K, Mizuta Y, Tanioka H, Imanishi T, Kohno S. Does the diagnosis of primary biliary cirrhosis or autoimmune cholangitis depend on the "phase" of the disease? *Liver Int* 2005: **25**: 317-324

5 **Moteki S**, Leung PSC, Coppel RL, Dickson ER, Kaplan MM, MunozS, Gershwin ME. Use of a designer triple expression hybrid clone for three different lipoyl domains for the detection of antimitochondrial autoantibodies. *Hepatology* 1996; **24**: 97-103

6 **Jensen WA**, Jois JA, Murphy P, De Giorgio J, Brown B, Rowley MJ, Mackay IR. Automated enzymatic mitochondrial antibody assay for the diagnosis of primary biliary cirrhosis. *Clin Chem Lab Med* 2000; **38**: 753-758

7 **Miyakawa H**, Tanaka A, Kikuchi K, Matsushita M, Kitazawa E, Kawaguchi N, Fujikawa H, Gershwin ME. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantigens. *Hepatology* 2001; **34**: 243-248

8 **Kadokawa Y**, Omagari K, Hazama H, Ohba K, Masuda J, Kinoshita H, Hayashida K, Isomoto H, Mizuta Y, Murase K, Murata I, Kohno S. Evaluation of newly developed ELISA using "MESACUP-2 Test Mitochondrial M2" kit for the diagnosis of primary biliary cirrhosis. *Clin Biochem* 2003; **36**: 203-210

9 **Takemura M**, Ohya K, Kojima K. Fundamental evaluation of "MESACUP-2 Test Mitochondrial M2". *Jpn J Med Pharm Sci* 2001; **46**: 809-816 (in Japanese)

10 **Kitami N**, Komada T, Ishii H, Shimizu H, Adachi H, Yamaguchi Y, Kitamura T, Oide H, Miyazaki A, Ishikawa M, Endo K, Watanabe S, Yokoi Y, Sato N. Immunological study of anti-M2 in antimitochondrial antibody-negative primary biliary cirrhosis. *Intern Med* 1995; **34**: 496-501

11 **Kinoshita H**, Omagari K, Matsuo I, Yamaguchi K, Ikuno N, Kohno S. Frequency of IgG, IgM, and IgA class autoantibodies against 2-oxo-acid dehydrogenase complex in 102 Japanese patients with primary biliary cirrhosis. *Hepatol Res* 1999; **15**: 163-171

12 **Masuda J**, Omagari K, Miyakawa H, Hazama H, Ohba K, Kinoshita H, Matsuo I, Isomoto H, Murata I, Kohno S. Clinical significance of positive immunoblotting but negative immunofluorescence for antimitochondrial antibodies in patients with liver diseases other than primary biliary cirrhosis. *Autoimmunity* 2002; **35**: 135-141

13 **Teoh K-L**, Rowley MJ, Zafirakis H, Dickson ER, Wiesner RH, Gershwin ME, Mackay IR. Enzyme inhibitory autoantibodies to pyruvate dehydrogenase complex in primary biliary cirrhosis: Applications of a semiautomated assay. *Hepatology* 1994; **20**: 1220-1224

14 **Schmit P**, Gilson G, Humbel RL. Evaluation of an automated enzyme inhibition assay for the detection of anti-mitochondrial M2 autoantibodies. *Clin Chem* 1999; **45**: 2287-2289

15 **Jois J**, Omagari K, Rowley MJ, Anderson J, Mackay IR. Enzyme inhibitory antibody to pyruvate dehydrogenase: diagnostic utility in primary biliary cirrhosis. *Ann Clin Biochem* 2000; **37**: 67-73

16 **Hazama H**, Omagari K, Masuda J, Ohba K, Kinoshita H, Matsuo I, Isomoto H, Mizuta Y, Murase K, Murata I, Kohno S. Automated enzymatic mitochondrial antibody assay for the diagnosis of primary biliary cirrhosis: applications of a routine diagnostic tool for the detection of antimitochondrial antibodies. *J Gastroenterol Hepatol* 2002; **17**: 316-323

17 **Bassendine MF**, Jones DEJ. Antimitochondrial and other autoantibodies in primary biliary cirrhosis. In: Krawitt EL, Wiesner RH, Nishioka M. Autoimmune Liver Diseases. *Amsterdam: Elsevier Science BV*, 1998; 287-304

18 **Teoh K-L**, Rowley MJ, Mackay IR. An automated microassay for enzyme inhibitory effects of M2 antibodies in primary biliary cirrhosis. *Liver* 1991; **11**: 287-291

19 **Hazama H**, Omagari K, Masuda J, Kinoshita H, Ohba K, Sakimura K, Matsuo I, Isomoto H, Murase K, Murata I, Kohno S. Serial changes in enzyme inhibitory antibody to pyruvate dehydrogenase complex during the course of primary biliary cirrhosis. *J Clin Lab Anal*. 2000; **14**: 208-213

20 **Omagari K**, Rowley MJ, Jois JA, Feeney SJ, Komatsu K, Maeda T, Onishi S, Yamazaki K, Suzuki K, Galperin C, Mackay IR. Immunoreactivity of antimitochondrial autoantibodies in patients with primary biliary cirrhosis. *J Gastroenterol* 1996; **31**: 61-68

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• *REVIEW*•

Loss of heterozygosity analyzed by single nucleotide polymorphism array in cancer

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Abstract
Neoplastic progression is generally characterized by the accumulation of multiple genetic alterations including loss of tumor suppression gene function. Loss of heterozygosity (LOH) has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages and progression. LOH pattern has been detected by allelotyping using restriction fragment length polymorphism, and later by simple sequence length polymorphisms (SSLPs or microsatellite) for 10 years. This paper reviews the detection of LOH by recently developed single nucleotide polymorphism (SNP) arrays (all analyzed by Affymetrix array); furthermore, its advantage and disadvantage were analyzed in several kinds of cancer.

Key words: Loss of heterozygosity; Single nucleotide polymorphism; Array; Cancer

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INTRODUCTION
Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes[1]. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations as the tumor progresses to advanced stages[2-6]. The classic mechanism of tumor suppressor gene inactivation is described by the two-hit mode in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in the loss of heterozygosity (LOH) at multiple loci[7-11]. Loss of heterozygosity is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of tumor suppressor genes predominates[12].

Loss of heterozygosity and studying methods
LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome nondisjunction, mitotic nondisjunction followed by republication of the remaining chromosomes, mitotic recombination, and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display complete loss. However, more than half of the losses are associated with the loss of only a part of the chromosome rather than the whole chromosome[13]. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages, and progression[14,15].

In addition to the inherited and sporadic polymorphisms, many tumors exhibit aneuploidy and chromosomal instability in which the diploid structure of the genome is corrupted. A modest increase in copy number (such as trisomy for a region) would not give rise to allelic imbalance in the SNP assay. Allelic imbalance in the SNP assay should thus usually indicate true LOH, except in the case of extreme amplification[16].

Global patterns of LOH can be analyzed through allelotyping of tumors with polymorphic genetic markers from each chromosomal arm[17]. Most investigations have concentrated on defining the minimal regions of loss of specific chromosomes in various cancers in an effort to identify the putative tumor suppressor genes targeted by the losses. Two allele RFLPs and Southern analysis give way to simple sequence length polymorphisms such as PCR-based microsatellite, and both have been proved to be reliable genetic markers for studying LOH[18]. RFLP markers have low heterozygosity rates and are available in small number, gel-based microsatellite assay is difficult to automate and not readily scalable[19]. Microsatellite markers are reliable genetic markers for studying LOH, but only

a modest number of SSLPs are used in LOH studies because the genotyping procedure is rather tedious and difficult to automate and are not readily scalable.

As a result, most genome-wide scans for LOH have been conducted at low resolution with a relatively small number of polymorphic markers. Previous allelotyping analysis of cancer by many groups was restricted to particular chromosomal regions or arms, or else used a relatively low density of markers. For example, an average of 120 microsatellites has been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype is ~280 polymorphic markers before the year 2000[20-24].

We conducted a genome-wide LOH study of 83 tumor samples obtained from Chinese patients in sporadic colorectal cancer. We employed 400 fluorescence-labeled microsatellite marker primers to amplify the corresponding loci of genomic DNA and then electrophoresed the polymerase chain reaction products and analyzed the fluorescent signals. The LOH frequencies were high (>35%) but not associated with the tumor stage and progression in 20 loci. Loss of other loci, including two narrow regions on chromosome 2, was related to the tumor stage[25,26]. In some loci, we performed detailed deletion mapping to narrow the loss region.

SNPs are the most common form of sequence variation in human genome, occurring approximately in every 1 200 bp[27]. SNPs may occur at more than 2 million sites in the genome, thus making it possible to place SNPs at high density along the genome[28].

High-throughput polymorphism detection technologies hold great promise for the characterization of complex diseases including cancer. High-density mapping of genetic losses reveals potential tumor suppressor loci and might be useful in the clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance.

Higher density SNP array can be used effectively to detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions. In addition, more markers increase confidence in a detected event. If multiple adjacent SNPs show a consistent change, the confidence in the call is much higher than when it is based on a single SNP[15].

HuSNP chip (the first generation of SNP chip), an array of oligonucleotide probes for 1494 SNP loci, is distributed in all human chromosomes with an average of 2.57 cm between each SNP markers. A recent study using microarray has demonstrated a 97% accuracy on 65% of the SNPs surveyed[29]. The Affymetrix 10K SNP array (the second generation) contains 11 560 SNP alleles with high frequency of heterozygosity (average 36% based on Affymetrix in-house data). The Affymetrix 100K SNP array, a new SNP array platform, provides a high accuracy (99.5%), a reproducibility (91.1%) and a high call (heterozygous or homozygous) rate (95%)[30]. The average accuracy is calculated as 81% at 95% significance with a median inter-SNP distance of 105 kb in osteosarcoma using 10K array[30].

The HuSNP chip call rate does not differ between normal and tumor samples[16]. The genotyping accuracy of the chip calls is estimated at 95.4% on the basis of validation of random SNPs in normal and tumor samples by gel-based length multiplex single-base extension (LM-SBE)[16].

Tumor sample purity mixing experiment

LOH involves complete loss of one of the two alleles at a locus, but normal cell contamination can confound the distinction between true LOH and other mechanisms of allelic imbalance[31]. However, studies using flow-cytometrically purified samples have shown that complete LOH can be detected in tissue samples[32,33].

Tumor sample purity mixing experiment showed that samples with 90% tumor purity give essentially identical results than those with 100% tumor purity, when the purity decreases to 80%; it results in an increase in "uncertain" calls and a few false positive "retention". Accuracy decreases steeply when the purity is 70% or lower, because the lost allele contains 15% or more of contaminated samples. Although tumor purity is dependent on tumor type, a purity of 80% can often be achieved using gross dissection or microdissection[16,34]. With the SNP arrays, Mei *et al*.[15] found that chromosomal changes are detectable in heterogeneous samples with a background of up to 50% normal DNA.

The DNA fragment that occurs with formalin fixation does not seem to affect HuSNP array analysis result[35]. However, it is believed that formalin reduces the size of PCR segments that may be amplified from a sample[36]. There is significant agreement between the LOH result obtained from formalin-fixed paraffin embedded prostate tumor sections and those for freshly cultured cancer cells[34].

SNP array principle and methods

DNA sample is subjected to 24 multiplex PCR reactions, the resulting products are pooled, hybridized to the SNP array, stained with streptavidin-phycoerythrin, and assayed by fluorescence detection.

Briefly, the detector for each SNP locus contains four rows of 25-mer oligonucleotides, two of which contain oligonucleotides that perfectly match either SNP allele A or SNP allele B, whereas the other two contain single-base mismatches at various positions. The allelotype at a locus is determined by fluorescence intensity ratios in an automated fashion. Affymetrix HuSNP mapping system is used to determine tumor and normal allelotypes.

A general scanner scans chips and genotyped "call" is made from the collected hybridization signals using Affymetrix HuSNP 3.1 software. Tumor and normal samples are allelotyped on separate chips. For each patient's tumor, each SNP locus is scored as LOH, retention of heterozygosity, uninformative, or uncertain by comparing the genotype calls for tumor and normal (autologous) pairs. The possible SNP calls made by Affymetrix genotyping software are A, B, AB, AB_A (i.e,

AB or A), AB_B (i.e, AB or B), and "no call". "no call", AB_A, AB_B calls are considered to be noninformative[15].

Both amplified and unamplified DNA give similar results in terms of SNP call and LOH[30]. LOH can be established or inferred from 10K SNP array data using only amplified tumor DNA with the Affymetrix Genechip chromosome copy number tool.

SNP array application in LOH detection

Using SNP detection array, Wang *et al*.[37] found that breast cancer is highly heterogeneous, with the proportion of LOH ranging widely from 0.3% to >60% of heterozygous markers.

The call rate is 74.9-83.2% over all samples, yielding 1 120–1 205 SNPs scored per sample using HuSNP array[8,14,34,37,38]. The median of heterozygous loci is 341-349 with an average coverage of one SNP per 7.9-8.7cm[14,37]. Using 10K SNP array, the call rate is 91.1% over all samples[39]. In lung and breast cancer, the average call rate does not vary significantly between the lymphoblastoid and tumor cell lines[8,37].

LOH result comparison between SNP array and microsatellite

Very few reports have presented allelotyping data on multiple sites in the same tumor using two different methods, LOH between SNP array and microsatellite is concordant in the majority of analyzed kinds of cancer samples[16,34,37]. Most affected LOH regions are consistent with those in previous LOH studies, lending validity to both the method and results[34].

The range of consistency between two methods in different loci varies from 50% to 100% in bladder cancer[14]. Moreover, when the two methods are compared by chromosome arms, the concordance is very robust[14]. In osteosarcoma assay, 14 of 18 microsatellite markers have associated SNPs with LOH[30].

By comparing the microsatellite results in selected areas of several chromosomes with SNP array-based detection of allelic imbalance, in 69 sites, 60 microsatellite markers correlate, but nine microsatellite markers do not correlate with adjacent HuSNP markers[35].

Janne *et al*.[8] found that neither HuSNP nor SSLP is perfect. Using two methods together, the combined informative rate is 84%, and both methods provide calls for loci that were not informative by the other methods. However, a combined analysis is unlikely to be practical for future studies.

The comparison shows that, given a sufficient number of polymorphic markers, the SNP array can be used to screen both small and large chromosomal losses. But neither technique is currently infallible in identifying LOH[14].

LOH conflict between SNP array and microsatellite

Lindblad-Toh *et al*.[16] examined a number of instances of apparent conflict between SSLPs and SNP-based analysis by repeating the analysis and found that discordance is slightly more often due to the errors in SSLP rather than in SNP genotyping. SNP genotyping thus appears to be at least as accurate as the SSLP approach.

SNPs associated with the remaining four microsatellite markers do not show any LOH[30]. Allelic imbalance has been detected in microsatellite analysis but not detected by the SNP, which is probably caused by a no-signal genotype call either in the tumor or in normal DNA or in both. This problem can be solved by increasing the number of SNPs for the specific loci and by developing a more sensitive method for the generation of calls[14].

Possible reason of discrepancy

Because of the lower average heterozygosity rate of SNPs (0.33) compared to microsatellite, approximately a threefold SNPs is required for an equivalent resolution.

It is difficult to determine whether the apparent discordance is due to the technical limitation or if the microsatellite markers recognize a smaller region with a different allelic loss pattern compared to the adjacent regions scored by SNP[35].

The possible reasons are as follows: limitation of mapping data; differences in resolution, amplification efficiency, and differential sensitivity between microsatellite and SNP, technical limitations such as a genotype call by the Affymetrix softwares, the presence of bad SNPs in the array[14,30].

Cancer classification by LOH pattern using SNP array

Finding unique LOH pattern by SNP array in different groups of breast cancer, in part defined by expression signature, adds confidence to newer schemes of molecular classification. Furthermore, exclusive association between biological subclasses and restricted LOH event provide rationale to search for targeted genes[37]. Janne *et al*.[8] demonstrate that clustering of LOH data can distinguish SCLC from NSCLC with reasonable accuracy.

Advantage of SNP array

SNP array assay is accurate, automatic, and readily adaptable to the clinical setting and high-density mapping. Analysis of genetic alterations with HuSNP assay saves considerable time over microsatellite analysis. The assay involves multiplex amplification and other methods that can be completed in one day. The SNP array method is also a molecular technique that allows the detection of chromosomal imbalance in tumor DNA. A minimal quantity (120-135 ng) of sample DNA is needed for each SNP assay. The amplification step makes it possible to use only a small amount of genomic DNA, which is often essential when working with limited clinical materials[14,15].

The 10K array also provides calls (either LOH or retention) for 71.7% and 22.3% of the loci identified as non-informative by HuSNP and SSLP, respectively. The proximal and distal ends of the deletion are clearly identified and single LOH events identified using SSLP fall within these regions. The mapping 10K array can identify more than twice the number of LOH regions compared to SSLP or HuSNP. The minimum, mean, and median sizes of these regions are substantially smaller by the mapping 10K array than by the other two methods. The maximum

size of the LOH regions is similar by the three methods[8]. Disadvantage of SNP array is.

SNP array is difficult to distinguish all polymorphisms and to detect low level polymorphism and requires PCR amplification.

The average proportion of LOH informative markers out of callable markers is 31–33%, which is a considerably lower heterozygosity rate than that of SSLPs (typically 70%), but can readily increase to about 50% by selecting SNP with higher heterozygosity[14,16].

High false positive rate (11-21%) and false negative rates (19.9%) have been observed with this technology, limiting its utility in both SNP and tumor analysis[30,41].

Array-based methods of SNP detection may have a certain degree of inaccuracy ("noise"), and moreover, the precise genomic mapping of each SNP is still not completely stable. Thus, "true" regions of LOH can be interrupted by apparently false positively "retained" SNP alleles. Conversely, true regions of retention of heterozygosity may be interrupted by false LOH calls[42].

In summary, with the increasing number of SNPs available and technical progression[43], it is possible to probe the entire genome, and specific regions at much higher resolution. SNP array hybridization is an accurate and efficient method for evaluating genome-wide tumor LOH at present.

REFERENCES

1 **Kirk BW**, Feinsod M, Favis R, Kliman RM, Barany F. Single nucleotide polymorphism seeking long term association with complex disease. *Nucleic Acids Res* 2002; **30:** 3295-3311
2 **Fults D**, Pedone CA, Thomas GA, White R. Allelotype of human malignant astrocytoma. *Cancer Res* 1990; **50:** 5784-5789
3 **Sato T**, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res* 1990; **50:** 7184-7189
4 **Tsuchiya E**, Nakamura Y, Weng SY, Nakagawa K, Tsuchiya S, Sugano H, Kitagawa T. Allelotype of non-small cell lung carcinoma--comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. *Cancer Res* 1992; **52:** 2478-2481
5 **Yamaguchi T**, Toguchida J, Yamamuro T, Kotoura Y, Takada N, Kawaguchi N, Kaneko Y, Nakamura Y, Sasaki MS, Ishizaki K. Allelotype analysis in osteosarcomas: frequent allele loss on 3q, 13q, 17p, and 18q. *Cancer Res* 1992; **52:** 2419-2423
6 **Thrash-Bingham CA**, Greenberg RE, Howard S, Bruzel A, Bremer M, Goll A, Salazar H, Freed JJ, Tartof KD. Comprehensive allelotyping of human renal cell carcinomas using microsatellite DNA probes. *Proc Natl Acad Sci U S A* 1995; **92:** 2854-2858
7 **Knudson AG Jr**, Nakahara memorial lecture. Hereditary cancer, oncogenes, and anti-oncogenes. *Princess Takamatsu Symp* 1989; **20:** 15-29
8 **Janne PA**, Li C, Zhao X, Girard L, Chen TH, Minna J, Christiani DC, Johnson BE, Meyerson M. High-resolution single-nucleotide polymorphism array and clustering analysis of loss of heterozygosity in human lung cancer cell lines. *Oncogene* 2004; **23:** 2716-2726
9 **Knudson AG Jr**, Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971; **68:** 820-823
10 **Hansen MF**, Cavenee WK. Genetics of cancer predisposition. *Cancer Res* 1987; **47:** 5518-5527
11 Brown MA, Tumor suppressor genes and human cancer. *Adv Genet* 1997; **36:** 45-135
12 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61:** 759-767
13 **Thiagalingam S**, Laken S, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B, Lengauer C. Mechanisms underlying losses of heterozygosity in human colorectal cancers. *Proc Natl Acad Sci U S A* 2001; **98:** 2698-2702
14 **Hoque MO**, Lee CC, Cairns P, Schoenberg M, Sidransky D. Genome-wide genetic characterization of bladder cancer: a comparison of high-density single-nucleotide polymorphism arrays and PCR-based microsatellite analysis. *Cancer Res* 2003; **63:** 2216-2222
15 **Mei R**, Galipeau PC, Prass C, Berno A, Ghandour G, Patil N, Wolff RK, Chee MS, Reid BJ, Lockhart DJ. Genome-wide detection of allelic imbalance using human SNPs and high-density DNA arrays. *Genome Res* 2000; **10:** 1126-1137
16 **Lindblad-Toh K**, Tanenbaum DM, Daly MJ, Winchester E, Lui WO, Villapakkam A, Stanton SE, Larsson C, Hudson TJ, Johnson BE, Lander ES, Meyerson M. Loss-of-heterozygosity analysis of small-cell lung carcinomas using single-nucleotide polymorphism arrays. *Nat Biotechnol* 2000; **18:** 1001-1005
17 **Vogelstein B**, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R. Allelotype of colorectal carcinomas. *Science* 1989; **244:** 207-211
18 **Weissenbach J**, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, Lathrop M. A second-generation linkage map of the human genome. *Nature* 1992; **359:** 794-801
19 **Gruis NA**, Abeln EC, Bardoel AF, Devilee P, Frants RR, Cornelisse CJ. PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. *Br J Cancer* 1993; **68:** 308-313
20 **Hatta Y**, Yamada Y, Tomonaga M, Said JW, Miyosi I, Koeffler HP. Allelotype analysis of adult T-cell leukemia. *Blood* 1998; **92:** 2113-2117
21 **Piao Z**, Park C, Park JH, Kim H. Allelotype analysis of hepatocellular carcinoma. *Int J Cancer* 1998; **75:** 29-33
22 **Shih YC**, Kerr J, Hurst TG, Khoo SK, Ward BG, Chenevix-Trench G. No evidence for microsatellite instability from allelotype analysis of benign and low malignant potential ovarian neoplasms. *Gynecol Oncol* 1998; ***69:*** 210-213
23 **Mao X**, Barfoot R, Hamoudi RA, Easton DF, Flanagan AM, Stratton MR. Allelotype of uterine leiomyomas. *Cancer Genet Cytogenet* 1999; **114:** 89-95
24 **Yustein AS**, Harper JC, Petroni GR, Cummings OW, Moskaluk CA, Powell SM. Allelotype of gastric adenocarcinoma. *Cancer Res* 1999; **59:** 1437-1441
25 **Peng Z**, Zhang F, Zhou C, Ling Y, Bai S, Liu W, Qiu G, He L, Wang L, Wei D, Lin E, Xie K. Genome-wide search for loss of heterozygosity in Chinese patients with sporadic colorectal cancer. *Int J Gastrointest Cancer* 2003; **34:** 39-48
26 **Peng Z**, Zhou C, Zhang F, Ling Y, Tang H, Bai S, Liu W, Qiu G, He L. Loss of heterozygosity of chromosome 20 in sporadic colorectal cancer. *Chin Med J (Engl)* 2002; **115:** 1529-1532
27 **Sachidanandam R**, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 2001; **409:** 928-933
28 **Marth G**, Yeh R, Minton M, Donaldson R, Li Q, Duan S, Davenport R, Miller RD, Kwok PY. Single-nucleotide polymorphisms in the public domain: how useful are they? *Nat Genet* 2001; **27:** 371-372
29 **Patil N**, Berno AJ, Hinds DA, Barrett WA, Doshi JM, Hacker CR, Kautzer CR, Lee DH, Marjoribanks C, McDonough DP, Nguyen BT, Norris MC, Sheehan JB, Shen N, Stern D, Stokowski RP, Thomas DJ, Trulson MO, Vyas KR, Frazer

KA, Fodor SP, Cox DR. Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. *Science* 2001; **294:** 1719-1723

30 **Wong KK**, Tsang YT, Shen J, Cheng RS, Chang YM, Man TK, Lau CC. Allelic imbalance analysis by high-density single-nucleotide polymorphic allele (SNP) array with whole genome amplified DNA. *Nucleic Acids Res* 2004; **32:** e69

31 **Hahn SA**, Seymour AB, Hoque AT, Schutte M, da Costa LT, Redston MS, Caldas C, Weinstein CL, Fischer A, Yeo CJ. Allelotype of pancreatic adenocarcinoma using xenograft enrichment. *Cancer Res* 1995; **55:** 4670-4675

32 **Barrett MT**, Galipeau PC, Sanchez CA, Emond MJ, Reid BJ. Determination of the frequency of loss of heterozygosity in esophageal adenocarcinoma by cell sorting, whole genome amplification and microsatellite polymorphisms. *Oncogene* 1996; **12:** 1873-1878

33 **Paulson TG**, Galipeau PC, Reid BJ. Loss of heterozygosity analysis using whole genome amplification, cell sorting, and fluorescence-based PCR. *Genome Res* 1999; **9:** 482-491

34 **Dumur CI**, Dechsukhum C, Ware JL, Cofield SS, Best AM, Wilkinson DS, Garrett CT, Ferreira-Gonzalez A. Genome-wide detection of LOH in prostate cancer using human SNP microarray technology. *Genomics* 2003; **81:** 260-269

35 **Zhao X**, Li C, Paez JG, Chin K, Janne PA, Chen TH, Girard L, Minna J, Christiani D, Leo C, Gray JW, Sellers WR, Meyerson M. An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. *Cancer Res* 2004; **64:** 3060-3071

36 **Greer CE**, Lund JK, Manos MM. PCR amplification from paraffin-embedded tissues: recommendations on fixatives for long-term storage and prospective studies. *PCR Methods Appl* 1991; **1:** 46-50

37 **Wang ZC**, Lin M, Wei LJ, Li C, Miron A, Lodeiro G, Harris L, Ramaswamy S, Tanenbaum DM, Meyerson M, Iglehart JD, Richardson A. Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. *Cancer Res* 2004; **64:** 64-71

38 **Lieberfarb ME**, Lin M, Lechpammer M, Li C, Tanenbaum DM, Febbo PG, Wright RL, Shim J, Kantoff PW, Loda M, Meyerson M, Sellers WR. Genome-wide loss of heterozygosity analysis from laser capture microdissected prostate cancer using single nucleotide polymorphic allele (SNP) arrays and a novel bioinformatics platform dChipSNP. *Cancer Res* 2003; **63:** 4781-4785

39 **Zhou X**, Mok SC, Chen Z, Li Y, Wong DT. Concurrent analysis of loss of heterozygosity (LOH) and copy number abnormality (CNA) for oral premalignancy progression using the Affymetrix 10K SNP mapping array. *Hum Genet* 2004; **115:** 327-330

40 **Primdahl H**, Wikman FP, von der Maase H, Zhou XG, Wolf H, Orntoft TF. Allelic imbalances in human bladder cancer: genome-wide detection with high-density single-nucleotide polymorphism arrays. *J Natl Cancer Inst* 2002; **94:** 216-223

41 **Halushka MK**, Fan JB, Bentley K, Hsie L, Shen N, Weder A, Cooper R, Lipshutz R, Chakravarti A. Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* 1999; **22:** 239-247

42 **Lieberfarb ME**, Lin M, Lechpammer M, Li C, Tanenbaum DM, Febbo PG, Wright RL, Shim J, Kantoff PW, Loda M, Meyerson M, Sellers WR. Genome-wide loss of heterozygosity analysis from laser capture microdissected prostate cancer using single nucleotide polymorphic allele (SNP) arrays and a novel bioinformatics platform dChipSNP. *Cancer Res* 2003; **63:** 4781-4785

43 **Lin M**, Wei LJ, Sellers WR, Lieberfarb M, Wong WH, Li C. dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. *Bioinformatics* 2004; **20:** 1233-1240

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Soluble adhesion molecules ICAM-1, VCAM-1, P-selectin in children with *Helicobacter pylori* infection

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Abstract

AIM: To assess the sICAM-1, sVCAM-1, and sP-selectin levels in children with *Helicobacter pylori* (*H pylori*) infection and to evaluate their significance for the morphological changes found in gastric mucosa.

METHODS: The study included 106 children: 59 children (55.7%) with chronic gastritis and positive IgG against *H pylori*, 29 children (27.3%) after previous *H pylori* infection without the bacterium colonization but with positive IgG against *H pylori*, and 18 children (17%) with functional disorders of the gastrointestinal system but with normal IgG against *H pylori*. Endoscopic and histopathological evaluation of gastric mucosa was performed based on the Sydney System classification. The evaluation of sP-selectin, sICAM-1, sVCAM-1 levels in the sera of children was carried out using ELISA test.

RESULTS: The assessment of gastritis activity degrees indicated statistically significant values in the antrum and corpus ($P<0.001$) of children examined. Serum sVCAM-1 levels were higher in group with gastritis due to *H pylori* infection than in group without infection and differed statistically ($P<0.05$). Serum sVCAM-1 levels proved to be the highest among other adhesive molecules in infected children and decreased after eradication of *H pylori*. Serum sICAM-1 levels were similar in all examined groups. Serum sP-selectin levels were similar in children with and without *H pylori* infection.

CONCLUSION: Assessment of adhesive molecules (sP-selectin, sICAM-1, sVCAM-1) in the sera of children with active *H pylori* infection can show the participation of sVCAM-1 in the pathogenesis of gastric mucosal inflammation. sP-selectin and sICAM-1 concentrations in the sera of children with *H pylori* infection after eradication cannot reveal any significant differences as compared to healthy children.

Key words: sVCAM-1; sICAM-1; sP-selectin; *Helicobacter pylori*

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INTRODUCTION

In the course of *Helicobacter pylori* (*H pylori*) infection, a selective recruitment of neutrophils, monocytes, macrophages, mast cells, T and B lymphocytes takes place[1]. Additionally, infiltrating cells synthesize and release mediating cells, which influence the recruitment and activation of further inflammatory cells and increase the triggering activity of cytokines and chemokines. For example, neutrophils are the source of IL-1, IL-8, TNF-α[2], and macrophages – MIP-1α[1]. Adhesion molecules (selectins, their ligands, integrins, immunoglobulin-like molecules) take part in a selective recruitment of leukocytes[3–8]. Selectins are transmembrane molecules with numerous extracellular domains, containing lectin domain at the N-end – hence their name, L selectin (CD62L) – leukocyte, E selectin (CD62E) – endothelial cell, P-selectin (CD62P) – platelet. Saccharic residues combined with sialic acid [blood group antigen Lewis X (CD15) and its isoforms] expressing numerous leukocytes are ligands for L, E, and P selectins.

Glycosaminoglycans, such as heparin sulfate and carbohydrate residues on platelets and neutrophils, are ligands for L- and P-selectin[9,10]. A soluble form of P-selectin binds to the same ligand on granulocytes as its membrane form. It does not inhibit intergrins of granulocytes but supports agonists stimulating polymorphonuclear leukocytes, their function of secretion, PAF synthesis and LTB4 synthesis, and secretion. Thus, a soluble form does not differ in its activity from a membrane form. Its high serum levels (1-20 μg/mL) are significantly higher in the inflammatory process than in a healthy condition (36-250 ng/mL)[11].

Soluble forms of ICAM-1 (sICAM) were described

in 1991 and are derived basically from mononuclear cells. Epithelial cells are unlikely to be their source[12]. They can be found in the serum, in molecular forms: 240 ku, 430 ku, and mainly 500 ku[13]. A functionally soluble ICAM-1 form can be regulated by cytokines and is able to bind to LFA-1 ligand. Thus, sICAM may compete with leukocytic ligands for binding and decrease leukocyte adhesion to endothelial cells and may even promote their de-adhesion. sICAM-1 may be regarded as a marker of inflammation, because its levels increase significantly in the serum in the course of inflammation, at tissue damage or during the activity of proteolytic enzymes[14]. In healthy individuals, ICAM-1 occurs in small amounts on surfaces of many cells, like leukocytes, endothelial vascular cells, fibroblasts, epithelial cells whereas in the course of inflammation correlates with chronic inflammatory phase as well as the occurrence of ulceration in the course of *H pylori* infection. ICAM-1 is considered to be a marker of chronic immunological stimulation and thus it is potentially responsible for chronic course of a disease. A vascular adhesion molecule (VCAM-1) requires about 8-96 h to be activated and expressed on cells *in vitro*. Its expression is enhanced especially by TNF-α, IL- 4, and IL-13[13]. Since VLA-4 is its ligand, it binds only to mononuclear leukocytes (lymphocytes and monocytes). Normal gastric mucosa is free of leukocytes. Abundant infiltrations of poly- and mono-nuclear cells and lymphatic follicles can be found in the course of *H pylori* infection.

The aim of the study was to evaluate the levels of adhesion molecules sICAM-1, sVCAM-1 and sP-selectin in the sera of children with *H pylori* infection and to determine their significance for morphological changes in gastric mucosa.

MATERIALS AND METHODS

Patients

The study included 106 patients, who were divided into three groups with regard to the presence and course of *H pylori* infection. Group I : 59 children (55.7%) with chronic gastritis in the course of *H pylori* infection with a positive titer of IgG antibodies against *H pylori*, including 29 girls (49.2%) and 30 boys (50.8%). The children's age ranged from 2 to 19 years, the mean age was 12.2±4.6 years.

Group II : 29 children (27.3%) after previous *H pylori* infection, without the bacterium colonization of the gastric mucosa but with a positive titer of IgG antibodies against *H pylori*, including 14 girls (14%) and 15 boys (51.7%). The children's age ranged from 3 to 19 years and the mean age was 11.0±4.1 years.

Group III : 18 children (17 %) with functional disorders of the gastrointestinal tract, without *H pylori* infection but with normal IgG level against *H pylori*, 12 girls (66.7%) and 6 boys (33.3%). The children's age ranged from 5 to 17 years, and the mean age was 10.7±3.6 years (Table 1).

Ethical approval for the research was obtained from local Ethics Committee in Medical University.

Table 1 Age of examined children (yr)

Groups	*n* (g/b)[1]	Min. value	Max. value	Mean arithmetic	Median	SD	Lower quartile	Upper quartile
Group I	46 (30/29)	2.0	19.0	12.2	13.0	4.6	9	16
Group II	17 (14/15)	3.0	19.0	11.0	11.0	4.2	8	14
Group III	18 (12/6)	5.0	17.0	10.7	10.0	3.6	8	13

[1]g/b – girls/boys.

Methods

Endoscopic examination of the upper gastrointestinal tract with gastric mucosa samples (corpus and antrum) was performed in 88 children with positive IgG against *H pylori* and 18 children with negative IgG against *H pylori*. Endoscopic and histopathological evaluation was performed based on the Sydney System[16]. Chronic stomachache indicated the need for endoscopy. The assessment of sP-selectin (sP-selectin, Bender MedSystems, Austria), sICAM-1 (sICAM, Bender MedSystem) and sVCAM-1 (sVCAM, Bender MedSystems) levels in the serum samples was performed using ELISA method. The materials for the examination and assessment of individual parameters were prepared according to the manufacturer's instructions. The results were read in a spectrophotometer at 450 nm wavelength. The minimum detection threshold in the method used equaled 1.3 ng/mL for sP-selectin, 0.5 ng/mL for sICAM-1, and 0.9 ng/mL for sVCAM-1.

Statistical analysis

The results of laboratory analysis were processed using appropriate calculating techniques and statistical tests. Descriptive statistics with central deviation measure including arithmetic mean (χ), median and mode, and measure of dispersion including standard deviation (SD), variations and values of the upper and lower quartiles were given to each variable (feature) measured and group of patients. The distribution of empirical data matching the normal distribution was checked using χ^2 test and the Kolmogorow–Smirnow test. The results enabled to determine the direction of further statistical analysis and to use parametric tests or their non-parametric equivalents in statistical analysis. Since in most cases, formal normality tests proved that variations differed significantly from assumed theoretical distribution, the Mann–Whitney *U* test was used to examine the significance of difference in the feature intensity between the groups examined. $P<0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curves were applied to the estimation of the usability of individual diagnostic parameters.

RESULTS

While evaluating the activity of antrum gastritis in groups, we showed the largest changes in children with *H pylori* infection (group I). The severe degree activity was found in 69.5% of this group and the moderate degree activity in 30.5% of the infected children. In children with previous infection and after bacteria eradication (group II), no severe or moderate degree activity was found, whereas mild degree activity was revealed only in 20.7% of this group (Figure 1A).

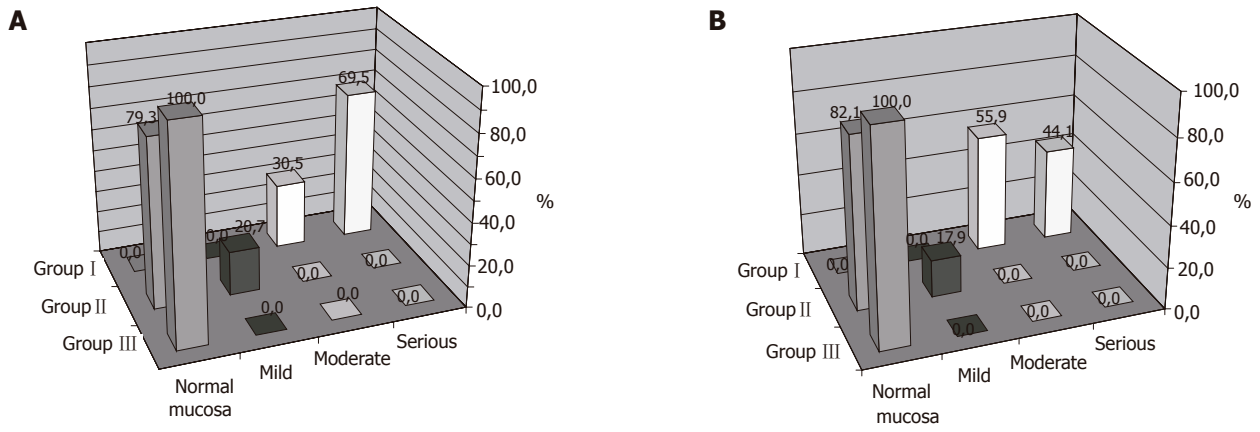

Figure 1 Gastritis activity in antrum (**A**) and corpus (**B**) of examined children (according to the Sydney System).

The analysis of antral gastritis activity by χ^2 test proved a statistical significance (P<0.001) in examined groups. While the corpus mucosa in children with *H pylori* infection (group I) was assessed, the moderate degree activity was found in 55.9% of children and the severe degree activity was revealed in 44.1% of children. In children after *H pylori* eradication (group II), the mild degree activity was established only in 17.9%. No severe or moderate degree activity was reported (Figure 1B). The histopathological evaluation of the corpus gastritis differed statistically significant in particular groups (P<0.001).

sP-selectin levels equaled 339.2±122.9 ng/mL in the sera of children with *H pylori* infection (group I). The similar levels of sP-selectin were observed in children after *H pylori* eradication (group II) and in controls. Therefore, no statistically significant differences were found between the groups examined (Table 2). Based on the results of ROC analysis of sP-selectin levels in serum, the usefulness of this parameter was not confirmed (AUC = 0.62±0.08). The highest accuracy was obtained when the level of 1 288.2 ng/mL was taken as the criterion of sP-selectin concentration. The sensitivity was 87.2% and the specificity was 52.9% for this value (Figure 2A).

Soluble ICAM level was 482.3±143.2 ng/mL in children with *H pylori* infection (group I). Similar sICAM-1 levels were observed in children after *H pylori* eradication (group II) and in controls. No statistically significant differences of sICAM levels were proved between/among the groups (Table 3). A linear correlation was proved between the age of children with *H pylori* infection (group I) and sICAM levels. A negative value of a slope of a line (b = –9.43±3.98) (according to the Sydney System) indicates that sICAM levels decreased in the serum as the age increased. The value of the line equaled 597.18±51.91 ng/mL. This correlation was statistically significant (P<0.05) (Figure 3A).

Soluble VCAM-1 levels in the sera of children with *H pylori* infection equaled 1 032.7±267.5 ng/mL. A statistically significant difference was proved between the levels of group I and group II (P<0.05) (Table 4). In children with *H pylori* infection, a statistically significant dependence (P<0.001) was revealed between the age of children examined and sVCAM-1 levels in their sera. This dependence can be described by a general formula: y =

Table 2 Serum sP-selectin levels in serum of examined children (ng/mL), Mann–Whitney *U* test

Groups	*n*	Min. value	Max. value	Mean arithmetic	Median	Mode	SD	Lower quartile	Upper quartile
Group I	46	158	612	339.2	312.0	343	122.9	248	380
Group II	17	127	624	389.1	375.2	–	151.5	283	509
Group III	3	215	330	283.7	306.0	–	60.7	261	318

Table 3 Serum sICAM-1 levels in examined children (ng/mL), Mann–Whitney *U* test

Groups	*n*	Min. value	Max. value	Mean arithmetic	Median	Mode	SD	Lower quartile	Upper quartile
Group I	55	232	887	482.3	478.0	514	143.2	399	548
Group II	23	176	930	490.5	487.7	–	164.7	412	580
Group III	5	304	705	475.3	446.0	–	168.1	394	527

Table 4 Serum sVCAM-1 levels in examined children (ng/mL), Mann–Whitney *U* test

Groups	*n*	Min. value	Max.value	Mean arithmetic	Median	Mode	SD	Lower quartile	Upper quartile
Group I	47	612	1 921	1 032.7	1 018.0	819	267.5	819.0	1 183.0
Group II	23	176	930	490.5	487.7	–	164.7	412.0	580.0
Group III	5	367	523	422.1	400.2	–	59.7	398.3	421.2

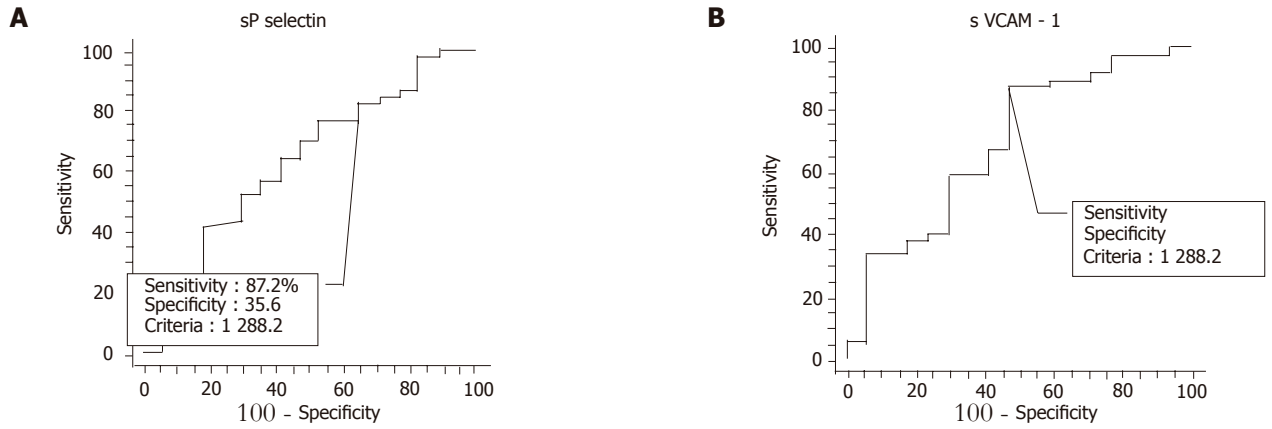

Figure 2 ROC curve for serum sP-selectin (**A**) and sVCAM-1 (**B**) levels.

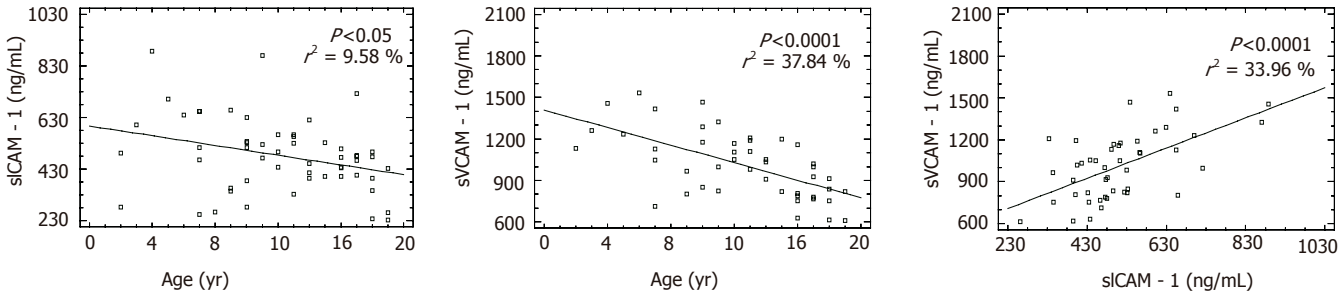

Figure 3 Correlation between age and sICAM-1 (**A**) between age and sVCAM-1 (**B**), and between sICAM-1 and sVCAM-1 (**C**) levels in serum of children with *H pylori* infection (group I).

$a+b\times x$. A negative value of a slope of a line ($b = -31.67\pm -5.18$) means that the value of sVCAM-1 levels decreased as the age of children increased (Figure 3B). Based on the results of ROC analysis of sVCAM levels in the serum, the usefulness of this parameter in *H pylori* infection and eradication was not confirmed (AUC = 0.69±0.08). In case of sVCAM-1 levels, the highest accuracy of diagnosis was obtained at the criterion of 1 288.2 ng/mL. The sensitivity was 87.2% and the specificity was 52.9% (Figure 2B).

In children with *H pylori* infection, a linear dependence ($y = a+b+x$) was proved between sICAM-1 and sVCAM-1 levels in the serum. A positive value of a slope of a line
(b = 1.08±0.23) indicates that sICAM-1 levels increased simultaneously with sVCAM-1 levels (Figure 3C).

DISCUSSION

The evaluation of soluble adhesion molecule levels in serum can confirm their presence and assess their levels quickly and therefore may be of better use in the diagnosis of *H pylori* infection or eradication than time-consuming immunohistochemical methods.

The levels of sVCAM-1 were higher in the sera of patients with gastritis of *H pylori* etiology than in patients without infection and differed significantly ($P<0.05$). The levels of sICAM in the sera were similar in all groups examined. The levels of sP-selectin were similar in the groups with or without *H pylori* infection but were twice as high as in controls. Similar results were presented in studies evaluating ICAM-1, VCAM-1 molecules on the surface of gastric epithelium[17-19].

Later studies in patients with chronic gastritis in the course of *H pylori* infection have proved that the predominant increase in ICAM-1 expression on vascular epithelial cells and inflammatory cells (lymphocytes, granulocytes) in lamina propria is connected with a massive inflammatory infiltrate and the expression of HLA-DR, LFA-1, and Mac-1 on cells presenting antigen[7,20]. No ICAM-1 expression was found on endothelial lymphocytes and epithelial cells. Similar to our study, no correlation with the degree of gastritis was proved. A decrease in the level of adhesion molecules examined was observed after effective eradication of *H pylori*.

Hatz *et al*.[21] have proved that ICAM-1 expression increases on endothelial cells. Moreover, they observed an increase in VCAM - expression in lymphatic follicles, though they neither found an increase in P-selectin expression nor any E-selectin expression. According to the authors, constant P-selectin levels (no increase) may be due to a quick metabolism of this molecule (*in vitro* it is decomposed after a few minutes after its exposition on epithelial cells) and undetectable changes of its levels in immunohistopathological examination. As stated by Hatz *et al*.[21], the increased levels of proinflammatory

cytokines such as IL-1b and TNF-α and the increased quantity of $CD4^+$ and CD45RO lymphocytes in lamina propria might contribute to the upregulated expression of ICAM-1 and VCAM-1. However, studies examining ICAM-1, VLA-4, and CD44 expressions on the surface of mononuclear cells in the serum have proved their increased quantity together with the expression of the molecules mentioned above in patients with *H pylori* infection but without ulceration and in healthy people[3,5]. Polymorphonuclear cells react similarly. The enhanced adhesion of these cells to the epithelial cells of the human navel vein exposed to *H pylori* antigens has been shown in laboratory tests evaluating ICAM-1, VCAM-1, E-selectin expression on neutrophils[6].

Innocenti *et al*.[4] found that not all *H pylori* strains are able to activate epithelial cells of gastric mucosa to the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) and chemokines for neutrophils. But the authors failed to prove whether combination of bacterial antigens (CagPaI, Lewis, BabA, VacA) could influence *H pylori* capability of activating epithelial cells. According to these authors, bacterial proteins not described so far take part in the activation of epithelial cells.

While the interdependence was evaluated between adhesion molecules, a strong correlation was found between the serum levels of sICAM and sVCAM-1 ($P<0.001$) in children with *H pylori* infection, indicating that the increase in sICAM-1 levels is accompanied with the increase in sVCAM-1 levels. Such a correlation may point to the simultaneous and proportional contribution of both adhesion molecules in inflammation.

In our study, while the correlation of sICAM-1 and sVCAM-1 levels with the age of children examined in groups I and II was evaluated, the highest levels were found in the youngest children, whereas they decreased gradually as the age of patients increased. The correlation was statistically significant in both groups (I and II) for sICAM ($P<0.05$), whereas for sVCAM in group I ($P<0.0001$) and in group II ($P<0.05$). The variability of sICAM-1 and sVCAM levels in serum regarding the age may suggest a greater maturity of children's immunological system and its reaction to bacterial antigens.

The results of our study and other studies indicate that adhesion molecules play an important role in immuno-inflammatory response in patients with gastritis due to *H pylori* infection. The levels of adhesion molecules increase in inflammatory process. In our study, such a correlation was proved for sP-selectin.

The quantity of adhesion molecules in inflammatory infiltrating cells or the increased levels of their soluble forms in the serum correlate with the intensity of inflammatory process.

REFERENCES

1 **Kusugami K**, Ando T, Imada A, Ina K, Ohsuga M, Shimizu T, Sakai T, Konagaya T, Kaneko H. Mucosal macrophage inflammatory protein-1alpha activity in Helicobacter pylori infection. *J Gastroenterol Hepatol* 1999; **14:** 20-26

2 **Kim JS**, Jung HC, Kim JM, Song IS, Kim CY. Interleukin-8 expression by human neutrophils activated by Helicobacter pylori soluble proteins. *Scand J Gastroenterol* 1998; **33:** 1249-1255

3 **Ohara T**, Arakawa T, Higuchi K, Kaneda K. Overexpression of co-stimulatory molecules in peripheral mononuclear cells of Helicobacter pylori-positive peptic ulcer patients: possible difference in host responsiveness compared with non-ulcer patients. *Eur J Gastroenterol Hepatol* 2001; **13:** 11-18

4 **Innocenti M**, Thoreson AC, Ferrero RL, Stromberg E, Bolin I, Eriksson L, Svennerholm AM, Quiding-Jarbrink M. Helicobacter pylori-induced activation of human endothelial cells. *Infect Immun* 2002; **70:** 4581-4590

5 **Fan XG**, Fan XJ, Xia HX, Keeling PW, Kelleher D. Up-regulation of CD44 and ICAM-1 expression on gastric epithelial cells by H. pylori. *APMIS* 1995; **103:** 744-748

6 **Byrne MF**, Corcoran PA, Atherton JC, Sheehan KM, Murray FE, Fitzgerald DJ, Murphy JF. Stimulation of adhesion molecule expression by Helicobacter pylori and increased neutrophil adhesion to human umbilical vein endothelial cells. *FEBS Lett* 2002; **532:** 411-414

7 **Dobretsov GE**, Kharitonenkov IG, Mishiev VE, Vladimirov IuA. [Relation between fluorescence and circular dichroism of the complex of the fluorescence probe 4-dimethylaminochalcone with serum albumin] *Biofizika* 1975; **20:** 215-221

8 **Hatz RA**, Rieder G, Stolte M, Bayerdorffer E, Meimarakis G, Schildberg FW, Enders G. Pattern of adhesion molecule expression on vascular endothelium in Helicobacter pylori-associated antral gastritis. *Gastroenterology* 1997; **112:** 1908-1919

9 **Mayadas TN**, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* 1993; **74:** 541-554

10 **Moore KL**, Structure and function of P-selectin glycoprotein ligand-1. *Leuk Lymphoma* 1998; **29:** 1-15

11 **Lorant DE**, Topham MK, Whatley RE, McEver RP, McIntyre TM, Prescott SM, Zimmerman GA. Inflammatory roles of P-selectin. *J Clin Invest* 1993; **92:** 559-570

12 **Marlin SD**, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 1987; **51:** 813-819

13 **Seth R**, Raymond FD, Makgoba MW. Circulating ICAM-1 isoforms: diagnostic prospects for inflammatory and immune disorders. *Lancet* 1991; **338:** 83-84

14 **Rothlein R**, Mainolfi EA, Czajkowski M, Marlin SD. A form of circulating ICAM-1 in human serum. *J Immunol* 1991; **147:** 3788-3793

15 **Kotowicz K**, Dixon GL, Klein NJ, Peters MJ, Callard RE. Biological function of CD40 on human endothelial cells: costimulation with CD40 ligand and interleukin-4 selectively induces expression of vascular cell adhesion molecule-1 and P-selectin resulting in preferential adhesion of lymphocytes. *Immunology* 2000; **100:** 441-448

16 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20:** 1161-1181

17 **Enders G**, Brooks W, von Jan N, Lehn N, Bayerdorffer E, Hatz R. Expression of adhesion molecules on human granulocytes after stimulation with Helicobacter pylori membrane proteins: comparison with membrane proteins from other bacteria. *Infect Immun* 1995; **63:** 2473-2477

18 **Scheynius A**, Engstrand L. Gastric epithelial cells in Helicobacter pylori-associated gastritis express HLA-DR but not ICAM-1. *Scand J Immunol* 1991; **33:** 237-241

19 **Fan X**, Long A, Fan X, Keeling PW, Kelleher D. Adhesion molecule expression on gastric intra-epithelial lymphocytes of patients with Helicobacter pylori infection. *Eur J Gastroenterol Hepatol* 1995; **7:** 541-546

20 **Archimandritis A**, Sougioultzis S, Foukas PG, Tzivras M, Davaris P, Moutsopoulos HM. Expression of HLA-DR, costimulatory molecules B7-1, B7-2, intercellular adhesion molecule-1 (ICAM-1) and Fas ligand (FasL) on gastric

epithelial cells in Helicobacter pylori gastritis; influence of H. pylori eradication. *Clin Exp Immunol* 2000; **119:** 464-471
21 **Hatz RA**, Meimarakis G, Bayerdorffer E, Stolte M, Kirchner T, Enders G. Characterization of lymphocytic infiltrates in Helicobacter pylori-associated gastritis. *Scand J Gastroenterol* 1996; **31:** 222-228

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Concentrations of gastric mucosal cytokines in children with food allergy and *Helicobacter pylori* infection

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Abstract

AIM: To measure the concentrations of chosen cytokines in the antrum mucosa depending on the kind of harmful pathogenic factors and to compare the concentrations with the values of controls without allergy and coexisting *Helicobacter pylori* (*H pylori*) infection.

METHODS: The patients (97 children) were divided into three groups according to the data obtained from the case history, to the main cause of the disease and to the dominant clinical symptoms. Group I: children with food allergy (Fa); group II: children infected with *H pylori*; group III (control group): children with functional disorders of the alimentary tract (without Fa and Hp infection). *H pylori* infection was determined by the presence of anti-Hp antibodies in serum (ELISA method) and urease test performed during endoscopic examination. Cytokine concentration in homogenates of gastric mucosa was detected by ELISA method.

RESULTS: The IL-2 concentration in gastric mucosa bioptates was the highest in children with Hp infection (116.5±179.5 pg/mg of the protein) and Fa and Hp infection (98.1±101.0 pg/mg), while decreased in children with Fa (44.8±50.3 pg/mg) and controls (45.7±23.5 pg/mg). The lowest mean concentration of IFN-γ was observed in children with Hp infection (18.9±16.4 pg/mg), with Fa and Hp infection (25.5±27.7 pg/mg), with Fa (40.6±39.7 pg/mg) and controls (49.9±33.4 pg/mg). The highest IL-4 concentrations were observed in children with Hp infection (35.3±52.8 pg/mg) and in children with Fa and Hp infection (37.2±51.7 pg/mg), while lower IL-4 concentration (23.6±35.8 pg/mg) was found in children with Fa compared to the controls (22.7±13.8 pg/mg). The analysis of IL-4 concentrations in children with Hp infection regarding the intensity of gastritis showed the highest value (62.2±61.2 pg/mg) in mild and moderate gastritis. The concentrations of IL-5 in the gastric mucosa of children with or without Fa did not differ significantly and were comparable to the control group. The highest mean IL-8 value was observed in Hp-infected children with or without Fa. The highest concentration of mucosal IL-10 was detected in children with Hp infection (79.3±41.2 pg/mg) and decreased in children with Fa and Hp infection (50.1±18.8 pg/mg) and in children with Fa (39.9±35.5 pg/mg). The intensity and activity of the inflammation did not affect IL-10 concentrations in the gastric mucosa. In children with Hp infection, TNF-α concentration was the highest (45.9±49.3 pg/mg) and in children with Fa and Hp infection was low (45.3±32.6 pg/mg), whereas decreased in children with Fa (21.7±34.2 pg/mg) and in controls (31.6±14.5 pg/mg).

CONCLUSION: The morphological changes of the gastric mucosa in children with Hp infection are comparable to those in children with Fa and coexisting Hp infection. Cytokine concentration in children with Fa and Hp infection is significantly different in IFN-γ, IL-2, IL-8, and TNF-α.

Key words: Mucosal cytokines; Food allergy; *Helicobacter pylori* dehydrogenase complex

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INTRODUCTION

Many medical research centers dealing with food allergy (Fa) have assessed the morphological changes of gastric mucosa in children with hypersensitivity. Endoscopic evaluation of the alimentary tract and allergical and immunological examinations of food hypersensitivity are valuable diagnostic examination and pathogenetic inquiry element[1,2]. Numerous mast cells releasing histamine and triptase as well as the phenomenon of the selective accumulation of eosinophils and neutrophils have been observed in various parts of the alimentary tract of patients sensitive to food[3-6]. Erosive gastritis and ulceration may occur periodically in the gastric and/or

duodenal mucosa, whereas chronic intestinal disorders are usually recurrent with stomachache or diarrhea with abundant mucus and sometimes bloody secretion.

Besides allergy, *Helicobacter pylori* (*H pylori)* infection is another factor triggering inflammatory changes in the gastric and duodenal mucosa of children. Czinn *et al*.[7] are the first to show the connection of inflammatory changes of the gastric pylorus mucosa with Hp in children. The presence of Hp in the stomach leads to diminishment of the mucous protective layer due to the inhibition of mucus production caused by epithelial cells. The bacteria settle in the stomach, locating in and under the layer of epithelial cells, around intercellular epithelial connections and on the surface by producing special adherence structures, the so-called "bridge attachment". The number of bacteria is the main factor conditioning the epithelial damage degree[8-10]. Inflammatory changes in the gastric mucosa dependent on Hp may persist for several years giving neutrophilic infiltrations in the acute phase and lymphoplasmatic, macrophagic, and eosinophilic infiltrations in the chronic phase of the inflammation. The size of leukocytic infiltration correlates with the degree of colonization and mucosal damage. A small amount of B lymphocytes can be observed in an inflammatory infiltrate. The inflammatory process is developed due to a smaller number of CD8+ lymphocytes when compared to helping lymphocytes CD4+[11-13].

Abnormal IgE production in response to allergens is a characteristic feature of atopy. IL-4, IL-13 and IFN-γ are the most important cytokines regulating IgE production. IL-4 and IL-13 are responsible for the change of immunoglobulins produced by B lymphocytes from IgG and IgM towards IgE and IgG4[14-16]. IFN-γ reacts adversely and inhibits IL-4 effect on B lymphocytes, which secretes specific anti-antigen antibodies. Other cytokines, IL-2, IL-5, and IL-6 have an adjunctive activity to IL-4. They increase IgE production induced by IL-4, whereas IL-12 blocks IgE production induced by IL-4[17-19].

In case of infectious factors or toxins, the first contact cells are the cells of the monocyte/macrophage system. After activation, the cells produce and secrete proinflammatory cytokines, namely IL-1, IL-6, and TNF-α. Biologically, the cell activity occurs through endocrine paths and concerns remote organs, i.e. the liver (acute phase protein production), hypothalamus (pyrogenic effect) and adrenal glands (inflammatory process eradication)[20]. In Hp infection, cytokines also play an important role in the pathogenesis of gastritis. Hp infection is connected with the upregulated production of mucosal inflammatory cytokines: TNF-α, IL-1β, IL-2, IL-6, IL-7, and IL-8[10,21–24].

The purpose of the present study was to assess the concentrations of chosen cytokines in the gastric mucosa of children with Fa and Hp infection and to compare the concentrations obtained by the group of children without Fa and coexisting Hp infection.

MATERIALS AND METHODS

Examinations were conducted in 97 patients with dyspeptic symptoms, including recurrent or chronic stomachache, disorexia, recurrent diarrhea, nausea, vomiting, and loss of body weight. The symptoms were indications for gastroscopy. The patients were divided into three groups according to the cause and clinical symptoms observed.

Group I consisted of 48 children (49.5%) with Fa including 22 girls (45.8%) and 26 boys (54.2%) aged 4.6-18.4 years (mean 10.6±3.6 years). The case history, clinical complaints and the results of the upper alimentary tract endoscopic and histopathological examinations were the qualifying criteria for the further morphologic and biochemical analysis of the patients. The children with Fa were chosen on the basis of the clinical picture, positive immunological and allergical examinations, and inflammatory changes of gastric mucosa after Hp and giardiasis exclusion.

Group II comprised 34 children (35%) with Hp infection including 17 girls (50%) and 17 boys (50%). Fa and simultaneous Hp infection (Fa+Hp) were revealed in 16 (47%) children aged 3.3-16.2 years (mean 11.0±3.8 years). The rest of the 18 patients (52.9%) suffered from Hp infection without Fa. The age of the patients ranged from 5.0 to 18 years (mean 12.8±4.1 years). Children with Fa or without Fa and Hp infection were chosen on the basis of the same criteria as in group I and the positive results urease test (CLO-test) performed during endoscopy. The presence of Hp in the gastric antrum and corpus mucosa specimens was confirmed by hematoxylin and eosin staining (H+E) and the Giemsa method. Moreover, anti-Hp antibodies were determined by ELISA method (RecomWell Helicobacter IgG, Mikrogen, Germany). The amount of the bacteria as well as gastric antrum and corpus inflammatory activities were the decisive factors defining the severity of infection[24].

Group III included 15 children (9 girls -60%, 6 boys-40%) aged 4.9-14.9 years (mean 10.1±3.2 years) with functional disorders of the alimentary tract. The patients did not complain of allergy and showed no Fa symptoms. Hp infection was also excluded and endoscopy was carried out due to clinical symptoms mentioned above. The group constituted the control group (C). During gastroscopy, three specimens were collected from the prepyloric part of the stomach. Biopsies were weighed on an analytical scale immediately after the collection and then put in 1 mL of phosphatic buffer (molality 0.05 and pH 7.4) and placed in a thermos with ice.

Biopsies were homogenized using a tissue homogenizer. The protein was determined by Lowry method (mg/100 mL). Homogenates were divided into portions of 200 mL each, then frozen and stored at -20 °C for further examinations. Determination was conducted separately for each homogenate sample after gradual warming up to the room temperature. The concentrations of tumor necrosis factor (TNF-α), IL-2, IL-4, IL-5, IL-8, IL-10, and INF-γ in homogenates of the gastric mucosa were determined by ELISA method using ENDOGEN standard kits (Cambridge, USA) according to the manufacturer's instructions. Absorbance reading was performed spectrophotometrically with the wavelength

recommended by the manufacturer. The concentrations of cytokines examined (in pg/mL) were calculated on the basis of the standard curve. The results were expressed in milligram of protein in homogenate of the tissue examined.

Statistical analysis

Statistical analysis included the arithmetic mean±SD, the minimum result (min), and the maximum result (max). The levels of parameters examined were compared using Student's *t*-test for independent or paired trials. The differences were significant at $P<0.05$. The interdependence between measurable features was evaluated with Pearson's linear correlation coefficient, of which significance was assessed using Student's *t*-test for each correlation. The interdependence between non-measurable features evaluated using an independence test χ^2 or Fisher's exact test is presented in the correlation tables.

RESULTS

The histopathological evaluation of the gastric mucosa in patients could distinguish the following categories of changes: normal mucous membrane, mucosa at the borderline of the norm (a slightly decreased number of mononuclear cells in superficial layers of the mucous membrane) and chronic inflammation. In children with Fa, normal gastric mucosa was observed in 43.7%, mucosa at the edge of the norm in 35.4% and chronic inflammation in 20.8% of children. In children with Fa and Hp infection and in those without Fa, morphological changes of the antrum mucosa resembled chronic inflammation in 100%. According to the Sydney System, three stages of gastritis could be distinguished: mild, moderate, and severe. The severe stage of antrum mucosal inflammation was observed in 55% of children with Hp infection and in 31.2% of children with Fa and Hp infection (Table 1). Moderate antrum gastritis concerned children with Fa and Hp infection (50%) and children with Hp infection (44.4%). Mild gastritis was revealed in 18.7% (group I) of children with Fa and in 18.7% of children with Fa coexisting Hp infection. Morphometric analysis regarding the severity of inflammation showed statistically significant pathological changes in the antrum ($P<0.001$). The evaluation of antral gastritis activity according to the Sydney System also presented a statistical significance ($P<0.001$). Severe antral gastritis was observed in 72.2% of children with Hp infection and in 68.7% of children with Fa and Hp infection (Table 2). The moderate activity was found in children with Fa and Hp infection (31.2%), in children with Hp infection (27.7%) and in children with Fa (6.2%). Group I showed the moderate activity in 6.2% of children. Histopathological examinations of the corpus mucosa were evaluated in the same way, i.e. in three categories of changes: normal mucosa, mucosa at the edge of the norm,and chronic inflammation.

Group I showed chronic corpus gastritis in 16.6% of children, whereas the second category of changes was observed in 37.7% and normal mucosa was reported in 45.8% of children in this group. Chronic corpus gastritis was diagnosed in 100% of children with Hp infection. In children with Fa and Hp infection, 93.7% of children had chronic inflammation and 6.2% had mucosa at the edge of the norm. Severe corpus gastritis was observed in 12.5% of children with Fa and Hp infection and in 5.5% of children without Fa but with Hp infection (Table 1). The moderate corpus gastritis occurred in 50% of children with Hp infection and in 50% of those with Fa and coexisting Hp infection. Only 2% of children with Fa had moderate corpus gastritis. Mild gastritis was observed in children with Hp infection (44.4%) and in 31.2% of children with Fa and Hp infection. Severe activity of the corpus mucosa was found in 38% of children infected with Hp and in 37.5% of allergic children infected with Hp. The moderate activity was observed in 61.1% of children with Hp infection, 56.2% of allergic children with Hp infection and 6.2% of children with Fa.

Gastric mucosal biopsies showed the highest IL-2 concentration in children infected with Hp (116.5±179.5 pg/mg of the protein) and slightly lower level in allergic children with Hp infection (98.1±101.0 pg/mg). The levels were statistically significantly different from those in the control group ($P<0.01$). The lowest IL-2 concentration was observed in children with Fa (44.8±50.3 pg/mg), which was comparable to that in the controls (45.7±23.5 pg/mg;

Table 1 Severity of corpus and antrum mucosa gastritis in children examined according to Sydney System[25] *n* (%)

Stage of corpus mucosa gastritis				
Group	*n*	Mild	Moderate	Severe
Fa	48	6 (12.5)	1 (2)	0 (0)
Hp	18	8 (44.4)	9 (50)	1 (5.5)
Fa+Hp	16	5 (31.2)	8 (50)	2 (12.5)
C	15	0 (0)	0 (0)	0 (0)
Stage of antrum mucosa gastritis				
Fa	48	9 (18.7)	1 (2)	0 (0)
Hp	18	1 (5.5)	8 (44.4)	9 (55)
Fa+Hp	16	3 (18.7)	8 (50)	5 (31.2)
C	15	0 (0)	0 (0)	0 (0)

Fa, food allergy; Hp, *H pylori*; C, control group.

Table 2 Activation of corpus and antrum mucosa gastritis in children examined according to Sydney System[25] *n* (%)

		Activity of corpus gastritis			
Group examined	*n*	Mild	Moderate	Severe	Lack
Fa	48	4 (8.3)	3 (6.2)	0 (0)	41 (85.4)
Hp	18	0 (0)	11 (61.1)	7 (38.8)	0 (0)
Fa+Hp	16	0 (0)	9 (56.2)	6 (37.5)	1 (6.2)
C	15	0 (0)	0 (0)	0 (0)	15 (100)
Activity of antrum gastritis					
Fa	48	7 (14.5)	3 (6.2)	0 (0)	38 (79.1)
Hp	18	0 (0)	5 (27.7)	13 (72.2)	0 (0)
Fa+Hp	16	0 (0)	5 (31.2)	11 (68.7)	0 (0)
C	15	0 (0)	0 (0)	0 (0)	15 (100)

Fa, food allergy; Hp, *H pylori*, C, control group.

Table 3 Cytokine concentration in gastric mucosa of children examined, according to Sydney System[25] (pg/mg)

Group	IL-2	IL-4	IL-5	IL-8	IL-10	IFN-γ	TNF-α
Fa	44.8	23.6	35.5	59.9	39.9	40.6	21.7
Hp	116.5[b]	35.3	34.1	88.0[b]	79.3[b]	18.9[b]	45.9[b]
Fa+Hp	98.1[d,f]	37.2	29.9	101.2[d]	50.1	25.5[f]	45.3[d,f]
C	45.7[h]	22.7	30.8	93.8[f]	40.4[h]	49.9[h]	31.6[j]

[b]P<0.01 *vs* Fa and Hp, [d]P<0.01 *vs* Fa and Fa+Hp, [f]P<0.01 *vs* Fa and C, [h]P<0.01 *vs* Hp and C, [j]P<0.01 *vs* Fa+Hp and C.

Table 3). The highest IL-2 concentration in the gastric mucosa with regard to the intensity of inflammation (mild+moderate) was observed in the antrum of children with Hp (184.4±227.8 pg/mg) and the allergic process with coexisting Hp infection (125.4±109.4 pg/mg). The highest IL-2 concentration in relation to gastric mucosa inflammation activity was found in children with severe Hp infection (187.6±232.6 pg/mg), being statistically significant when compared to the control group (P<0.01; Tables 4 and 5). IFN-γ concentrations in the gastric mucosa ranged from undetermined levels to 188.2 pg/mg of the protein. The lowest mean concentration of IFN-γ was observed in children infected with Hp (18.9±16.4 pg/mg of the protein) and differed significantly when compared to the controls (P<0.01). In allergic children with Hp infection, the mean concentration of IFN-γ in the gastric mucosa was slightly higher (25.5±27.7 pg/mg, P<0.01; Table 3).

The mean concentration of IFN-γ was 40.6±39.7 pg/mg of the protein in children with Fa and was close to that in the controls (49.9 ±33.4 pg/mg). In Hp infected children with mild and moderate gastric mucosal inflammation, IFN-γ concentration was the lowest (13.1±16.5 pg/mg), being significantly different from that in the controls (P<0.01; Tables 4 and 5).

The values of IL-4 ranged from undetectable to 208.4 pg/mg in biopsies of the gastric mucosa. The highest IL-4 concentration was observed in Hp infected children. IL-4 concentration was 37.2±51.7 pg/mg in allergic and Hp infected children and 35.3±52.8 pg/mg in those without Fa.

The mean concentration of IL-4 was 23.6±35.8 pg/mg in children with Fa, being comparable with the control group (22.7±13.8 pg/mg). The highest IL-4 concentration in children with Hp infection regarding the intensity of gastritis was 62.2±61.2 pg/mg in mild and moderate gastritis (P<0.01).

The IL-5 concentration ranged from 6.7 to 250.9 pg/mg in the biopsies and was 35.5±50.9 pg/mg in allergic children, being not significantly different from that in the controls. The mean concentration of IL-5 in the gastric mucosa of children with or without Fa did not differ significantly and was comparable to that in the control group.

IL-8 concentration ranged from 10.5 to 618.8 pg/mg in

Table 4 Concentrations of cytokines in gastric mucosa of children examined depending on inflammation stage according to Sydney System[25]

Cytokines (pg/mg of the protein)	Fa		Hp				Fa+Hp			
	Antrum	Corpus	Antrum		Corpus		Antrum		Corpus	
	Mild+moderate	Mild+moderate	Mild+moderate	Severe	Mild	Moderate+severe	Mild+moderate	Severe	Mild	Moderate+severe
IL-2	17.7	17.9	184.4[b]	38.8	77.7	150.4[b]	125.4[b]	38.3	106.7 [b]	98.6
IFN-γ	54.9	46.6	13.1[b]	24.1	17.8[b]	19.7[b]	21.9	34.3	23.1	27.1
IL-4	15.4	6.7[b]	62.2[b]	3.9[a]	37.6	32.7	44.3	1.7	32.1	46.4
IL-5	24.5	25.5	–	–	–	–	29.7	–	–	33.0
IL-8	37.5	37.5	101.2	72.9	93.6	83.1	98.0	108.2	71.1	118.3
IL-10	30.4	24.4	–	–	–	–	44.6	–	–	58.9
TNF-α	20.6	4.8	65.2	24.1	48.7	43.3	54.8[b]	24.3	49.0	44.8

[b]P<0.01, cytokine concentrations statistically significantly different from controls. Fa, food allergy; Hp, *H pylori*; C, control group.

Table 5 Cytokine concentrations in gastric mucosa of children examined depending on the inflammation activity according to Sydney System[25]

Cytokines (pg/mg protein)	Fa		Hp				Fa+Hp			
	Antrum	Corpus	Antrum		Corpus		Antrum		Corpus	
	Mild+moderate	Mild+moderate	Mild+moderate	Severe	Mild+moderate	Severe	Mild+moderate	Severe	Mild	Moderate+severe
IL-2	17.7	17.9	204.3[b]	72.5	69.0	187.6[b]	70.1	110.9	119.3	74.3
IFN-γ	54.9	46.6	20.4	18.4	21.8[b]	14.6[b]	25.6	25.4	22.4	33.4
IL-4	15.4	6.7[b]	81.1[b]	6.7[b]	30.0	47.8	52.0	29.8	39.9	39.9
IL-5	24.5	25.0	–	–	–	–	–	–	–	–
IL-8	37.5	37.5	83.5	90.2	86.9	89.6	69.8	115.5	83.2	131.6
IL-10	30.4	24.0	–	–	–	–	–	–	–	–
TNF-α	20.6	4.8	76.0	33.3	37.4	57.9	24.6	54.6	60.4	24.9

[b]P<0.01 *vs* cytokine concentrations statistically significantly different from controls. Fa, food allergy; Hp, *H pylori*; C, control group.

gastric mucosal biopsies before the treatment. The highest mean IL-8 value was observed in Hp infected children (with or without Fa). The mean IL-8 concentration was 101.2 ±68.3 pg/mg in allergic children with Hp infection. This value was statistically significant ($P<0.005$) in comparison to that in allergic children. In children with Hp infection, the mean gastric mucosa IL-8 concentration was 88.0± 40.4 pg/mg, being statistically significant ($P<0.005$) in comparison to that in children with Fa. The evaluation of mucosa IL-8 concentration with regard to the intensity and activity of inflammatory process in the gastric mucosa did not show any statistically significant dependence (Tables 4 and 5).

In gastric mucosal biopsies, IL-10 concentration ranged from undetectable to 141.8 pg/mg. The highest mean concentration of mucosa IL-10 was detected in children with Hp infection (79.3±41.2 pg/mg) and in allergic children with Hp infection (50.1±18.8 pg/mg).

The mean IL-10 concentration was 39.9±35.5 pg/mg in children with Fa, being statistically significantly different from that in children with Hp infection ($P<0.01$, Table 3). The intensity and activity of inflammation did not affect IL-10 concentration in the gastric mucosa (Tables 4 and 5).

TNF-α concentration ranged from undetectable to 208.4 pg/mg in gastric mucosal biopsies. The mean TNF-α concentration was the highest in children with Hp infection and 45.9±49.3 pg/mg, in children without Fa compared to that in the controls ($P<0.06$). Infection revealed the mean TNF-α concentration of 45.3±32.6 pg/mg in allergic children with coexisting Hp infection, being statistically different from controls ($P<0.01$). The mean TNF-α concentration was 21.7±34.2 pg/mg in allergic children and showed statistically significant difference ($P<0.003$) in comparison to children with Hp infection or with Fa and coexisting Hp infection ($P<0.004$). The analysis of TNF-α concentration in the gastric mucosa with regard to the intensity and activity of the inflammation showed no statistically significant differences.

DISCUSSION

Our study proved that the pathogenic factors like harmful food could induce certain morphological changes of the gastric mucosa. They are smaller with regard to the percentage and "depth" than in the combined activity of allergy and Hp. It seems essential to check the intensity of the changes and their dynamics by assessing their characteristics and size as well as local properties of cytokines produced. The concentration of chosen cytokines in the antrum mucosa in children with Fa was comparable to that in the controls. However, IL-4 concentration was statistically significantly different from the control group. IL-4 is secreted by antigen- or mitogen-inactivated lymphocytes Th2 and by mast cells in the gastric mucosa infiltrates in children with Fa.

Another frequent etiological factor, Hp infection leading to gastritis, was observed besides the allergic one affecting the gastric mucosa. Histopathological changes induced by the activity of this factor were far greater than those observed in children with Fa.

It should be assumed that the intensified local production of many inflammatory cytokines, including IL-2, takes place in Hp infection where gastric mucosa is infiltrated by such cells as neutrophils, lymphocytes, monocytes/macrophages and cytoplasmic cells. The IL-2 concentration was the highest in the gastric mucosa of children with Hp infection (116.5 pg/mg) and slightly lower in children with Fa and Hp infection (98.1 pg/mg). The values were statistically significant when compared to the control group.

IL-8 is another cytokine, whose chemotactic properties are directed selectively towards neutrophils. The activity of IL-8 on neutrophils is triggered by binding to a specific receptor, which can be found on the surface of the cells (5 ku)[10,25-27]. A population of children with Fa and coexisting Hp infection was included additionally in our study. The mean concentration of IL-8 in the gastric mucosa of children with Hp infection was 88.0 pg/mg, being statistically significantly different ($P<0.01$) in comparison to 59.9 pg/mg in allergic children. The mean IL-8 concentration was even higher (101.2 pg/mg) in allergic children with Hp infection.

Another cytokine with a wide range of biological actions is IL-4 secreted by antigen-inactivated Th lymphocytes (Th2) and mast cells[28-31]. We evaluated IL-4 concentration in particular groups of children and found IL-4 levels were increased in allergic patients, though the values were not statistically different from those in the controls.

TNF-α is one of the most potent cytokines activating numerous functions of neutrophils. It stimulates directly oxygenic metabolite release by neutrophils and the effectiveness of this phenomenon depends on the doses used[32-34]. Isolauri *et al*.[35] and Majamaa *et al*.[36] showed that TNF-α is elevated in feces of allergic children, which is thought to be a delayed reaction type to food challenge. Children with immediate reaction have a significant increase in α1-antitripsin and eosinophil cationic protein (ECP) in their feces as compared to patients who do not respond to food challenge.

The relatively low TNF-α concentration in the antrum mucosa (21.7 pg/mg) of allergic children may be due to the fact that a marked percentage of the patients revealed IgE-dependent mediators engaged in allergic reactions. TNF-α assessed in biopsies of the alimentary tract mucosa or excrements feces seems to be a good factor for active immunological reactions to food allergens ingested by children who react to food without IgE participation. Isolauri *et al*.[35] showed that TNF-α can be equally used as a factor of pathogenic process activation and food hypersensitivity as cationic protein (ECP) and α1-antitripsin in case of patients whose cytokines secreted by T lymphocytes are responsible for clinical symptoms.

In conclusion, the pathogenic process is individually different with regard to morphological examinations and proinflammatory and proallergic cytokine production. Concentrations of IFN-γ, IL-2, IL-8, and TNF-α in the gastric mucosa of children with Fa and those with Hp infection (without Fa) are different.

REFERENCES

1 **Burks AW**, Sampson HA. Diagnostic approaches to the patient with suspected food allergies. *J Pediatr* 1992; **121:** S 64-S 71
2 **Hourihane JO**. Prevalence and severity of food allergy--need for control. *Allergy* 1998; **53**: (suppl. 46): 84-88
3 **Leal-Berumen I**, Conlon P, Marshall JS. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *J Immunol* 1994; **152:** 5468-5476
4 **Schwartz LB**. Tryptase, a mediator of human mast cells. *J Allergy Clin Immunol* 1990; **86:** 594-598
5 **Tharp MD**, Thirlby R, Sullivan TJ. Gastrin induces histamine release from human cutaneous mast cells. *J Allergy Clin Immunol* 1984; **74:** 159-165
6 **Katz AJ**, Goldman H, Grand RJ. Gastric mucosal biopsy in eosinophilic (allergic) gastroenteritis. *Gastroenterology* 1977; **73:** 705-709
7 **Czinn SJ**, Dahms BB, Jacobs GH, Kaplan B, Rothstein FC. Campylobacter-like organisms in association with symptomatic gastritis in children. *J Pediatr* 1986; **109:** 80-83
8 **Chan WY**, Hui PK, Leung KM, Thomas TM. Modes of Helicobacter colonization and gastric epithelial damage. *Histopathology* 1992; **21:** 521-528
9 **Crabtree JE**. Immune and inflammatory responses to Helicobacter pylori infection. *Scand J Gastroenterol Suppl* 1996; **215:** 3-10
10. **Crabtree JE**, Peichl P, Wyatt JI, Stachl U, Lindley IJ. Gastric interleukin-8 and IgA IL-8 autoantibodies in Helicobacter pylori infection. *Scand J Immunol* 1993; **37:** 65-70
11 **Atherton JC**, Peek RM Jr, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* 1997; **112:** 92-99
12 **Harris PR**, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology* 1996; **111:** 419-425
13 **Mai UE**, Perez-Perez GI, Allen JB, Wahl SM, Blaser MJ, Smith PD. Surface proteins from Helicobacter pylori exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. *J Exp Med* 1992; **175:** 517-525
14 **Andre F**, Pene J, Andre C. Interleukin-4 and interferon-gamma production by peripheral blood mononuclear cells from food-allergic patients. *Allergy* 1996; **51:** 350-355
15 **Kiniwa M**, Gately M, Gubler U, Chizzonite R, Fargeas C, Delespesse G. Recombinant interleukin - 12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J Clin Invest* 1992; **90:** 262-266
16 **Zurawski G**, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol Today* 1994; **15:** 19-26
17 **Chandra RK**, Kumari S. Nutrition and immunity: an overview. *J Nutr* 1994; **124:** 1433-1435
18 **Pene J**, Rousset F, Briere F, Chretien I, Wideman J, Bonnefoy JY, De Vries JE. Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells. The role of soluble CD23 antigen. *Eur J Immunol* 1988; **18:** 929-935
19 **Schroeder JT**, MacGlashan DW Jr. New concepts: the basophil. *J Allergy Clin Immunol* 1997; **99:** 429-433
20 **Baggiolini M**, Dahinden CA. CC chemokines in allergic inflammation. *Immunol Today* 1994; **15:** 127-133
21 **Yamaoka Y**, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. Expression of cytokine mRNA in gastric mucosa with Helicobacter pylori infection. *Scand J Gastroenterol* 1995; **30:** 511-519
22 **Yamaoka Y**, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. Expression of cytokine mRNA in gastric mucosa with Hp infection. *Scand J Gastroenterol* 1995; **30:** 1153-1159
23 **Ishihara S**, Fukuda R, Fukumoto S. Cytokine gene expression in the gastric mucosa: its role in chronic gastritis. *J Gastroenterol* 1996; **31:** 485-490
24 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20:** 1161-1181
25 **Abreu-Martin MT**, Vidrich A, Lynch DH, Targan SR. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-alpha and ligation of Fas antigen. *J Immunol* 1995; **155:** 4147-4154
26 ***Nishi Y***, Isomoto H, Uotani S, Wen CY, Shikuwa S, Ohnita K, Mizuta Y, Kawaguchi A, Inoue K, Kohno S. Enhanced production of leptin in gastric fundic mucosa with Helicobacter pylori infection. *World J Gastroenterol* 2005; **11:** 695-699
27 **Wang KX**, Chen L. Helicobacter pylori L-form and patients with chronic gastritis. *World J Gastroenterol* 2004; **10:** 1306-1309
28 **Chen CC**, Manning AM. TGF-beta 1, IL-10 and IL-4 differentially modulate the cytokine-induced expression of IL-6 and IL-8 in human endothelial cells. *Cytokine* 1996; **8:** 58-65
29 **Huang H**, Hu-Li J, Chen H, Ben-Sasson SZ, Paul WE. IL-4 and IL-13 production in differentiated T helper type 2 cells is not IL-4 dependent. *J Immunol* 1997; **159:** 3731-3738
30 **Joyce DA**, Steer JH. IL-4, IL-10 and IFN-gamma have distinct, but interacting, effects on differentiation-induced changes in TNF-alpha and TNF receptor release by cultured human monocytes. *Cytokine* 1996; **8:** 49-57
31 **Ji SL**, Cui HF, Shi F, Chi YQ, Cao JC, Geng MY, Guan HS. Inhibitory effect of heparin-derived oligosaccharides on secretion of interleukin-4 and interleukin-5 from human peripheral blood T lymphocytes. *World J Gastroenterol* 2004; **10:** 3490-3494
32 **Gordon JR**, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* 1990; **346:** 274-276
33 **Hernandez-Pando R**, Rook GA. The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology* 1994; **82:** 591-595
34 **Marie C**, Pitton C, Fitting C, Cavaillon JM. IL-10 and IL-4 synergize with TNF-alpha to induce IL-1ra production by human neutrophils. *Cytokine* 1996; **8:** 147-151
35 **Isolauri E**, Suomalainen H, Kaila M, Jalonen T, Soppi E, Virtanen E, Arvilommi H. Local immune response in patients with cow milk allergy: follow-up of patients retaining allergy or becoming tolerant. *J Pediatr* 1992; **120:** 9-15
36 **Majamaa H**, Miettinen A, Laine S, Isolauri E. Intestinal inflammation in children with atopic eczema: faecal eosinophil cationic protein and tumour necrosis factor-alpha as non-invasive indicators of food allergy. *Clin Exp Allergy* 1996; **26:** 181-187

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• *BASIC RESEARCH* •

Effect of bombesin and neurotensin on gut barrier function in partially hepatectomized rats

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Abstract

AIM: To investigate the effect of regulatory peptides bombesin (BBS) and neurotensin (NT) on intestinal barrier function in partially hepatectomized rats.

METHODS: Ninety male Wistar rats were randomly divided into five groups: I (*n* = 10): controls, II (*n* = 20): sham operated, III (*n* = 20): partial hepatectomy 70% (PHx), IV (*n* = 20): PHx+BBS (30 μg/kg/d), V (*n* = 20): PHx+NT (300 μg/kg/d). Groups IV and V were treated for 8 days before PHx and 48 h post surgery. At the end of the experiment, on day 10, intestinal barrier function was assessed by measuring endotoxin concentrations in portal and aortic blood. Tissue sections of the terminal ileum were examined histologically and villus density, mucosal thickness, mitotic activity and apoptosis in crypts were assessed. In addition, ileal mucosa was analyzed for DNA and protein content and microbiological analysis was performed in cecal contents. To estimate intestinal oxidative stress, lipid peroxidation was determined on tissue homogenates from terminal ileum.

RESULTS: BBS or NT administration significantly reduced portal and systemic endotoxemia observed 48 h after partial hepatectomy. In hepatectomized rats (group III), a trend towards induction of mucosal atrophy was observed, demonstrated by the reduction of villus density, mucosal thickness, protein content and significant reduction of DNA, while these alterations were reversed by regulatory peptides administration. This trophic effect of BBS and NT was accompanied by induction of mitoses above control levels and a significant reduction of apoptosis in intestinal crypts. Intestinal lipid peroxidation was found significantly lower in PHx group and regulatory peptides exerted an antioxidant action, further decreasing this parameter of oxidative stress. The bacterial population of *E. coli* and aerobic Gram (+) cocci was increased in cecal content of hepatectomized rats, while this parameter was not affected by the administration of BBS or NT.

CONCLUSION: Gut regulatory peptides BBS and NT improve intestinal barrier function and reduce endotoxemia in experimental partial hepatectomy. This effect is, at least in part, mediated by their trophic, antiapoptotic, mitogenic, and antioxidant effect on the intestinal epithelium. This observation might be of potential value in patients undergoing liver resection.

Key words: Hepatectomy; Rats; Bombesin; Neurotensin; Intestinal barrier; Apoptosis; Oxidative stress

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INTRODUCTION

Despite current advances in the safety of hepatic resection and perioperative care, which have led to a significant reduction of perioperative mortality, septic events remain still an important problem complicating the outcome of hepatectomized patients[1]. It has been previously shown that enteric bacteria and endotoxins passing through the intestinal barrier to extraintestinal sites and the systemic circulation result in intra-abdominal abscess formation, pulmonary infections, sepsis and multiple organ dysfunction in patients and rats after liver resection[1,2]. A

compromised gut barrier function promotes the escape of enteric bacteria and endotoxins into portal circulation, while the reduction of the functional reticuloendothelial volume after hepatectomies permits their systemic spread[3].

Bombesin (BBS), a tetradecapeptide originally isolated from the skin of the European frog *Bombina bombina* is analogous to gastrin-releasing peptide found in mammalians[4]. BBS stimulates the release of various gut hormones and peptides e.g, gastrin, cholecystokinine, insulin, glucagons, somatostatin, motilin, pancreatic polypeptide, neurotensin and exerts a trophic effect on intestinal and gastric mucosa and pancreas, while it stimulates intestinal motility[5,6]. It has been previously shown that BBS improves intestinal integrity in experimental models of gut barrier dysfunction, such as after elemental diets, methotrexate administration, chemically induced colitis and burns[5,7-9].

Neurotensin (NT), a tridecapeptide originally isolated from the bovine hypothalamus, is additionally found in the gut mucosal endocrine cells (N cells), especially in the ileum[10]. NT stimulates pancreaticobiliary secretions, intestinal blood flow and colonic motility, while it inhibits small intestinal and gastric motility. It is a potent trophic agent for small and large intestine, gastric mucosa and pancreas[11-14]. NT has been shown to prevent intestinal atrophy induced by feeding rats an elemental diet, restores mucosal ulceration after radiation therapy and enhances intestinal regeneration after small bowel resection[7,15,16].

We have recently shown that BBS and NT improve intestinal barrier function in experimental obstructive jaundice by exerting mitogenic, antiapoptotic, and antioxidant effects on the intestinal epithelium[17]. The present study was undertaken to investigate the effect of exogenous administration of BBS and NT on gut barrier function after partial hepatectomy in rats.

MATERIALS AND METHODS

Animals

Ninety male albino Wistar rats, weighing 250-320 g, were used. They were housed in stainless-steel cages, three rats per cage, under controlled temperature (23 °C) and humidity conditions, with 12-h dark/light cycles, and maintained on standard laboratory diet with tap water *ad libitum* throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to the guidelines set forth by the Ethics Committee of Patras University Hospital, Patras, Greece.

Experimental design

Animals were divided randomly into five groups: Group I (n = 10): non-operated controls, group II (n = 20): sham operated, group III (n = 20): partial hepatectomy (70%), group IV (n = 20): partial hepatectomy and BBS administration, group V (n = 20): partial hepatectomy and NT administration.

Starting on d 0, the animals of groups IV and V were treated daily with BBS (10 μg/kg, subcutaneously, thrice a day) and NT (300 μg/kg, intraperitoneally, once a day), respectively, while the animals of groups I, II, and III were divided to receive daily either three subcutaneous or one intraperitoneal injection of 0.5 mL normal saline. Previous pilot studies showed that the way of saline administration does not affect the results. On the 8th day, animals from groups III, IV, and V underwent laparotomy and partial hepatectomy (almost 70%) as described by Higgins and Andersson[18], while animals in group II underwent laparotomy and mobilization of the liver. The abdominal incision was closed in two layers with chromic 4-0 cat gut and 4-0 silk. All surgical procedures were performed under strict sterile conditions, using light ether anesthesia. Administration of BBS, NT, and normal saline was continued for 48 h after surgery. On the 10th d, all animals were operated (group I) or reoperated (groups II, III, IV, and V), again under strict sterile conditions. Samples were obtained according to the experimental protocol, after which the rats were killed by exsanguination.

Peptides preparation

A stock solution of BBS (Sigma Chemical Co, St. Louis, MO, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in plastic tubes at -20 °C. At the time of administration, in order to prolong absorption, each aliquot was mixed with 0.4 mL of a solution of 8% (w/v) hydrolyzed gelatin (Sigma Chemical Co, St. Louis, MO, USA). A final volume of 0.5 mL, containing 10 μg BBS/kg body weight, was injected subcutaneously thrice daily.

A stock solution of NT (Sigma Chemical Co, St. Louis, MO, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 0.1% (w/v) bovine serum albumin, so that the dose of NT needed for each injection to be contained in a volume of 0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in glass vials at -20 °C. At the time of administration, each aliquot was further diluted with 0.4 mL sterile saline to a final volume of 0.5 mL and was given intraperitoneally as a bolus injection containing 300 μg NT/kg body weight.

Endotoxin measurements

For the determination of endotoxin concentrations, a laparotomy was performed in all groups, the portal vein and the abdominal aorta were punctured and samples of 1 and 2 mL of blood were obtained, respectively. Endotoxin concentration was determined by the quantitative chromogenic Limulus amebocyte lysate test according to the manufacturer's instructions (QCL-1 000, BioWhittaker, Walkersville, USA) and expressed in EU/mL.

Measurements of mucosal DNA and protein

DNA and protein content in the terminal ileal mucosa were determined in all animals. A 1-cm long sample of the terminal ileum was excised, opened by longitudinal incision and washed with cold normal saline. Using a clean glass slide the mucosa was removed and homogenized in 1 mL NaOH 1 N, by means of a polytron homogenizer. The protein was measured according to Lowry's method[19] using a commercial kit (Sigma Diagnostics, Deisenhofen, Germany) and the DNA was determined according to a modified Barton technique[20].

Histological evaluation

For histological examination, tissue samples from the terminal ileum were obtained from all animals. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. In each ileal specimen, several histologic features were evaluated and recorded. These features included architectural distortion, villous blunting, surface and crypt epithelial injury, presence and cell type of inflammation of the lamina propria, surface and cryptal intraepithelial infiltration, lamina propria fibrosis and granulation tissue formation. Ileal mucosal morphometric characteristics were studied by measurements of villus density, defined as the number of villi per centimeter (V/cm), and villus height (Vh) in micrometers (μm). Villus height was measured with a micrometer eyepiece affixed to a Reichert–Jung light microscope and at least 20 well-preserved villi were estimated in each sample. In addition, the number of mitoses and apoptotic bodies per crypt were also counted. Apoptotic bodies of the cryptal epithelium were identified and tallied using a morphometric analysis, which has been described in detail elsewhere[21]. Apoptotic bodies were defined as rounded vacuoles with fragments of karyorrhectic nuclear debris and were differentiated from small isolated fragments of nuclear chromatin and intraepithelial neutrophils. Apoptotic bodies and mitoses were counted in all architecturally successive crypts included in the specimen, regardless of crypt orientation, and their total number was divided by the number of the crypts. The number of apoptotic bodies per crypt is referred to as the apoptotic body count (ABC).

Cecal bacterial population

Collection of cecal contents for microbiological analysis, was performed by the following technique: the ascending colon was ligated below the hepatic flexure, and a 21-gauge needle mounted on a 5 mL syringe was introduced into the cecum through the ileocecal valve, after puncturing the terminal ileum. Two milliliters of sterile saline was infused in the cecum, after which the needle was withdrawn and the terminal ileum was ligated. The cecal content was manually mollified for 2 min and after a good mixture was achieved, 1 mL of colonic content was removed; serial tenfold dilutions were performed and 0.001 mL of each sample was inoculated into 5% blood and McConkey's agar plates for recovery of aerobic bacteria and Gram-negative/nonspore-forming agar plates for anaerobes. After 24 and 48 h of incubation at 37 °C, for aerobic and anaerobic cultures respectively, colonies were identified and counted. Quantitative culture results were expressed as the number of colony-forming units (CFU) per milliliter or per gram of the cecal samples, calculated from the dilutions of colonic content. *E coli* was identified by Gram stain and a standard biochemical identification system (API-20E, Biomérieux, Marcy-l'Etoile, France, according to the analytical profile index).

Intestinal lipid peroxidation

A 1-cm long tissue sample of the terminal ileum of each animal was excised, washed in 9 g/L of NaCl and was homogenized in a porcelain mortar in liquid nitrogen. Intestinal homogenates were processed for the determination of lipid peroxidation, according to a modified [2-thiobarbituric acid (TBA)]-based method, as reported previously[22]. Lipid peroxidation was expressed in pmoles malondialdehyde (MDA)/mg total protein.

Statistical analysis

The results are expressed as mean (SD). Comparisons among multiple groups were performed using the one-way ANOVA, followed by Bonferroni's *post hoc* test, when variances across groups were equal or by Dunnett's T3 *post hoc* test, when variances were not equal. Variance equality was tested by Levene statistical analysis. Differences were considered significant, when $P<0.05$.

RESULTS

Portal and aortic endotoxin concentrations

Hepatectomized animals (group III) presented significantly elevated endotoxin concentrations in portal and aortic blood compared with groups I and II ($P<0.001$, respectively). Treatment with BBS or NT led to significantly lower endotoxin values both in portal vein ($P<0.001$ *vs* group III, respectively) and aorta ($P<0.001$ and $P<0.01$ *vs* group III, respectively) (Figure 1).

Mucosal DNA and protein

DNA content of the intestinal mucosa was significantly decreased in partially hepatectomized rats as compared to controls ($P<0.01$) and increased in BBS and NT treated rats ($P<0.05$ and $P<0.01$ *vs* group III, respectively)

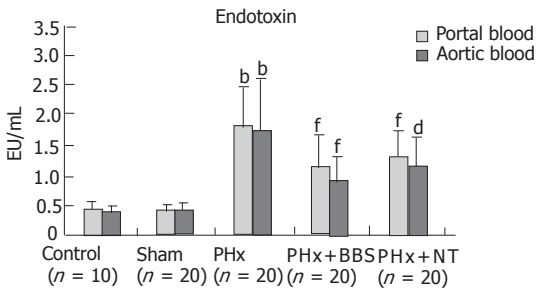

Figure 1 Portal and aortic endotoxin concentrations. Values are mean±SD. [b]$P<0.001$ *vs* sham, [d]$P<0.01$ and [f]$P<0.001$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

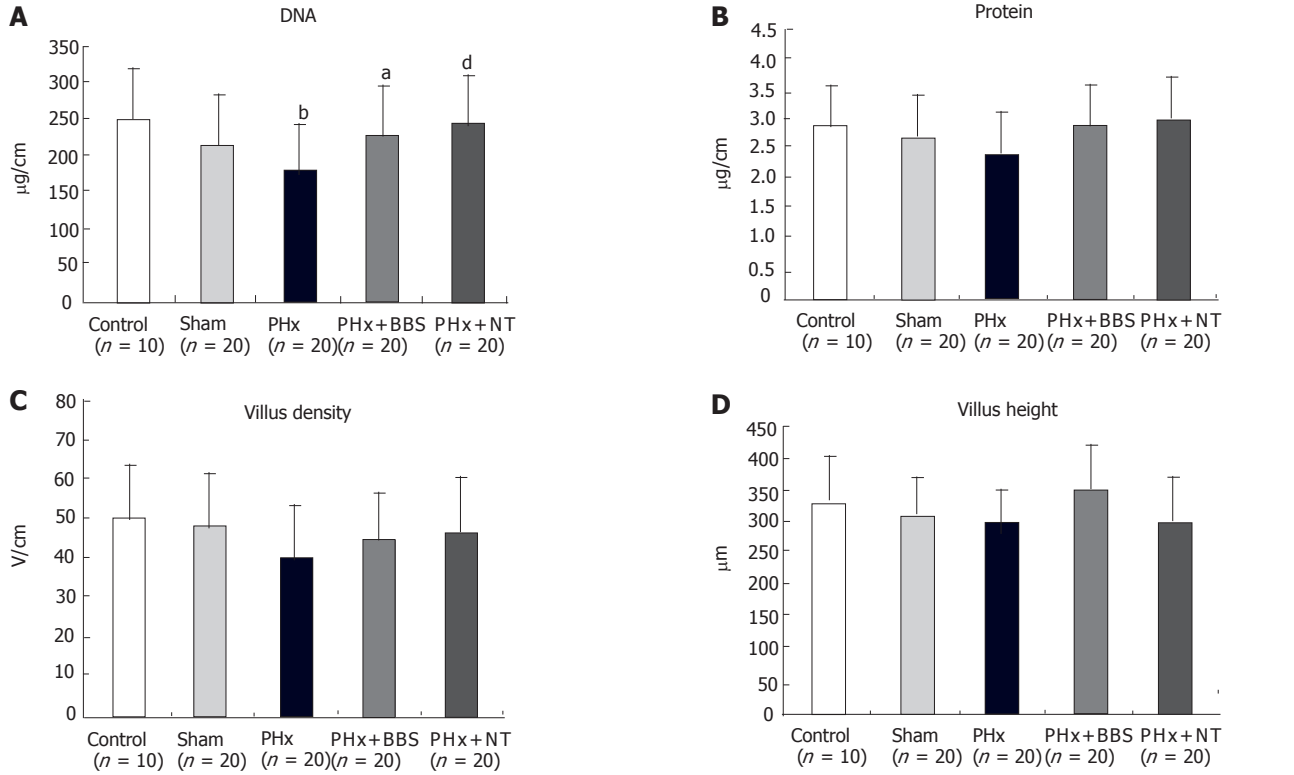

Figure 2 Indices of ileal mucosa trophic state. Values are mean±SD. [b]*P*<0.01 *vs* control, [a]*P*<0.05 and [d]*P*<0.01 *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin(**A-D**).

(Figure 2A). Protein content was also decreased in hepatectomized rats and increased to normal levels after peptides administration, but these differences did not reach statistical significant levels (Figure 2B).

Intestinal morphology

Overall, the ileal architecture remained intact and epithelial continuity was retained, in all specimens studied. The ileal biopsies from group III demonstrated a reduction in the number of villi per centimeter (Figure 2C) and in villus height (Figure 2D), which were increased towards control values in rats treated with BBS or NT; however, the differences among groups were not significant. Intestinal crypt mitotic activity was reduced in hepatectomized rats, though not to significant levels, and increased above normal levels after BBS or NT administration (Figure 3A). Crypt epithelial apoptosis was present in all intestinal samples evaluated. The ABC in ileal specimens from control group was significantly lower compared with group III ($P<0.001$) (Figure 3B). After BBS or NT administration the ABC was significantly reduced ($P<0.05$ and $P<0.01$, compared to PHx, respectively).

Cecal bacterial population

Table 1 provides the quantitative culture results of the cecal contents for aerobic and anaerobic bacteria. In partially hepatectomized rats (group III) there was a significant increase in Gram (+) cocci and *E coli* cecal count as compared with group I ($P<0.05$, respectively). Neurotensin presented a trend towards reduction of aerobic and anaerobic bacterial populations, which reached significant levels for Gram (+) anaerobic bacteria ($P<0.001$ *vs* group III).

Table 1 Bacterial cecal population (CFU×10^5/mL)

Groups	*n*	Aerobic bacteria		Anaerobic bacteria	
		Gram (+) cocci	*E coli*	Gram (+)	Gram (–)
Control	10	6.70 (1.62)	1.04 (0.88)	9 (2.74)	4.64 (1.99)
Sham	20	9.45 (4.92)	1.60 (2.07)	13.34 (5.62)	6.5 (4.35)
PHx	20	11.09 (5.71)[a]	6.43 (6.38)[a]	13.17 (3.88)	5.48 (4.57)
PHx+BBS	20	11.70 (6.41)	6.31 (6.20)	10 (4.29)	4.45 (2.86)
PHx+NT	20	6.76 (4.31)	2.77 (3.92)	6.76 (5.64)[b]	3.08 (4.48)

Data expressed as mean (SD). [a]*P*<0.05 *vs* control, [b]*P*<0.001 *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

Intestinal lipid peroxidation

Liver resection resulted in decreased intestinal lipid peroxidation ($P<0.001$ *vs* group II), while administration of BBS or NT led to further significant decrease of this index of oxidative stress ($P<0.01$ and $P<0.001$ *vs* group III, respectively, Figure 4).

DISCUSSION

An intact intestinal barrier function effectively separates potentially harmful intraluminal elements such as bacteria and endotoxins from extraintestinal tissues and the systemic circulation. Major liver resection has been shown to compromise the anatomic and functional integrity of the gut barrier resulting in the translocation of indigenous bacteria and endotoxins to remote organs and

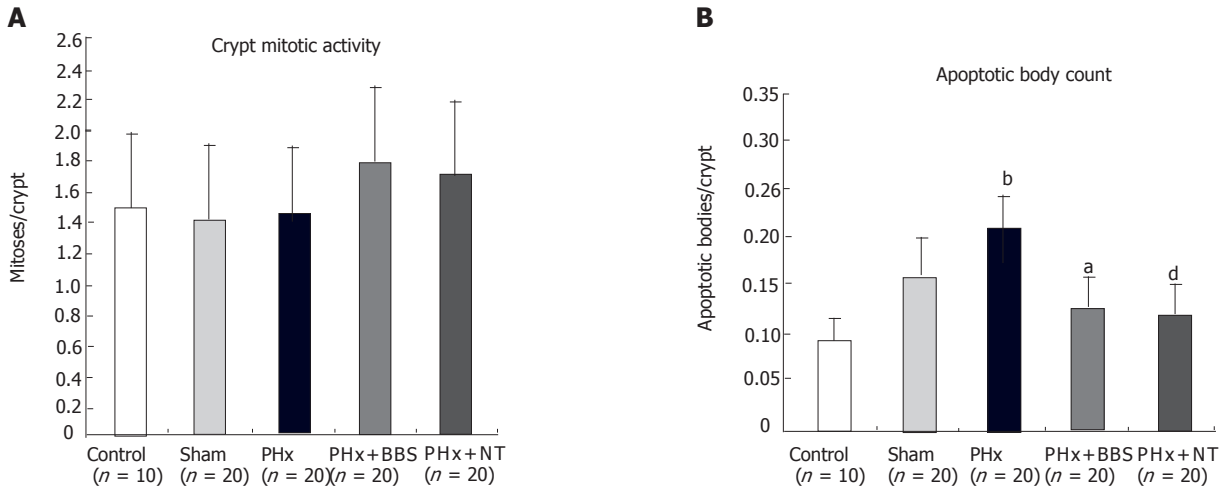

Figure 3 Crypt epithelial cell proliferation and apoptosis. Values are mean±SD. $^{b}P<0.001$ *vs* control, $^{a}P<0.05$ and $^{d}P<0.01$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin(**A**, **B**).

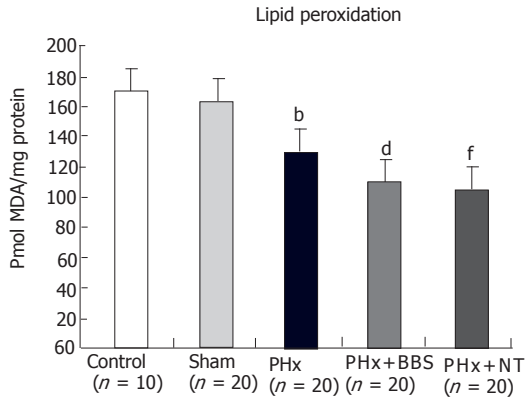

Figure 4 Lipid peroxidation in the intestine. Values are mean±SD. $^{b}P<0.001$ *vs* sham, $^{d}P<0.01$ and $^{f}P<0.001$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

tissues[3,23]. Systemic endotoxemia plays a pivotal role in the development of septic complications and dysfunction of remote organs after liver resections through activation of a systemic inflammatory response, which is associated with structural and functional deleterious effects on vital organs[24]. Failure of the gut barrier leads to bacterial and endotoxin translocation and the pathogenesis of the so-called "gut-derived sepsis"[25]. However, the mechanisms implicated in intestinal barrier failure after liver resection have not been fully elucidated.

Physical injury to the intestinal mucosa is one of the mechanisms postulated to promote bacterial translocation. In our study, the ileal architecture remained intact and epithelial continuity was retained in hepatectomized rats. Previous experimental studies have shown that 70% hepatectomy induces trophic changes on the distal ileum after 30 d, causing atrophy of the ileum wall and a drop in villus thickness[26]. The present study shows that 48 h after partial liver resection there is a trend for decreased villous density and mucosal thickness that does not reach statistical significant levels. Diminished bile production and secretion to the duodenum, after partial hepatectomy, deprives partly the gut from its trophic action thus promoting mucosal atrophy. On the other hand, the plethora of enterotrophic growth factors and cytokines that are elaborated after liver resection exert the opposite effect[27]. The combinatory action of these factors seems to produce a balanced adaptive intestinal response, 48 h after partial hepatectomy.

The balance between cell proliferation and death in intestinal crypts is crucial for epithelial homeostasis because the total of epithelial cells lining villi originate from stem cells located in the proliferation zone of the crypt. Apoptosis was the only histologic parameter that altered significantly in the present study. Definite evidence of apoptosis is seen in the crypt, where apoptotic cells are seen once in every 5^{th}-10^{th} crypt section[28]. Occasional apoptotic bodies found in the rapidly proliferating epithelial cells of the normal gastrointestinal mucosa, help to maintain a steady-state in cellular populations[29]. Control animals presented this basal level of apoptotic activity known as spontaneous apoptosis, which serves to remove either occasionally overproduced stem cells or stem cells with minor DNA damage caused by external factors. Two-thirds hepatectomy resulted in a significant increase of apoptosis in the crypt, which may explain the trend for induction of mucosal atrophy without alterations in the mitotic activity of the intestinal epithelium. In addition, increase of the apoptotic process in the intestinal epithelium may partly contribute to the decrease of mucosal DNA content. Apoptotic cells have a lower DNA content than the G0/G1 value and this hypo-diploid DNA is a hallmark to show apoptosis[30].

Apoptosis is a morphologically distinct, gene-directed, form of cell death that contributes to both physiological and pathological processes[31]. Although in gastrointestinal epithelium it has been associated with several conditions[17,21,32], the biological significance of intestinal apoptosis after liver resection is not clear till

date. The responsible mechanism could reflect primary immunologic events following hepatectomy (apoptosis has been shown to be induced by a variety of triggers, including proinflammatory cytokines such as TNF, IL-1, and IL-6, or by cytotoxic T lymphocytes that act through either granzyme B or Fas receptor pathways) or a direct action of bacterial toxins[31,33,34].

Another factor associated with bacterial translocation is intestinal flora disturbances. In our study hepatectomized rats presented a significant increase of cecal aerobic bacteria [*E coli* and Gram (+) cocci]. Diminished bile production and disturbances of interdigestive motility may have led to bacterial overgrowth. Bile salts have a constraining role on the indigenous microflora, while secretory *IgA* contained in bile modulates the local immunological milieu[35]. In addition, delayed intestinal transit time in experimental liver resection seem to be implicated in intestinal bacterial overgrowth and increased bacterial translocation[36]. A correlation between cecal overgrowth of a specific organism and bacterial translocation of the same organism is well documented[37]. Overgrowth of *E coli* detected in our study and diminished biliary *IgA* may lead to increased attachment of this bacterial strain to the intestinal mucosa, predisposing to bacterial translocation and previous studies have shown that *E coli* is usually cultured from the mesenteric lymph nodes of hepatectomized rats[2].

Another parameter we have addressed in our study is intestinal oxidative stress estimated by quantification of lipid peroxidation, which is a common indicator of oxidative stress. Our data show that 48 h after partial hepatectomy decreased levels of lipid peroxidation were measured in the intestine. This may be attributed to liver regeneration and release of several growth factors and hormones that may influence intestinal oxidative stress[27]. In addition, previous studies demonstrate evidence for activation of the intestinal cytosolic antioxidant enzyme glutathione *S*-transferase after hepatectomy[38]. Another explanation might be that the removal of a large portion of the liver could result in a decrease of oxidants originating from the liver, therefore lowering lipid peroxidation. The liver and gut are considered as an anatomic and functional unit with inseparable and interdependent functions and from this point of view liver regeneration under low oxidative stress[39] may be associated with similar oxidant alterations in the intestine. A question that rose is if low oxidative stress is compatible with increased apoptosis. Whether a cell will enter or not the apoptotic process is determined by a variety of stimuli and intrinsic pathways and it seems that apoptosis of enterocytes after partial hepatectomy is not mediated by oxidative stress.

The present study has further investigated the role of gut regulatory peptides BBS and NT on gut barrier function in experimental partial hepatectomy. To the best of our knowledge, our results demonstrate for the first time that administration of BBS or NT improves gut barrier function after partial hepatectomy, leading to significantly lower portal and aortic endotoxin concentrations. Treatment with these factors reversed the decrease of intestinal morphometric characteristics and preserved mucosal DNA and protein content to control levels. Although these alterations were statistically significant only for DNA, they show a trend for induction of a trophic effect on the intestinal mucosa. This effect was accompanied by the increase of cell proliferation beyond control levels and inhibition of programmed cell death in intestinal crypts. The mitogenic effect of BBS and NT may be a direct receptor-mediated effect, since intestinal epithelial cells express receptors for both peptides[40-42] and it is known that BBS and NT are potent cellular growth factors whose binding to their receptors activates a mitogenic signal to the nucleus[43]. An indirect mechanism may be related to a further reduction of intestinal lipid peroxidation since oxidative state is involved in the modulation of cell proliferation and death[44]. The antiapoptotic effect of BBS and NT may be mediated by the reduction of endotoxemia and amelioration of the subsequent systemic inflammatory response, characterized by the release of numerous cytokines and proinflammatory mediators, such as tumor necrosis factor-alpha, which may activate the apoptotic process. Another explanation of their antiapoptotic action may be provided through induction of vasodilation[45,46], which improves intestinal microcirculation thus preventing enterocytes hypoxia, energy depletion and activation of apoptotic pathways.

Apart from their antiapoptotic action on the intestinal epithelium, BBS and NT exerted an antioxidant effect as well, further decreasing intestinal lipid peroxidation. The compromise of the intestinal barrier function, after partial hepatectomy, shows that intestinal compensatory response, which may be partly expressed by low oxidative stress, is relatively insufficient to overwhelm the noxious effect of hepatectomy on the intestinal epithelium. From this point of view, the additional decrease of intestinal oxidative stress achieved by peptides treatment may contribute to the enhancement of intestinal barrier. The results of this study are consistent with our previous work demonstrating the antioxidant effect of BBS and NT on the intestinal epithelium of bile duct ligated rats[17]. Possible explanations of their antioxidant action is the reduction of systemic endotoxemia, which is associated with generation of oxygen free radicals via a xanthine oxidase depended pathway, improvement of oxygen supply to enterocytes through vasodilation, or receptor-mediated activation of intracellular antioxidant pathways. It has been shown that binding of regulatory peptides to their specific G-protein coupled receptors is followed by a pivotal activation of protein kinase C[43]. Increased cellular PKC activity promotes the mitochondrial translocation and organization in a catalytically active form of a representative isoform of glutathione *S*-transferases, which are an important antioxidant enzyme system[47].

In conclusion, the present study shows that the gut regulatory peptides BBS and NT improve the intestinal barrier function and reduce endotoxemia, in experimental partial hepatectomy. This effect is, at least in part, mediated by their antiapoptotic, mitogenic and antioxidant effect on the intestinal epithelium. Although laboratory results

should not be easily extrapolated to the clinical situation, we feel that BBS and NT merit consideration, as potential therapeutic agents, for the improvement of gut barrier function in cases of liver resection.

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REFERENCES

1 **Jarnagin WR**, Gonen M, Fong Y, DeMatteo RP, Ben-Porat L, Little S, Corvera C, Weber S, Blumgart LH. Improvement in perioperative outcome after hepatic resection: analysis of 1,803 consecutive cases over the past decade. *Ann Surg* 2002; **236:** 397-406; discussion 406-407
2 **Wang X**, Andersson R, Soltesz V, Bengmark S. Bacterial translocation after major hepatectomy in patients and rats. *Arch Surg* 1992; **127:** 1101-1106
3 **Wang XD**, Parsson H, Andersson R, Soltesz V, Johansson K, Bengmark S. Bacterial translocation, intestinal ultrastructure and cell membrane permeability early after major liver resection in the rat. *Br J Surg* 1994; **81:** 579-584
4 **Anastasi A**, Erspamer V, Bucci M. Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. *Experientia* 1971; **27:** 166-167
5 **Chu KU**, Evers BM, Ishizuka J, Townsend CM Jr, Thompson JC. Role of bombesin on gut mucosal growth. *Ann Surg* 1995; **222:** 94-100
6 **Chu KU**, Higashide S, Evers BM, Ishizuka J, Townsend CM, Jr., Thompson JC. Bombesin stimulates mucosal growth in jejunal and ileal Thiry-Vella fistulas. *Ann Surg* 1995; **221:** 602-609; discussion 609-611
7 **Evers BM**, Izukura M, Townsend CM, Jr., Uchida T, Thompson JC. Differential effects of gut hormones on pancreatic and intestinal growth during administration of an elemental diet. *Ann Surg* 1990; **211:** 630-636; discussion 636-638
8 **Gulluoglu BM**, Kurtel H, Gulluoglu MG, Aktan AO, Yegen BC, Dizdaroglu F, Yalin R, Yegen BC. Bombesin ameliorates colonic damage in experimental colitis. *Dig Dis Sci* 1999; **44:** 1531-1538
9 **Alican I**, Unluer EE, Yegen C, Yegen BC. Bombesin improves burn-induced intestinal injury in the rat. *Peptides* 2000; **21:** 1265-1269
10 **Polak JM**, Sullivan SN, Bloom SR, Buchan AM, Facer P, Brown MR, Pearse AG. Specific localisation of neurotensin to the N cell in human intestine by radioimmunoassay and immunocytochemistry. *Nature* 1977; **270:** 183-184
11 **Chung DH**, Evers BM, Shimoda I, Townsend CM Jr, Rajaraman S, Thompson JC. Effect of neurotensin on gut mucosal growth in rats with jejunal and ileal Thiry-Vella fistulas. *Gastroenterology* 1992; **103:** 1254-1259
12 **Wood JG**, Hoang HD, Bussjaeger LJ, Solomon TE. Neurotensin stimulates growth of small intestine in rats. *Am J Physiol* 1988; **255:** G813-G817
13 **Evers BM**, Izukura M, Chung DH, Parekh D, Yoshinaga K, Greeley GH Jr, Uchida T, Townsend CM Jr, Thompson JC. Neurotensin stimulates growth of colonic mucosa in young and aged rats. *Gastroenterology* 1992; **103:** 86-91
14 **Feurle GE**, Muller B, Rix E. Neurotensin induces hyperplasia of the pancreas and growth of the gastric antrum in rats. *Gut* 1987; **28 Suppl:** 19-23
15 **Vagianos C**, Karatzas T, Scopa CD, Panagopoulos C, Tsoni I, Spiliopoulou I, Kalfarentzos F. Neurotensin reduces microbial translocation and improves intestinal mucosa integrity after abdominal radiation. *Eur Surg Res* 1992; **24:** 77-83
16 **Olsen PS**, Pedersen JH, Poulsen SS, Yamashita Y, Kirkegaard P. Neurotensin-like immunoreactivity after intestinal resection in the rat. *Gut* 1987; **28:** 1107-1111
17 **Assimakopoulos SF**, Scopa CD, Zervoudakis G, Mylonas PG, Georgiou C, Nikolopoulou V, Vagianos CE. Bombesin and neurotensin reduce endotoxemia, intestinal oxidative stress, and apoptosis in experimental obstructive jaundice. *Ann Surg* 2005; **241:** 159-167
18 **Higgins G**, Andersson RM. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 1931; **12:** 186-202
19 **LOWRY OH**, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193:** 265-275
20 **Giles K**, Myers M. Improved diphenylamine method for estimation of DNA. *Nature* 1965; **206:** 93-95
21 **Lee RG**, Nakamura K, Tsamandas AC, Abu-Elmagd K, Furukawa H, Hutson WR, Reyes J, Tabasco-Minguillan JS, Todo S, Demetris AJ. Pathology of human intestinal transplantation. *Gastroenterology* 1996; **110:** 1820-1834
22 **Georgiou CD**, Zervoudakis G, Tairis N, Kornaros M. beta-Carotene production and its role in sclerotial differentiation of Sclerotium rolfsii. *Fungal Genet Biol* 2001; **34:** 11-20
23 **Kakkos SK**, Kirkilesis J, Scopa CD, Arvaniti A, Alexandrides T, Vagianos CE. Nonabsorbable antibiotics reduce bacterial and endotoxin translocation in hepatectomised rats. *HPB Surg* 1997; **10:** 283-289; discussion 289-291
24 **Boermeester MA**, Houdijk AP, Meyer S, Cuesta MA, Appelmelk BJ, Wesdorp RI, Hack CE, Van Leeuwen PA. Liver failure induces a systemic inflammatory response. Prevention by recombinant N-terminal bactericidal/permeability-increasing protein. *Am J Pathol* 1995; **147:** 1428-1440
25 **Deitch EA**. The role of intestinal barrier failure and bacterial translocation in the development of systemic infection and multiple organ failure. *Arch Surg* 1990; **125:** 403-404
26 **Rodriguez Sanz MB**, Alarcon Garcia J, del Riego Tomas FJ, Vaquero Puerta C. Effects of partial hepatectomy on the distal ileum in rats. *Rev Esp Enferm Dig* 2004; **96:** 185-190
27 **Michalopoulos GK**, DeFrances MC. Liver regeneration. *Science* 1997; **276:** 60-66
28 **Pritchard DM**, Watson AJ. Apoptosis and gastrointestinal pharmacology. *Pharmacol Ther* 1996; **72:** 149-169
29 **Lee FD**. Importance of apoptosis in the histopathology of drug related lesions in the large intestine. *J Clin Pathol* 1993; **46:** 118-122
30 **Suen YK**, Fung KP, Choy YM, Lee CY, Chan CW, Kong SK. Concanavalin A induced apoptosis in murine macrophage PU5-1.8 cells through clustering of mitochondria and release of cytochrome c. *Apoptosis* 2000; **5:** 369-377
31 **Cummings MC**, Winterford CM, Walker NI. Apoptosis. *Am J Surg Pathol* 1997; **21:** 88-101
32 **Potten CS**, Merritt A, Hickman J, Hall P, Faranda A. Characterization of radiation-induced apoptosis in the small intestine and its biological implications. *Int J Radiat Biol* 1994; **65:** 71-78
33 **Bemelmans MH**, Gouma DJ, Greve JW, Buurman WA. Cytokines tumor necrosis factor and interleukin-6 in experimental biliary obstruction in mice. *Hepatology* 1992; **15:** 1132-1136
34 **Thompson CB**, Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267:** 1456-1462
35 **Deitch EA**, Sittig K, Li M, Berg R, Specian RD. Obstructive jaundice promotes bacterial translocation from the gut. *Am J Surg* 1990; **159:** 79-84
36 **Wang XD**, Soltesz V, Andersson R. Cisapride prevents enteric bacterial overgrowth and translocation by improvement of intestinal motility in rats with acute liver failure. *Eur Surg Res* 1996; **28:** 402-412
37 **Guarner C**, Runyon BA, Young S, Heck M, Sheikh MY. Intestinal bacterial overgrowth and bacterial translocation in cirrhotic rats with ascites. *J Hepatol* 1997; **26:** 1372-1378
38 **Carnovale CE**, Monti JA, Favre C, Scapini C, Carrillo MC. Is

intestinal cytosolic glutathione S-transferase an alternative detoxification pathway in two-thirds hepatectomized rats? *Life Sci* 1995; **57:** 903-910

39 **Alexandris IH**, Assimakopoulos SF, Vagianos CE, Patsoukis N, Georgiou C, Nikolopoulou V, Scopa CD. Oxidative state in intestine and liver after partial hepatectomy in rats. Effect of bombesin and neurotensin. *Clin Biochem* 2004; **37:** 350-356

40 **Chang EB**, Brown DR, Wang NS, Field M. Secretagogue-induced changes in membrane calcium permeability in chicken and chinchilla ileal mucosa. Selective inhibition by loperamide. *J Clin Invest* 1986; **78:** 281-287

41 **Seybold VS**, Treder BG, Aanonsen LM, Parsons A, Brown DR. Neurotensin binding sites in porcine jejunum: biochemical characterization and intramural localization. *Synapse* 1990; **6:** 81-90

42 **Seybold VS**, Parsons AM, Aanonsen LM, Brown DR. Characterization and autoradiographic localization of gastrin releasing peptide receptors in the porcine gut. *Peptides* 1990; **11:** 779-787

43 **Rozengurt E**, Signal transduction pathways in the mitogenic response to G protein-coupled neuropeptide receptor agonists. *J Cell Physiol* 1998; **177:** 507-517

44 **Schafer FQ**, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001; **30:** 1191-1212

45 **Heuser M**, Pfaar O, Gralla O, Grone HJ, Nustede R, Post S. Impact of gastrin-releasing peptide on intestinal microcirculation after ischemia-reperfusion in rats. *Digestion* 2000; **61:** 172-180

46 **Harper SL**, Barrowman JA, Kvietys PR, Granger DN. Effect of neurotensin on intestinal capillary permeability and blood flow. *Am J Physiol* 1984; **247:** G161-G166

47 **Robin MA**, Prabu SK, Raza H, Anandatheerthavarada HK, Avadhani NG. Phosphorylation enhances mitochondrial targeting of GSTA4-4 through increased affinity for binding to cytoplasmic Hsp70. *J Biol Chem* 2003; **278:** 18960-18970

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• *BASIC RESEARCH* •

Expression of Ki-67, p53, and K-*ras* in chronic pancreatitis and pancreatic ductal adenocarcinoma

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Abstract

AIM: To examine surgical specimens of pancreas with either chronic pancreatitis or pancreatic cancer in order to study whether ductal hyperplasia and dysplasia in pancreas represent precursor lesions for pancreatic cancer.

METHODS: We examined expression of Ki-67, CEA, p53, and K-*ras*, in the surgical specimens of pancreas with adenocarcinomas (n = 11) and chronic pancreatitis (n = 12). Cellular proliferation was assessed by Ki-67 proliferation index using the proliferation marker Ki-67. In specimens with pancreas cancer, we divided pancreas epithelium into normal (n = 7), ductal hyperplasia (n = 3), dysplasia (n = 4), and cancerous lesion (n = 11) after hematoxylin and eosin staining, Ki-67, and CEA immunohistochemical staining. In cases with chronic pancreatitis, the specimen was pathologically examined as in cases with pancreas cancer, and they were also determined as normal (n = 10), ductal hyperplasia (n = 4), or dysplasia (n = 5). p53 and K-*ras* expression were also studied by immunohistochemical staining.

RESULTS: In pancreatic cancer, the Ki-67 index was 3.73±3.58 in normal site, 6.62±4.39 in ductal hyperplasia, 13.47±4.02 in dysplasia and 37.03±10.05 in cancer tissue, respectively. Overall, p53 was positive in normal ducts, ductal hyperplasia, dysplasia, and carcinoma cells in 0 of 14 (0%), 0 of 7 (0%), 7 of 9 (78%), and 10 of 11 (91%), respectively, and K-*ras* was positive in 0 of 8 (0%), 1 of 3 (33%), 4 of 6 (67%), 4 of 5 (80%), respectively.

CONCLUSION: Our results favorably support the hypothesis that ductal hyperplasia and dysplasia of the pancreas might be precursor lesions for pancreas cancer. Further evaluation of oncogenes by the molecular study is needed.

Key words: Ki-67; p53; K-*ras*; Chronic pancreatitis; Pancreatic ductal adenocarcinoma

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INTRODUCTION

The pancreatic cancer has the poorest prognosis among various cancers with a 5-year survival rate of 3%, making it the fourth most common cause of cancer-related mortality rate[1-3]. Although the surgical resection offers the only chance for cure, only 10-30% of patients have resectable tumor[4]. Since early diagnosis followed by complete resection is the only way to the complete recovery, understanding the pathogenesis of pancreatic cancer seems to be of great value.

The ductal hyperplasia of pancreas is defined by abnormally increased number of epithelial cells of pancreatic duct. It may be detected in both normal and chronically inflamed pancreatic tissues, and its incidence is generally increased with age[5]. However, the patients with chronic pancreatitis and ductal hyperplasia have been reported to be associated with the development of pancreas cancer[5-8]. In addition, there has been an investigation suggesting three times higher incidence of papillary hyperplasia of pancreatic duct epithelium in patients with pancreatic cancer compared with the control group[6]. There is also a report that 41% of patients with pancreatic cancer showed hyperplasia of pancreatic duct epithelium when 9% of the control group have that pathological finding[7].

In an effort to search for the pathogenesis of pancreatic cancer, mutations of K-*ras*, p53, and overexpression of HER-2/neu were found in human pancreatic ductal adenocarcinoma[9].

Currently, the hypothesis of multistep carcinogenesis

that states cumulative genetic mutations in the normal pancreas might have progressed into pancreatic cancer is mostly accepted. However, detailed explanation for each step of carcinogenesis needs further investigation. In the current study, we performed histological examination of pancreas and detected normal epithelium and that with ductal hyperplasia, dysplasia, or cancerous lesions. Then we immunohistochemically determined the expression of Ki-67, K-*ras*, and p53 to identify if ductal hyperplasia and dysplasia of pancreas might be precancerous lesion.

MATERIALS AND METHODS

Subjects

The study was performed in 11 patients with pancreatic ductal adenocarcinoma and 12 patients with chronic pancreatitis, the total 23 patients went through surgical pancreatic resection at Inha University Hospital. The mean age of patients was 67 years (range 40-74 years) for pancreatic cancer and 61 years (range 37-67 years) for chronic pancreatitis. Male to female ratio was 6:5 for pancreatic cancer and 10:2 for chronic pancreatitis.

Histologic classification

The degree of pancreatic ductal lesion was classified morphologically according to Cubilla and Fitzgerald[6], and the pancreatic carcinoma according to Kloppel[10]. Most pancreatic duct epitheliums are composed of cuboidal and low columnar cells, their cytoplasm filled with mucous components. They are surrounded by loose fibrous connective tissue. Hyperplasia of pancreatic duct epithelium is divided into either flat or papillary type. However, recent studies show that there is no difference in genetic mutations between these two forms of hyperplasia[9]. Therefore, we considered these two different forms of hyperplasia in the same tissue in this study. Dysplasia was classified according to flattening, papillary hyperplasia of ductal epithelium, increased nucleus/cytoplasm ratio as the epithelium changes into atypical form, loss of nuclear polarity, morphologic form, and aggregation. A little evidence of cellular division was also considered as dysplasia. The degree of hyperplasia was evaluated objectively by counting the numbers of hyperplasia using the method of Ki-67 (Immunotec, France) immunohistochemical staining. To see whether dysplasia develops or not, we performed CEA immunohistochemical staining in the area of hyperplasia stained with Ki-67 using double staining method and classified them into different categories, so that we can minimize the errors arising from differential classification of pancreatic tissue.

Immunohistochemical staining

After 10% of surgically resected pancreatic tissue was fixed in neutral formalin, paraffin-embedded tissue was sliced to the thickness of 4 μm continuously. One slice was stained with hematoxylin-eosin (H&E), and the others were stuck to the slide with poly-L-lysine for immunohistochemical staining. After all the slices went through autoclave (120 °C, 15 lb) for 15 min to expose the antigens fixed by formalin, we treated with H_2O_2 for 30 min to eliminate intrinsic peroxidase. For immunohistochemical staining, CEA monoclonal antibody (Ab) (DAKO, Carpenteria, CA, USA) and Ki-67 monoclonal Ab (Immunotech, Marseille, Cedex, France) were diluted to 1:150 and 1:100, respectively, then incubated at room temperature for 3 h. Labeled streptavidin biotin kit (LSAB kit, DAKO) and 3',3-diaminobenzidine (DAB) were used for staining. Also p53 monoclonal Ab (Novocastra, Newcastle, UK) was diluted to 1:150 and incubated at 4 °C for one day. Then alkaline phosphatase, anti-alkaline phosphatase kit (APAAP kit, DAKO) was used for staining and fast red (DAKO) for expression. For K-*ras* staining, $p21^{ras}$ monoclonal Ab (Oncogene Research Products, Calbiochem, Germany) was diluted to 1:50. The serially sliced tissues were examined morphologically at the same area using H&E staining for histologic diagnosis, and then were compared with the results of immunohistochemical staining in response to CEA, p53, K-*ras*, and Ki-67. The following Ki-67 proliferation index was expressed as the percent of Ki-67-positive cells of 1 000 pancreatic duct epithelial cells. The double staining was performed using Ki-67 and CEA monoclonal Ab to ascertain the presence of dysplasia. Dysplasia was classified using CEA+p53 and CEA+Ki-67 staining.

RESULTS

Histology

Hematoxylin and eosin staining Normal pancreatic duct epithelium is composed of mainly cuboidal and low columnar cells and surrounded by fibrous connective tissues (Figure 1A). We detected basally located nuclei, aggregation and intraductal papillary proliferation of pancreatic duct epithelial cells in ductal hyperplasia (Figure 1C). Dysplasia of the pancreatic duct showed an

Table 1 Summary of immunohistochemical staining in each group of patients

	Case of study *n* (%)	Ki-67 index	*p*53 *n* (%)	K-*ras* *n* (%)
Overall				
Normal	17/23 (74)	2.35±2.79	0/14 (0)	0/8 (0)
Hyperplasia	7/16 (44)	9.07±5.39	0/7 (0)	1/3 (33)
Dysplasia	9/16 (56)	10.79±5.51	7/9 (78)	4/6 (67)
Carcinoma	11/23 (48)	37.03±10.05	10/11 (91)	4/5 (80)
Pancreatic cancer				
Normal	7/11 (64)	3.73±3.58	0/6 (0)	0/5 (0)
Hyperplasia	3/7 (43)	6.62±4.39	0/3 (0)	1/3 (33)
Dysplasia	4/7 (57)	13.47±4.02	4/4 (100)	2/3 (67)
Carcinoma	11/11 (100)	37.03±10.05	10/11 (91)	4/5 (80)
Chronic pancreatitis				
Normal	10/13 (77)	2.35±2.79	0/8 (0)	0/3 (0)
Hyperplasia[1]	4/9 (44)	7.66±5.58	0/4 (0)	
Dysplasia	5/9 (56)	8.64±5.51	3/5 (60)	2/3 (67)

[1]Fails to stain.

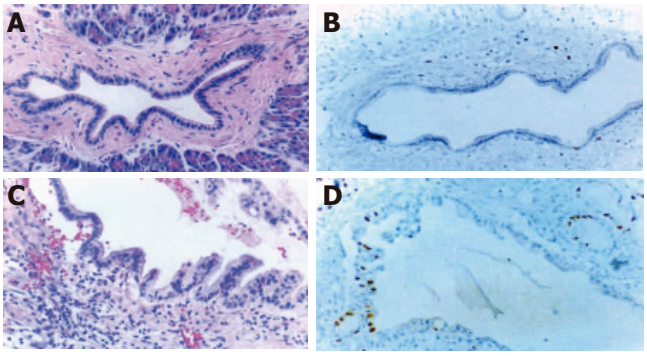

Figure 1 H&E and immunohistochemical staining of normal pancreatic and ductal hyperplasia tissues. **A:** Normal interlobular duct surrounded by fibrous tissue is illustrated. Small cuboidal cells with basally located nuclei having intact nuclear polarity are seen in the inset. H&E stain, ×100; **B:** Ki-67 monoclonal Ab does not show reaction to almost epithelial cells. DAB and hematoxylin counter stain, ×100; **C:** Pancreatic duct hyperplasia with uniformly large columnar epithelial cells, which are more than twice as long as cytoplasm of normal cells and have mucinous metaplasia of the cytoplasm. The nuclei are small and basally located without atypia. H&E stain, ×100; **D:** Ki-67 monoclonal Ab shows a few proliferating nuclei of hyperplastic ductal epithelium. DAB and hematoxylin counter stain, ×100.

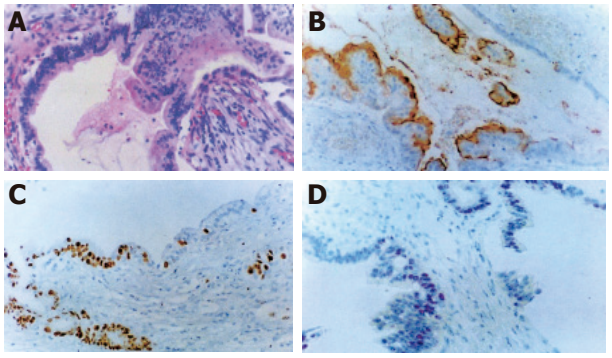

Figure 2 H&E and immunohistochemical staining of dysplastic tissues of the pancreatic duct. **A:** The nuclear enlargement, increased nuclear-to-cytoplasmic ratio, loss of nuclear polarity, pleomorphism, and nuclear overcrowding are seen. Several mitotic figures including atypical form are present. H&E stain, ×100; **B:** CEA monoclonal antibody shows positive reaction in the cytoplasmic membranes of dysplastic cells. DAB and hematoxylin counter stain, ×100; **C:** Ki-67 monoclonal antibody shows focally increased proliferating nuclei of dysplastic cells. DAB and hematoxylin counter stain, ×100; **D:** p53 monoclonal antibody shows focally positive reaction in the nuclei of dysplastic cells. AEC and hematoxylin counter stain, × 100.

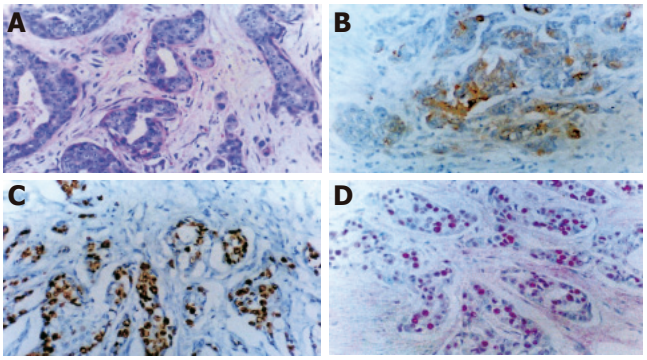

Figure 3 H&E and immunohistochemical staining of pancreatic cancer. **A**: Moderate to poorly differentiated pancreatic adenocarcinoma with desmoplastic background. H&E stain, ×200; **B**: Immunohistochemical staining using CEA monoclonal antibody shows positive reaction in the cytoplasms of tumor cells. DAB and hematoxylin counter stain, ×200; **C**: Ki-67 monoclonal antibody shows significantly increased proliferation of nuclei of tumor cells. DAB and hematoxylin counter stain, ×200; **D**: Intense nuclear immunohistochemical staining of p53 in an invasive pancreatic adenocarcinoma. AEC and hematoxylin counter stain, ×200.

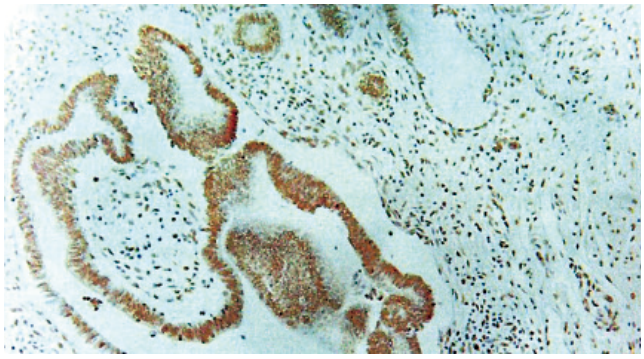

Figure 4 Cytoplasmic staining with K-*ras* is illustrated in both ductal hyperplasia and adjacent invasive adenocarcinoma. K-*ras* immunohistochemical stain, ×200.

increased nuclear to cytoplasm ratio and loss of nuclear polarity (Figure 2A). The tissue of pancreatic duct showed numerous cell divisions, an increased nuclear-to-cytoplasm ratio, and loss of cellular polarity (Figure 3A).

Immunohistochemical staining Normal pancreatic duct showed negative reaction to Ki-67, CEA, p53, K-*ras* staining (Figure 1B), whereas in hyperplasia, the nuclei were stained by Ki-67 (Figure 1D), cellular membrane by CEA and cytoplasm by K-*ras*, respectively. In dysplasia, the nuclei were stained by Ki-67, p53, and cell membrane by CEA respectively (Figures 2B-D). Focal, weak positive reaction to CEA was also detected in the cytoplasm. In pancreatic cancer, the nuclei were stained by Ki-67 and p53, whereas both cell membrane and cytoplasm showed strong positive reaction to CEA (Figures 3B-D). K-*ras* showed negative reaction to K-*ras* (Table 1, Figure 4).

Ki-67 proliferation index

Pancreatic cancer tissue Ki-67 proliferation index of pancreatic cancer tissue based on the histologic classification was 3.73±3.58, 6.62±4.39, 13.47±4.02, and 37.03±10.05 in normal, ductal hyperplasia, dysplasia, and cancer site respectively.

Chronic pancreatitis tissue Ki-67 proliferation index based on the histologic classification in chronic pancreatitis tissue was 2.35±2.79, 7.66±5.78, and 8.64±5.51 in normal, ductal hyperplasia, and dysplasia, respectively.

Results based on random histologic classification Ki-67 proliferation index based on random histologic classification was 2.35±2.79, 9.07±5.39, 10.79±5.51, and 37.03±10.05 in normal, hyperplasia, dysplasia, and cancer area, respectively.

Expression of p53 protein

In all 11 pancreatic cancer tissues, the expression of p53 protein was observed in all dysplasia (4/4), but not in all normal sites. The expression of p53 protein was also observed in 91% of cancer area that involved lesions.

In 12 samples of all chronic pancreatitis tissues, the expression of p53 protein was negative in both normal and hyperplasia sites, whereas positive in 60% of dysplasia site. Overall, the expression of p53 protein was negative in all normal sites and pancreatic epithelial hyperplasia, whereas positive in 78% of dysplasia and 91% of cancer sites.

Expression of K-ras protein

The positive reaction to K-*ras* protein seen in cytoplasm was observed in 33% of hyperplasia sites and 67% of dysplasia sites, but not in normal tissue in the pancreatic cancer patients. The positive staining to K-*ras* was in 80% of cancer tissue. The character and intensity in staining was not proportional to the differentiation of cancer. In chronic pancreatitis, K-*ras* was expressed in 67% of dysplasia, but not in normal sites. Overall, K-*ras* was expressed in 33% of pancreatic duct hyperplasia, 67% of dysplasia and 80% of cancerous sites, but no expression of K-*ras* could be detected in any normal sites.

DISCUSSION

It is generally accepted that three to seven accumulated mutations might be required for a normal cell to transform into a neoplastic cell[11]. A famous example of multistep development of malignancy has been reported in colorectal carcinoma[12], and similar multiple mutational steps seem to be associated in the development of pancreatic cancer[13]. Chronic pancreatitis is accepted as a precancerous lesion of pancreatic cancer, although a detailed mechanism regarding this is yet to be delineated. Several possible mechanisms have been proposed. There has been a study suggesting that ductal hyperplasia might play an important role[6,8]. Unfortunately, this hypothesis is hard to be tested since ductal hyperplasia could not be objectively determined on general means of pathological examination. Moreover, morphologically and functionally different kinds of pancreatic tissue tend to be mixed together which makes statistical analysis difficult. All these problems are partly due to the ambiguous pathologic terms and definitions; for example, the pancreatic duct hyperplasia, dysplasia, atypical ductal hyperplasia, intraductal tumor, carcinoma *in situ*, adenomatous hyperplasia, and atypical adenomatous hyperplasia[7,14].

In the present study, we only used the term, hyperplasia and dysplasia, since there are similar lesions, morphologically and cytologically, in other parts of the gastrointestinal tract. In order to objectively determine pancreatic duct hyperplasia, we used Ki-67 proliferation index. In evaluation of atypical hyperplasia, we used CEA immunohistochemical study.

The p53 gene is a tumor suppressor gene that involves regulation of the cell cycle and its mutation is most common in human cancers[15]. Mutated p53 in tumor cell accumulates nuclear p53 proteins and this overexpression is used as a marker for immunohistochemical staining[15,18]. It is reported that mutation of p53 has been reported to be detected in at least 50-70% of pancreatic adenocarcinoma[15-17]. In this study, p53 overexpression was most frequently shown in the cancerous lesion, rarely in the dysplastic cells and not at all in hyperplastic and normal cells. Therefore, it seems that the overexpression of p53 is implied to be the last phenomenon in the tumorigenesis. The fact that overexpression of p53 is shown more frequently in cancer tissues, regardless of the cell differentiation, suggests that it would be a useful marker in detecting activated dysplasia from well-differentiated cancer.

$P21^{K\text{-}ras}$ protein, synthesized by K-*ras* gene, accumulates in the cytoplasm[12], and found to be strongly stained in cytoplasm of most of the cancer cells[19]. The pancreas cancer originated from the exocrine gland showed 75-95% incidence of K-*ras* activation[20-24]. High incidence of K-*ras* mutation in pancreatic cancer implicates that this mutation might be a basic step of carcinogenesis. However, the earliest pancreatic lesion in which K-*ras* mutation can be found is not clearly known. As for the reports on the detection of K-*ras* mutation in the hyperplastic pancreatic duct of chronic pancreatitis patients, there have been some discrepancies. The current study immunohistochemically examined oncogene protein expressions in various pancreatic lesions. Although the presence of mutated gene needs to be confirmed at the molecular level, we found the possible continuity between pancreas diseases through protein expressions of oncogene. K-*ras* was strongly and frequently expressed in dysplastic and cancerous tissues compared with that of hyperplastic tissue. These results indicate that the overexpression of K-*ras* is associated with the pancreatic duct dysplasia in the carcinogenesis. Results in chronic pancreatitis were also similar to pancreatic cancer, suggesting that ductal dysplasia may be the precancerous lesion.

In our study, we found the possibility of the existence of stepwise mutation in the progression of advanced cancer from normal pancreatic cell. First, in the pancreatic cancer tissues, we found tissues presumed to be precancerous lesion, histologically different from normal epithelial cells. Second, this premalignant lesion was found more frequently in the tissues of pancreatic cancer compared with that of other disease states. Third, in the dysplastic and cancer tissues, the excessive expression of oncogene product was shown more frequently than in normal tissues. Fourth, K-*ras* protein overexpression developed in earlier stage of carcinogenesis, while the overexpression of p53 protein mostly occurred in advanced steps such as dysplasia or cancer. Finally, in chronic pancreatitis, some hyperplastic and dysplastic tissues showed the overexpression of oncogenic proteins similar to pancreatic cancer, although at low percentage. Therefore, chronic pancreatitis patients with protein overexpression of oncogene would have higher risk of cancer. Consequently, these results suggest that there is a correlation between the protein overexpression by genetic alteration and histological changes. Although we investigated mutation of only two important genes using low-sensitive immunohistochemical staining method, we believe that this study would be useful in the future

researches on the pathogenesis of pancreatic cancer.

REFERENCES

1 **Warshaw AL,** Fernandez-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465
2 **Boyle P,** Hsieh CC, Maisonneuve P, La Vecchia C, Macfarlane GJ, Walker AM, Trichopoulos D. Epidemiology of pancreas cancer (1988). *Int J Pancreatol* 1989; **5**: 327-346
3 **Wingo PA,** Tong T, Bolden S. Cancer statistics, 1995. *CA Cancer J Clin* 1995; **45:** 8-30
4 **Crist DW,** Cameron JL. The current status of the Whipple operation for periampullary carcinoma. *Adv Surg* 1992; **25**: 21-49
5 **Lowenfels AB,** Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andren-Sandberg A, Domellof L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med* 1993; **328**: 1433-1437
6 **Cubilla AL,** Fitzgerald PJ. Morphological lesions associated with human primary invasive nonendocrine pancreas cancer. *Cancer Res* 1976; **36**: 2690-2698
7 **Kozuka S,** Sassa R, Taki T, Masamoto K, Nagasawa S, Saga S, Hasegawa K, Takeuchi M. Relation of pancreatic duct hyperplasia to carcinoma. *Cancer* 1979; 43: 1418-1428
8 **SOMMERS SC,** MURPHY SA, WARREN S. Pancreatic duct hyperplasia and cancer. *Gastroenterology* 1954; **27:** 629-640
9 **Apple SK,** Hecht JR, Lewin DN, Jahromi SA, Grody WW, Nieberg RK. Immunohistochemical evaluation of K-*ras*, p53, and HER-2/neu expression in hyperplastic, dysplastic, and carcinomatous lesions of the pancreas: evidence for multistep carcinogenesis. *Hum Pathol* 1999; **30**: 123-129
10 **Kloppel G.** Pancreatic Pathology. Edinburg, Scotland: *Churchill Livingstone,* 1984: 79-113
11 **Fearon ER,** Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767
12 **Vogelstein B,** Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; **319**: 525-532
13 **Chen J,** Baithun SI, Ramsay MA. Histogenesis of pancreatic carcinomas: a study based on 248 cases. *J Pathol* 1985; **146**: 65-76
14 **Kloppel G.** The pancreas: biology, pathobiology, and disease. 2nd ed. New York: *Raven Press,* 1993: 871-898
15 **Greenblatt MS,** Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54**: 4855-4878
16 **DiGiuseppe JA,** Hruban RH, Goodman SN, Polak M, van den Berg FM, Allison DC, Cameron JL, Offerhaus GJ. Overexpression of p53 protein in adenocarcinoma of the pancreas. *Am J Clin Pathol* 1994; **101**: 684-688
17 **Pellegata NS,** Sessa F, Renault B, Bonato M, Leone BE, Solcia E, Ranzani GN. K-*ras* and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. *Cancer Res* 1994; **54**: 1556-1560
18 **Yokoyama M,** Yamanaka Y, Friess H, Buchler M, Korc M. p53 expression in human pancreatic cancer correlates with enhanced biological aggressiveness. *Anticancer Res* 1994; **14**: 2477-2483
19 **Sakorafas GH,** Lazaris A, Tsiotou AG, Koullias G, Glinatsis MT, Golematis BC. Oncogenes in cancer of the pancreas. *Eur J Surg Oncol* 1995; **21**: 251-253
20 **Berrozpe G,** Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the p53 and K-*ras* genes in pancreatic cancer. *Int J Cancer* 1994; **58**: 185-191
21 **Almoguera C,** Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell* 1988; **53**: 549-554
22 **Motojima K,** Tsunoda T, Kanematsu T, Nagata Y, Urano T, Shiku H. Distinguishing pancreatic carcinoma from other periampullary carcinomas by analysis of mutations in the Kirsten-*ras* oncogene. *Ann Surg* 1991; **214**: 657-662
23 **Motojima K,** Urano T, Nagata Y, Shiku H, Tsurifune T, Kanematsu T. Detection of point mutations in the Kirsten-*ras* oncogene provides evidence for the multicentricity of pancreatic carcinoma. *Ann Surg* 1993; **217**: 138-143
24 **Tabata T,** Fujimori T, Maeda S, Yamamoto M, Saitoh Y. The role of *Ras* mutation in pancreatic cancer, precancerous lesions, and chronic pancreatitis. *Int J Pancreatol* 1993; **14**: 237-244

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• *BASIC RESEARCH* •

Effect of IL-4 on altered expression of complement activation regulators in rat pancreatic cells during severe acute pancreatitis

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Abstract

AIM: To investigate the effect of IL-4 on the altered expression of complement activation regulators in pancreas and pancreatic necrosis during experimental severe acute pancreatitis (SAP).

METHODS: SAP model of rats was established by retrograde injection of 5% sodium taurocholate (1 mL/kg) into the pancreatic duct. We immunohistochemically assayed the expression of three complement activation regulators: decay accelerating factor (DAF; CD55), 20 ku homologous restriction factor (HRF20; CD59) and membrane cofactor protein (MCP; CD46), in the pancreatic acinar cells of rats at 0, 3, 6, 12, and 24 h after the induction of SAP model. Meanwhile the levels of amylase and lipase were determined, and morphological examination was performed. Then, 61 rats were randomly divided into three groups. Group A (n = 21) received no treatment after the SAP model was established; group B (n = 20) was given IL-4 (8 μg/animal) intraperitoneally 0.5 h before the SAP model was established; group C (n = 20) was given IL-4 (8 μg/animal) intraperitoneally 0.5 h after the SAP model was established. Plasma amylase and lipase, extent of pancreatic necrosis and expression of complement activation regulators were investigated 6 h after the induction of SAP model.

RESULTS: Three complement activation regulators were all expressed in pancreatic acinar cells. MCP was not found on the basolateral surface as reported. Contrary to the gradually increasing plasma level of amylase and lipase, expression of complement activation regulators decreased after SAP model was set up. At the same time, the severity of pancreatic necrosis was enhanced. A strong negative correlation was found between the expression of MCP, DAF, CD59 in pancreatic acinar cells and the severity of pancreatic necrosis (r = –0.748, –0.827, –0.723; P<0.01). In the second series of experiments, no matter when the treatment of IL-4 was given (before or after the induction of SAP model), the serum level of amylase or lipase was decreased and the extent of pancreatic necrosis was ameliorated significantly. Compared to SAP control group, the expression of DAF and CD59 in pancreas was reinforced when IL-4 was given before the induction of SAP model (P<0.01, P<0.05), but the expression of MCP was not influenced (P>0.05). The expression of DAF was enhanced, when IL-4 was given after the induction of SAP model (P<0.05), but the expression of CD59 and MCP did not change (P>0.05).

CONCLUSION: Complement activation regulators may participate in the pathogenesis of pancreatic inflammation. Downregulation of complement activation regulators expression may be one of the causes of pancreatic necrosis. IL-4 treatment may control SAP aggravation by enhancing expression of DAF and CD59 in pancreas and decreasing pancreatic necrosis. Moreover, DAF and CD59 may play an important role in the regulation of complement activation regulators during SAP.

Key words: Severe acute pancreatitis; Complement activation regulators; Interleukin-4

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INTRODUCTION

During activation of complement, fragments of complement proteins may be deposited on non-target cells[1], thereby causing tissue injury. In order to prevent this, several proteins on cell membranes inhibit the activation of the complement cascade[2]. These proteins include decay accelerating factor (DAF; CD55) which inhibits the formation of C3 and C5 convertases and promotes their catabolism[3,4], 20 ku homologous restriction factor (HRF20; CD59) which inhibits the formation

of terminal complement complexes by preventing the polymerization of C9 on C5b-8[5,6], and membrane cofactor protein (MCP; CD46) which functions as a cofactor in the factor I-mediated inactivation of C3b and C4b[7]. These proteins, which are widely distributed in normal tissues[8-10], are thought to protect host tissues from autologous complement-mediated damage.

Expression of these three proteins has been described in the gastrointestinal tract of human beings[11-14]. In normal colonic and gastric mucosa, DAF and CD59 have been found only on the apical surface of epithelial cells, whereas MCP is localized on the basolateral surface of these cells[11,13-15]. In patients with ulcerative colitis (UC) and chronic gastritis, the expression of DAF in colonic and gastric epithelial cells was markedly enhanced and was proportional to the severity of mucosal inflammation[14,15]. Expression of these complement activation regulators has not been assayed in more severe types of inflammation. Being one of the main causes of pancreatic necrosis, activation of complement in the process of SAP has been proved by other authors[16,17]. We therefore examined the distribution of DAF, CD59, and MCP in pancreatic acinar cells and the relationship between the expression of these proteins and the severity of pancreatic necrosis during SAP. In addition, we investigated the therapeutic effects of IL-4 on the expression of complement activation regulators and pancreatic necrosis in order to clarify the role of IL-4 and three membrane inhibitors of complement during SAP.

MATERIALS AND METHODS

Animals and model of severe acute pancreatitis

Male Wistar rats (250-300 g) were used in this study. Animals were allowed to have free access to water before the experiment. Anesthesia was induced with an intraperitoneal injection of 6% sodium pentobarbital (Northeast Drug Manufactory, China) (0.1 mL/100 g body weight). After midline laparotomy and transduodenal cannulation of the pancreatic duct, the hepatic duct was closed by a small bulldog clamp, and then 5% sodium taurocholate (Sigma, USA) in saline was infused at a rate of 0.1 mL/min. Control animals received an infusion of 0.9% saline solution.

Experimental design

In the first series of experiments, animals were killed at 3, 6, 12, and 24 h after the induction of pancreatitis (n = 10 per group). Pancreatic tissues were removed and processed as indicated below.

In the second series of experiments, to test the effect of IL-4 on complement activation regulators, 61 rats were randomly divided into three groups. Group A (n = 21) received no treatment after the SAP model was established, group B (n = 20) was given IL-4 (Peprotech, UK) (8 μg/animal)[18-20] intraperitoneally 0.5 h before the SAP model was established, group C (n = 20) was given IL-4 (8 μg/animal) intraperitoneally 0.5 h after the SAP model was established. Animals were killed at 6 h after the induction of pancreatitis model.

Pancreatic tissue was immediately frozen in liquid N_2 and embedded in OCT embedding medium. All blood samples were centrifuged at 3 000 r/min for 5 min. Supernatant was stored at -70 ℃.

The serum levels of amylase and lipase were determined by a colorimetric kinetic method and nephelometry method respectively.

Tissue immunohistochemistry staining

Cryostat sections were serially cut at 6 μm, and fixed in acetone for 10 min. After being washed with phosphate-buffered saline (PBS), the sections were treated with 30 mL/L H_2O_2 for 15 min at room temperature to inactivate endogenous peroxidase, and then with a 1:10 dilution of non-immune goat serum for 20 min to block non-specific immunoglobulin binding sites. After the excess serum was blotted, the sections were incubated with each of the primary antibodies (dilution 1:150 in PBS) [MCP (sc-7056), DAF (sc-9156), CD59 (sc-9157), Santa Cruz, USA] for 2 h at room temperature, control sections were incubated with PBS. Unbound antibody was washed from the tissue thrice in PBS for 5 min, incubated with the secondary biotin-labeled goat anti-mouse IgG antibody for 20 min, and then washed thrice for 5 min with PBS. Subsequently, the sections were incubated with peroxidase-labeled streptavidin-biotin for 20 min (ABC kit; Boster, Wuhan, China). After being washed with PBS for four times, the sections were stained with 0.02% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (Boster, Wuhan, China). The sections were counterstained with hematoxylin, washed with PBS, dehydrated in graded concentrations of ethanol, and mounted.

Vascular endothelial cells were used as a positive control for DAF, MCP, and CD59. Expression of complement activation regulators in pancreatic acinar cells was scored on a scale of (-) to (+3)[15], where (-) denotes faint or no staining; (1+) specific staining in 10-50% of cells; (2+) specific staining of 50-90% of the cells; (3+) specific staining of 90-100% of cells.

Evaluation of pancreatitis severity

The extent of pancreatic acinar cell necrosis was quantitated morphometrically by an observer who was not aware of the sample identity, as described by Kusske *et al.* [21]. For these studies, cryostat sections were stained with hematoxylin and eosin. Ten randomly chosen microscopic fields (×200) were examined for each tissue sample, and the extent of acinar cell injury/necrosis was expressed as a percentage of total acinar tissue.

Statistical analysis

Data were presented as mean±SE. Differences between groups were compared using analysis of variance followed by Student's t-test. Correlation between the expression of complement activation regulators and pancreatic necrosis was analyzed using Spearman's rank correlation test. Correlation between IL-4 intervention and the expression of complement membrane inhibitors was analyzed using

Table 1 Serum amylase and lipase activities (u/L) and histological scores of pancreatic necrosis in the first series of experiment (mean±SE)

	Control group	Pancreatitis group			
		3 h	6 h	12 h	24 h
Serum amylase	1360±259	4715±1 120[b]	7527±1 595[b]	13321±3 848[b]	13686±4 175[b]
Serum lipase	2.52±0.35	11.09±2.93[b]	31.17±5.66[b]	40.39±9.04[b]	38.06±9.67[b]
Scores of pancreatic necrosis	0.00±0.00	0.30±0.48[b]	1.40±0.70[b]	2.70±0.48[b]	2.80±0.79[b]

[b]P<0.01 *vs* control.

Table 3 Serum amylase and lipase activities (u/L) and histological scores of pancreatic necrosis in the second series of experiment (mean±SE)

Group	n	Serum amylase	Serum lipase	Scores of pancreatic necrosis
A	21	7 393±1 433	32.15±5.42	1.52±0.75
B	20	4 482±1 224[a]	11.75±4.42[a]	0.45±0.69[a]
C	20	5 947±1 503[a]	22.28±7.10[a]	0.95±0.76[a]

[a]P<0.05 *vs* group A.

the χ^2-test. P<0.05 was considered statistically significant.

RESULTS

Serum levels of amylase and lipase

Tables 1 and 3 summarize the changes of serum amylase and lipase activities in experiments 1 and 2.

Evaluation of morphology

In the first series of experiments, the morphological changes varied with the time elapsed after sodium taurocholate injection. By gross examination, the ductal tree became hemorrhagic immediately after injection, and the whole pancreas became edematous and brownish red in color within the next 5-10 min. As time elapsed, edema, hemorrhages, acinar cell necrosis (Table 1), amount of ascitic fluid and inflammatory cell infiltration aggravated gradually. At 24 h, the color of pancreas was pale and slightly yellowish. Many animals had marked distension of the stomach.

In the second series of experiments, pancreatic necrosis was significantly reduced, when IL-4 was administered either prophylactically or therapeutically (P<0.01), (Table 3).

Expression of complement activation regulators

At the microscopic level, we observed the expression of DAF, CD59, and MCP on the luminal surface of pancreatic acinar cells. We did not find positive staining of MCP on the basolateral surface of pancreatic acinar cells, though staining for MCP was positive on the basolateral surface of gastric and colonic epithelial cells[14,15]. Although DAF, CD59, and MCP were well recognized as membrane binding proteins, immunostaining of these proteins was also detected in the cytoplasm[15]. No staining was observed when samples were treated with control PBS. Expression of complement activation regulators decreased gradually after SAP model was set up. We also examined the relationship between the expression of three complement inhibitors and the pancreatic necrosis. We found a strong negative correlation between the expression of MCP, DAF, and CD59 in pancreatic acinar cells and the severity of pancreatic necrosis (r = -0.748, -0.827, and -0.723; P<0.01), (Table 2). In the second series of experiments, compared to SAP control group, the expression of DAF and CD59 in pancreas was reinforced when IL-4 was given before the induction of SAP model (P<0.01, P<0.05), but the expression of MCP was not influenced (P>0.05). The expression of DAF was enhanced when IL-4 was given after the induction of SAP model (P<0.05), but the expression of CD59 and MCP did not change (P>0.05) (Table 4).

Table 2 Correlation between expression of DAF, CD59, MCP, and pancreatic necrosis

		Histological scores of pancreatic necrosis				
		0	1	2	3	4
MCP	–	1	5	6	12	2
	+	8	3	3	0	0
	++	6	2	0	0	0
	+++	2	0	0	0	0
DAF	–	0	6	7	12	2
	+	6	4	2	0	0
	++	10	0	0	0	0
	+++	1	0	0	0	0
CD59	–	2	6	6	12	2
	+	5	3	3	0	0
	++	7	1	0	0	0
	+++	3	0	0	0	0

Table 4 Effects of IL-4 on expression of MCP, DAF, and CD59 in pancreas during SAP

Group	n	Expression intensity of DAF			Expression intensity of CD59			Expression intensity of MCP		
		–	+	++	–	+	++	–	+	++
A	21	12	7	2	12	6	3	11	6	4
B	20	2	7	11[b]	3	7	10[a]	5	8	7[c]
C	20	4	8	8[a]	9	6	5[c]	7	7	6[c]

[a]P<0.05, [b]P<0.01, [c]P>0.05 *vs* group A.

DISCUSSION

Severe acute pancreatitis is a common acute abdominal disorder. Since angiorrhexis and hemorrhage in pancreas are frequent during severe acute pancreatitis, and the complement system is activated by trypsin[16,17], it is possible that inflammation may result in constant exposure of pancreatic acinar cells to activated complement

fragments in the blood. Then, severe pancreatic necrosis is inevitable. Being the membrane inhibitors, complement activation regulators in the acinar cells may be important for local defense system during SAP. We investigated the role of RCA in the process of SAP and then attempted to attenuate inflammatory reaction by regulating the expression of complement activation regulators.

It has been reported that expression of DAF on gastric epithelial cells and colonic epithelial cells is strongly enhanced during gastritis and ulcerative colitis, and a strong positive correlation between DAF expression and degree of inflammation (extent of inflammatory cell infiltration) has been found[14,15]. At the same time, other researches revealed that expression of MCP and CD59 in alveolar epithelial cells is conspicuously enhanced during acute lung injury, but is greatly decreased during acute respiratory distress syndrome[22]. These results suggest that complement activation regulators play a different role in these inflammatory diseases.

We utilized immunohistochemical methods to assay the expression of DAF, CD59, and MCP in normal and pathological pancreatic specimens. We found that MCP, DAF, and CD59, which are glycosyl phosphatidylinositol (GPI) anchored membrane proteins, were present on the apical side of pancreatic acinar cells[23], whereas MCP, a transmembrane protein, was not distributed in these cells as in epithelial cells of the colonic and gastric mucosa[14,15]. While complement activation regulators were slightly or moderately expressed in the acinar cells of normal pancreatic tissue, their expression in pancreatic acinar cells decreased gradually after the model of SAP was induced. These phenomena are similar to those in ARDS[22]. Although there are differences in the inflammatory process between pancreatic and pulmonary epithelial cells, the lower expression of complement activation regulators detected in the inflammatory cells suggests that complement activation regulators play an important role in the regulation of complement activation in epithelia during severe inflammation. In this study, a strong negative correlation was observed between the expression of complement activation regulators and inflammation (pancreatic necrosis). It is not clear, which of MCP, DAF, and CD59 is the most important factor in the regulation of complement activation during SAP. Although the precise mechanism by which the repression of complement activation regulators is regulated remains to be elucidated, it is clear that these proteins are important in the regulation of the inflammatory process of the pancreas.

In general, the expression of membrane inhibitors of complement is mediated by various cytokines[24-27]. Being an anti-inflammatory mediator, IL-4 has been proven to enhance the expression of DAF in small intestine epithelial cells and vascular endothelial cells *in vitro*[24-28]. We applied IL-4 to rats with severe acute pancreatitis to testify its therapeutic effects on the expression of complement activation regulators and pancreatic necrosis *in vivo*. We found that the expression of DAF and CD59 was upregulated when IL-4 was administered prophylactically (given before the model was established), but the expression of MCP did not change significantly. When IL-4 was administered therapeutically (given after the model was established), only DAF was upregulated. Compared to control group, the serum amylase and lipase activities and the extent of pancreatic necrosis were significantly reduced when IL-4 was administered either prophylactically or therapeutically. These findings suggest that activation of complement during severe acute pancreatitis may be mediated primarily by DAF and CD59, and IL-4 treatment may be a valuable strategy for severe acute pancreatitis. Considering the therapeutic value of IL-4, some other biological activities of IL-4 should be mentioned. It has been reported that IL-4 displays its anti-inflammatory feature by inhibiting the activation and accumulation of macrophages[29], preventing the production of TNF-α and IL-1β[30], upregulating the expression of IL-1 receptor antagonist[31], stimulating 15-lipoxidase activity of macrophages[32]. These functions are important to control systemic inflammation reaction that is common during SAP.

In conclusion, complement activation regulators may play an important role in the regulation of complement activation, and downregulation of complement activation regulators may be one of the causes of pancreatic necrosis. IL-4 treatment provides a new therapeutic strategy for controlling systemic inflammatory reaction during severe acute pancreatitis.

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REFERENCES

1 **Davitz MA.** Decay-accelerating factor (DAF): a review of its function and structure. *Acta Med Scand Suppl* 1987; **715:** 111-121
2 **Kinoshita T**. Biology of complement: the overture. *Immunol Today* 1991; **12:** 291-295.
3 **Nicholson-Weller A,** Burge J, Fearon DT, Weller PF, Austen KF. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol* 1982; **129:** 184-189
4 **Fujita T, Inoue T,** Ogawa K, Iida K, Tamura N. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med* 1987; **166:** 1221-1228
5 **Okada N,** Harada R, Fujita T, Okada H. A novel membrane glycoprotein capable of inhibiting membrane attack by homologous complement. *Int Immunol* 1989; **1:** 205-208
6 **Rollins SA, Sims PJ**. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J Immunol* 1990; **144:** 3478-3483.
7 **Seya T**, Turner JR, Atkinson JP. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J Exp Med* 1986; **163:** 837-855.
8 **Nose M,** Katoh M, Okada N, Kyogoku M, Okada H. Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression on endothelial cells in vivo. *Immunology* 1990; **70:** 145-149
9 **McNearney T,** Ballard L, Seya T, Atkinson JP. Membrane

cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells. *J Clin Invest* 1989; **84:** 538-545

10 **Medof ME,** Walter EI, Rutgers JL, Knowles DM, Nussenzweig V. Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J Exp Med* 1987; **165:** 848-864

11 **Koretz K,** Bruderlein S, Henne C, Moller P. **Decay-accelerating** factor (DAF, CD55) in normal colorectal mucosa, adenomas and carcinomas. *Br J Cancer* 1992; **66:** 810-814

12 **Koretz K,** Bruderlein S, Henne C, Moller P. **Expression of** CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium. *Br J Cancer* 1993; **68:** 926-931.

13 **Inoue H,** Mizuno M, Uesu T, Ueki T, Tsuji T. Distribution of complement regulatory proteins, decay-accelerating factor, CD59/homologous restriction factor 20 and membrane cofactor protein in human colorectal adenoma and cancer. *Acta Med Okayama* 1994; **48:** 271-777

14 **Uesu T,** Mizuno M, Inoue H, Tomoda J, Tsuji T. Enhanced expression of decay accelerating factor and CD59/homologous restriction factor 20 on the colonic epithelium of ulcerative colitis. *Lab Invest* 1995; **72:** 587-591

15 **Sasaki M,** Joh T, Tada T, Okada N, Yokoyama Y, Itoh M. Altered expression of membrane inhibitors of complement in human gastric epithelium during Helicobacter-associated gastritis. *Histopathology* 1998; **33:** 554-560

16 **Roxvall L,** Bengtson A, Sennerby L, Heideman M. Activation of the complement cascade by trypsin. *Biol Chem Hoppe Seyler* 1991; **372:** 273-278

17 **Bhatia M,** Saluja AK, Singh VP, Frossard JL, Lee HS, Bhagat L, Gerard C, Steer ML. Complement factor C5a exerts an anti-inflammatory effect in acute pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol* 2001; **280:** G974-978

18 **Tam FW,** Smith J, Karkar AM, Pusey CD, Rees AJ. Interleukin-4 ameliorates experimental glomerulonephritis and up-regulates glomerular gene expression of IL-1 decoy receptor. *Kidney Int* 1997; **52:** 1224-31

19 **He XY,** Chen J, Verma N, Plain K, Tran G, Hall BM. Treatment with interleukin-4 prolongs allogeneic neonatal heart graft survival by inducing T helper 2 responses. *Transplantation* 1998; **65:** 1145-1152

20 **Erwig LP,** Stewart K, Rees AJ. Macrophages from inflamed but not normal glomeruli are unresponsive to anti-inflammatory cytokines. *Am J Pathol* 2000; **156:** 295-301

21 **Kusske AM,** Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. *Surgery* 1996; **120:** 284-289

22 **Yang Kang,** LIU Wei-Yong. **CD59 and CD46 expression in** lungs of cats with traumatic ALI or ARDS. *Disi Junyi Daxue Xuebao* 2000; **21**: 624-626.

23 **Johnstone RW**, Loveland BE, McKenzie IF. Identification and quantification of complement regulator CD46 on normal human tissues. *Immunology* 1993; **79**: 341-347

24 **Moutabarrik A,** Nakanishi I, Namiki M, Hara T, Matsumoto M, Ishibashi M, Okuyama A, Zaid D, Seya T. Cytokine-mediated regulation of the surface expression of complement regulatory proteins, CD46(MCP), CD55(DAF), and CD59 on human vascular endothelial cells. *Lymphokine Cytokine Res* 1993; **12:** 167-172

25 **Mason JC,** Lidington EA, Yarwood H, Lublin DM, Haskard DO. Induction of endothelial cell decay-accelerating factor by vascular endothelial growth factor: a mechanism for cytoprotection against complement-mediated injury during inflammatory angiogenesis. *Arthritis Rheum* 2001; **44:** 138-150

26 **Spiller OB,** Criado-Garcia O, Rodriguez De Cordoba S, Morgan BP. Cytokine-mediated up-regulation of CD55 and CD59 protects human hepatoma cells from complement attack. *Clin Exp Immunol* 2000; **121:** 234-241

27 **Bjorge L**, Jensen TS, Matre R. Characterisation of the complement-regulatory proteins decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) on a human colonic adenocarcinoma cell line. *Cancer Immunol Immunother* 1996; **42:** 185-192.

28 **Andoh A,** Fujiyama Y, Sumiyoshi K, Sakumoto H, Bamba T. Interleukin 4 acts as an inducer of decay-accelerating factor gene expression in human intestinal epithelial cells. *Gastroenterology* 1996; **111:** 911-918

29 **Cook HT,** Singh SJ, Wembridge DE, Smith J, Tam FW, Pusey CD. Interleukin-4 ameliorates crescentic glomerulonephritis in Wistar Kyoto rats. *Kidney Int* 1999; **55:** 1319-1326

30 **Hart PH**, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E_2. *Proc Natl Acad Sci U S A* 1989; **86**: 3803-3807

31 **Vannier E**, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. *Proc Natl Acad Sci U S A* 1992; **89**: 4076-4080

32 **Katoh T,** Lakkis FG, Makita N, Badr KF. Co-regulated expression of glomerular 12/15-lipoxygenase and interleukin-4 mRNAs in rat nephrotoxic nephritis. *Kidney Int* 1994; **46**: 341-349

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• *BASIC RESEARCH* •

Effects of nuclear factor-kappaB on rat hepatocyte regeneration and apoptosis after 70% portal branch ligation

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Abstract

AIM: To detect the DNA binding activity of nuclear factor-kappaB (NF-κB) in rat hepatocyte and to investigate the effects of NF-κB on rat hepatocyte regeneration and apoptosis after 70% portal branch ligation.

METHODS: Sixty Wistar rats were randomly divided into control group and portal branch ligation group. The animals were killed 12 h, 1, 2, 3, 7, and 14 d after surgery to determine the contents of plasma ALT. Hepatocytes were isolated and nuclear protein was extracted. DNA binding activity of NF-κB was measured by EMSA. Hepatocyte regeneration and apoptosis were observed under microscope by TUNEL staining. The ultrastructural changes of liver were observed under electron microscope.

RESULTS: Seventy percent portal branch ligation produced atrophy of the ligated lobes and the perfused lobes underwent compensatory regeneration, the total liver weight and plasma ALT levels were maintained at the level of sham-operated animals throughout the experiment. After 2 d of portal branch ligation, DNA binding activity of NF-κB in hepatocyte increased and reached its peak, the number of apoptotic hepatocyte in the ligated lobes and the number of mitotic hepatocyte in the perfused lobes also reached their peak. Typical apoptotic changes and evident fibrotic changes in the ligated lobes were observed under electron microscope.

CONCLUSION: After 70% portal branch ligation, DNA binding activity of NF-κB in hepatocyte is significantly increased and NF-κB plays an important role in hepatocyte regeneration and apoptosis.

INTRODUCTION

Portal branch ligation (PBL) or embolization is widely used in the treatment of liver carcinoma, especially in the treatment of patients who have already missed the surgical opportunity[1-3]. PBL or embolization could produce atrophy of the ligated lobes and the perfused lobes undergo compensatory regeneration, while the liver structure and function maintained normal. But the mechanism is still unclear.

It is demonstrated that nuclear factor-kappaB (NF-κB) plays an important role in cell regeneration and apoptosis after partial hepatectomy[4-6]. To study its effects on hepatocyte regeneration and apoptosis, we observed the changes of DNA binding activity of NF-κB in rat liver and its relations to hepatocyte regeneration and apoptosis after 70% PBL.

MATERIALS AND METHODS

Animals

Sixty Wistar rats, weighing 200-240 g, were obtained from the Animal Center of the Third Military University and used in all experiments. All animals were kept in a temperature- and humidity-controlled environment in a 12-h light/dark cycle and allowed free access to water and standard food-pellet diet.

Surgical procedure and experimental design

All surgical procedures were carried out under sodium pentobarbital (40 mg/kg intraperitoneally) anesthesia at room air between 9:00 and 12:00 a.m. with a clean but not sterile technique. The 70% PBL model used was based on the Bilodeau method[7]. In 70% PBL, a median laparotomy was performed, and the branch of the portal vein feeding the anterior and lateral lobes was carefully dissected under

an operating microscope and completely ligated with a 7-0 suture. Care was taken not to injure the hepatic artery and the bile duct and to avoid hemorrhage. In sham-operated rats, a laparotomy followed by dissection of the relevant ligaments without ligature was performed. The animals had free access to water and food after surgery. The animals were killed 12 h, 1, 2, 3, 7, and 14 d after surgery.

Electrophoretic mobility shift assay

Nuclear extracts were prepared separately from the anterior (ligated) and posterior (nonligated) lobes as previously described[8]. Protein concentration was determined using the Bradford method. Double-stranded NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5', Promega Co., USA) were end-labeled[5] with [γ-^{32}P] ATP (Beijing Yahui Biomed Inc., Beijing, China) using T4 polynucleotide kinase (Promega Co.). After the probe was purified, 5 μg of nuclear proteins was preincubated for 10 min at room temperature with 2 μg poly (dI-dC) (Sigma Co., USA) in the binding buffer. Double-stranded oligonucleotides were ^{32}P end-labeled with [γ-^{32}P] ATP and added to the extracts. The mixture was further incubated for 30 min at room temperature and then electrophoresed (200 V, 2 h) on a 5% polyacrylamide gel in a 0.5× TBE buffer. Then the gel was subjected to gamma autoradiography at -70 ℃ for 12 h, and analyzed with gel imaging system (Biorad Co., USA).

Liver morphologic structure

The rat liver color and quality were observed. Both ligated and nonligated lobes were weighed separately for measurement of their absolute and relative weights (the ratio of liver weight/body weight), and their percent in the whole liver was calculated.

Serum ALT level

Serum ALT level was measured with the biochemical multi-analyzer in Biochemistry Department of our hospital.

Histological studies and mitotic activity

Liver sections were derived from formaldehyde-fixed tissues embedded in paraffin and stained with hematoxylin –phloxin–saffron. Mitotic activity in stained sections was determined. Mitotic hepatocytes were sought in 100 consecutive high-power fields (×400), and mitotic index was expressed as per 1 000 hepatic nuclei. Prophases before dissolution of nuclear membrane and late telophases were excluded.

In situ determination of hepatocyte apoptosis (TUNEL stain)

Tissue sections of rat liver were dewaxed in toluene and alcohol. After rehydration with phosphate-buffered saline, they were incubated with proteinase K (25 μg/mL) for 15 min at room temperature and then with terminal deoxyribonucleotidyl transferase (1 U/mL) and digoxigenin-tagged dUTP (10 μmol/L) at 37 ℃ for 1 h in 0.2 mol/L sodium cacodylate, 30 mmol/L Tris (pH 7.2), 1 mmol/L $CoCl_2$, and 0.25 mg/mL bovine serum albumin. The sections were exposed to anti-digoxigenin antibodies labeled with alkaline phosphatase for 30 min at room temperature in 50 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl. The binding of the antibody was revealed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate reagents. Slides were counterstained with 1% eosin.

Ultrastructural study

The rat liver was cut into sections as large as 1 mm× 1 mm×1 mm, which were embedded in Epon618 resin and stained with uranyl acetate and lead citrate. H-2000 transmission electron microscope was used to study the ultrastructure.

Statistical analysis

Results were expressed as mean±SD. The statistical difference between the groups was tested using the one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

Activation of NF-κB in hepatocyte after 70% PBL

After 12 h of 70% PBL, DNA binding activity of NF-κB both in ligated and nonligated lobes increased and reached its peak on d 2, and returned to normal on d 7. The changes of DNA binding activity of NF-κB in the ligated lobes were more obvious than those in the perfused lobes. No NF-κB binding was observed in nuclear extracts from control animals (Figure 1).

Changes in liver morphologic structure

The liver lobes deprived of portal flow were darkened, rapid and progressive atrophy occurred, with their proportion being reduced gradually in the whole liver. At the same time, the nonligated lobes were progressively enlarged, the total liver weight was maintained at the level of sham-PBL controls at each time interval (Figure 2A).

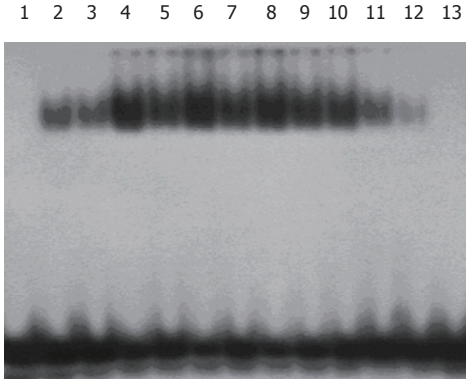

Figure 1 Changes of NF-κB binding activity in rat liver at different time points after 70% PBL by EMSA. Lane 1: control group; lanes 2, 4, 6, 8, 10, 12: 0.5, 1, 2, 3, 7, 14 d ligated liver lobes in PBL group; lanes 3, 5, 7, 9, 11, 13: 0.5, 1, 2, 3, 7, 14 d nonligated liver lobes in PBL group.

Changes in serum ALT level

There were no changes of serum ALT level in control group. After 70% PBL, the serum ALT level only increased slightly 1 d after surgery and then returned to normal (Table 1).

Table 1 Changes of serum ALT level after 70% PBL (U/L) (mean ± SD)

Group	0.5 d	1 d	2 d	3 d	7 d	14 d
Control	55.6±8.9	43.7±10.4	38.5±7.8	40.1±9.4	36.4±11.3	42.0±13.7
PBL	59.7±17.3	67.9±15.7[a]	44.6±9.8	42.6±7.8	38.9±12.1	41.3±7.9

[a]$P<0.05$ *vs* control group.

Histological studies and mitotic activity quantification

No apparent changes were found at light microscopic examination in the control liver. One day after 70% PBL, mild necrosis was found in the ligated lobes, mainly around the central vein. The cytoplasm was homogenous, and the nuclear pyknosis inside the necrosis and neutrophils was seen in some lesions. After 3 d, the necrosis was partly resorbed, and many monocytes were seen inside them. The lobules were small, with portal areas lying near each other. One to two weeks after ligation, the necrosis was completely disappeared, and the lobules became small. Fibrosis appeared around larger portal and hepatic veins, and bile ducts were collapsed with low epithelium.

Remarkable hepatocyte regeneration was observed in the nonligated lobes 12 h after 70% PBL and reached its peak 2 d later, and was still high after 7 d. The liver structure was almost normal (Figure 2B).

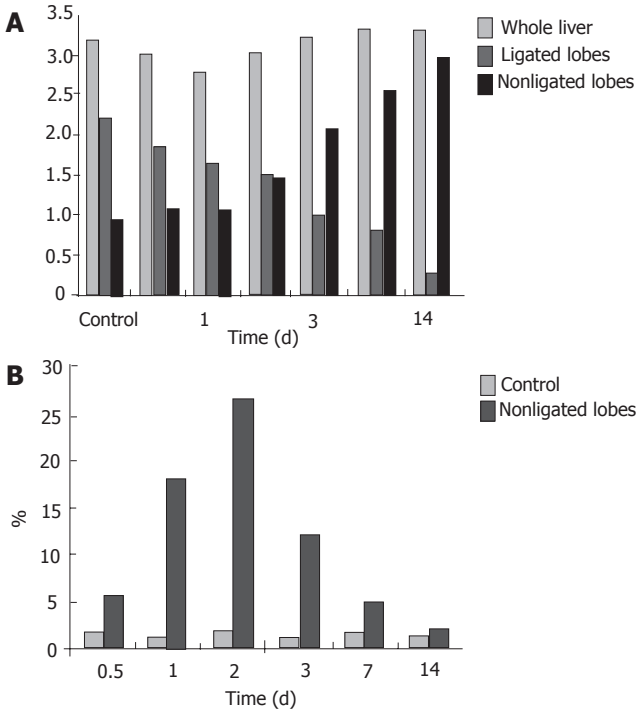

Figure 2 Changes in weight of rat liver (**A**), Changes of mitogenetic index of rat hepatocyte (**B**) after 70% PBL.

Hepatocyte apoptosis assay

There were only few apoptotic hepatocytes in the liver of control animals and in the nonligated lobes after 70% PBL. Many apoptotic hepatocytes were found in the ligated lobes 1 d after 70% portal branch ligation, and reached its peak 2 d later. These cells were mainly present around the central veins with necrosis (Figures 3A-E).

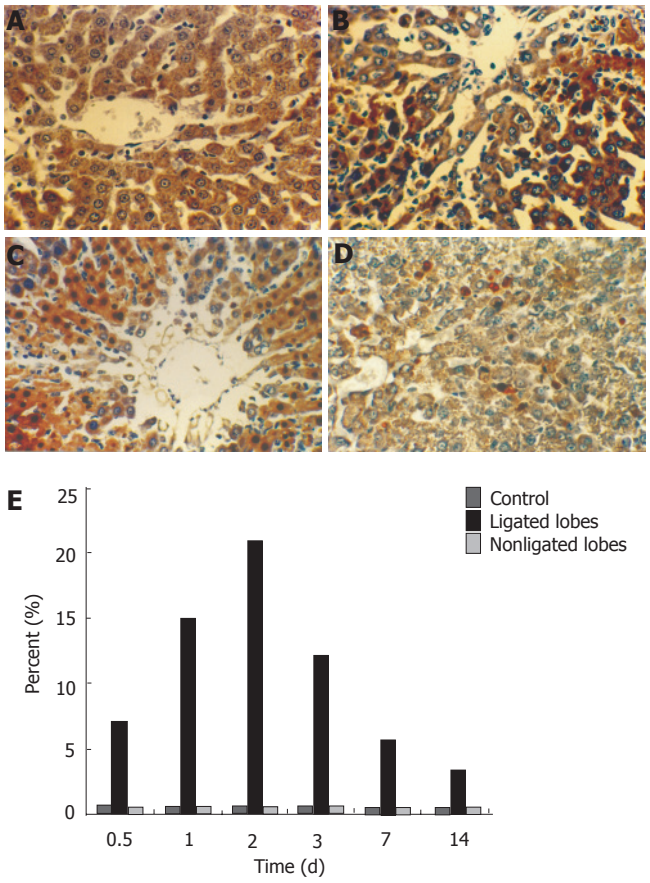

Figure 3 Apoptotic hepatocytes in control group (**A**), in rat ligated liver lobes 1 d (**B**), 2 d (**C**), 7 d (**D**), and hepatocyte apoptotic index (**E**) after 70% PBL.

Ultrastructural changes

The liver ultrastructure was normal in control animals and was almost normal in the ligated lobes early after 70% PBL, and only wild necrosis was found in some local areas. One day after 70% PBL, many apoptotic hepatocytes were found in the ligated lobes. Histological evidence for apoptosis included disappearance of the nuclear membrane, condensation, and margin of karyoplasms or chromatin, pieces of nuclei. There were no morphological changes in the mitochondriae and other intracellular structures (Figure 4). A number of apoptotic hepatocytes reached its peak 2 d later. Collagen was deposited in the Disse space and hepatic sinus became narrow 7 d after 70% PBL. Evident fibrotic changes were found in the ligated lobes 14 d after 70% PBL. A lot of collagens were deposited in the Disse space and portal areas, between hepatocytes and in hepatocytes.

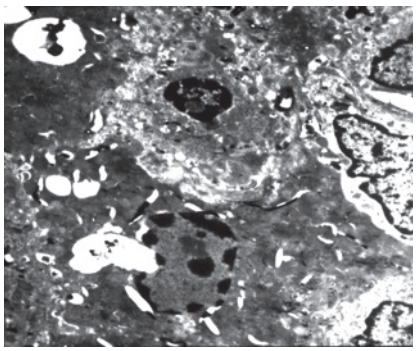

Figure 4 Ultrastructure of rat ligated liver lobes 2 d after 70% PBL (EM ×6 000)

DISCUSSION

PBL or embolization is widely used in the treatment of liver carcinoma, especially in the treatment of patients who have missed the opportunity of surgery[1-3]. It was verified in our experiment that PBL could produce atrophy of the ligated lobes, whereas the perfused lobes underwent compensatory regeneration, the liver structure and function maintained normal. Therefore, it is safe and practicable to ligate 70% portal vein branch in normal rat liver.

The 70% PBL could produce atrophy of the ligated lobes through hepatocyte apoptosis; whereas the perfused lobes undergo compensatory regeneration through hepatocyte mitosis. The total liver weight and function maintained normal. The mechanism of rat liver is still unclear. We observed the changes of DNA binding activity of NF-κB in liver through EMSA. After 70% PBL, DNA binding activity of NF-κB significantly increased both in ligated lobes and nonligated lobes, which was positively correlated with hepatocyte regeneration and apoptosis. Therefore, we could conclude that NF-κB plays an important role in hepatocyte regeneration and apoptosis after 70% PBL.

NF-κB, as a universal nuclear transcriptional factor, plays an important role in the regulation of genes relative to cell regeneration and apoptosis[9,10]. In most cells, NF-κB heterodimers are present in the cytoplasm forming an active complex by interacting with the IκB family of proteins. In response to a variety of activators, IκB-α, the prototypic member of this family of inhibitors, is phosphorylated at series 32 and 36, rendering the factor susceptible to proteolysis via the ubiquitin–proteasome pathway. This event unmasks a nuclear localization sequence of the transactivating heterodimers, allowing NF-κB translocation to nuclei. Therefore, the complex binds to κB consensus motifs in DNA, upregulating the transcription of many genes[11-14].

NF-κB plays an important role on hepatocyte regeneration in nonligated lobes by inhibiting hepatocyte apoptosis and accelerating hepatocyte regeneration. NF-κB can inhibit hepatocyte apoptosis by regulating relative cytokine transcription and expression[15-17], inducing antiapoptotic genes in Bcl-2 family[18], regulating the expression of TRAF and IAP at transcription and translation level and inhibiting the activation of caspase-8, a key enzyme in cell apoptosis[19-21], activating the apoptotic inhibitors[22,23]. NF-κB, which takes part in hepatocyte regeneration, may be mediated by regulating the transcription and expression of relative genes. NF-κB can activate the transcription and expression of TNF-α, increased TNF-α can stimulate secretion of IL-6, which can activate STAT3 through combining with IL-6R on the surface of hepatocyte, and accelerate hepatocyte regeneration[24-27].

We observed that the changes of DNA binding activity of NF-κB in the ligated lobes were more obvious than those in the perfused lobes. One reason is that compensatory response to the excessive hepatocyte apoptosis could maintain the relative weight and function of the liver. The other reason is that NF-κB plays an important role in accelerating hepatocyte apoptosis. Kuhnel *et al*.[28] reported that NF-κB mediates hepatocyte apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis. Recently the induction of NF-κB through the double-stranded RNA-dependent protein kinase has been suggested as a principal mechanism of virus-mediated apoptotic cell death[29].

In conclusion, NF-κB plays a completely different role in the ligated lobes and nonligated lobes through different stimulating factors and different signal transduction pathways. This may have important significance in maintaining liver structure and function after 70% PBL.

REFERENCES

1 **Tanaka H**, Hirohashi K, Kubo S, Shuto T, Higaki I, Kinoshita H. Preoperative portal vein embolization improves prognosis after right hepatectomy for hepatocellular carcinoma in patients with impaired hepatic function. *Br J Surg* 2000; **87:** 879-882

2 **Hatsuno T**, Kaneko T, Inoue S, Sugimoto H, Takeda S, Nakao A. Changes in hepatic lobe volume in hepatocellular carcinoma after transcatheter arterial and percutaneous transhepatic portal embolization. *Hepatogastroenterology* 2004; **51:** 1820-1824

3 **Kokudo N**, Makuuchi M. Current role of portal vein embolization/hepatic artery chemoembolization. *Surg Clin North Am* 2004; **84:** 643-657

4 **Riggins RB**, Zwart A, Nehra R, Clarke R. The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. *Mol Cancer Ther* 2005; **4:** 33-41

5 **Singh RP**, Mallikarjuna GU, Sharma G, Dhanalakshmi S, Tyagi AK, Chan DC, Agarwal C, Agarwal R. Oral silibinin inhibits lung tumor growth in athymic nude mice and forms a novel chemocombination with doxorubicin targeting nuclear factor kappaB-mediated inducible chemoresistance. *Clin Cancer Res* 2004; **10:** 8641-8647

6 **Djavaheri-Mergny M**, Javelaud D, Wietzerbin J, Besancon F. NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells. *FEBS Lett* 2004; **578:** 111-115

7 **Bilodeau M**, Aubry MC, Houle R, Burnes PN, Ethier C. Evaluation of hepatocyte injury following partial ligation of the left portal vein. *J Hepatol* 1999; **30:** 29-37

8 **Kawamura I**, Morishita R, Tsujimoto S, Manda T, Tomoi M, Tomita N, Goto T, Ogihara T, Kaneda Y. Intravenous injection of oligodeoxynucleotides to the NF-kappaB binding site inhibits hepatic metastasis of M5076 reticulosarcoma in mice. *Gene Ther* 2001; **8:** 905-912

9 **Tang JB**, Xu Y, Wang XT. Tendon healing in vitro: activation

of NIK, IKKalpha, IKKbeta, and NF- kappaB genes in signal pathway and proliferation of tenocytes. *Plast Reconstr Surg* 2004; 113: 1703-1711

10 **Sanchez A**, Factor VM, Schroeder IS, Nagy P, Thorgeirsson SS. Activation of NF-kappaB and STAT3 in rat oval cells during 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. *Hepatology* 2004; **39:** 376-385

11 **Buss H**, Dorrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M. Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity. *J Biol Chem* 2004; **279:** 49571-39574

12 **Natarajan R**, Fisher BJ, Jones DG, Fowler AA 3rd. Atypical mechanism of NF-kappaB activation during reoxygenation stress in microvascular endothelium: a role for tyrosine kinases. *Free Radic Biol Med* 2002; **33:** 962

13 **Li Q**, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002; **2:** 725-734

14 **Schooley K**, Zhu P, Dower SK, Qwarnstrom EE. Regulation of nuclear translocation of nuclear factor-kappaB relA: evidence for complex dynamics at the single-cell level. *Biochem J* 2003; **369:** 331-339

15 **Zong WX**, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev* 1999; **13:** 382-387

16 **Shah RD**, Gonzales F, Golez E, Augustin D, Caudillo S, Abbott A, Morello J, McDonough PM, Paolini PJ, Shubeita HE. The antidiabetic agent rosiglitazone upregulates SERCA2 and enhances TNF-alpha- and LPS-induced NF-kappaB-dependent transcription and TNF-alpha-induced IL-6 secretion in ventricular myocytes. *Cell Physiol Biochem* 2005; **15:** 41-50

17 **Takada I**, Suzawa M, Kato S. Nuclear receptors as targets for drug development: crosstalk between peroxisome proliferator-activated receptor gamma and cytokines in bone marrow-derived mesenchymal stem cells. *J Pharmacol Sci* 2005; **97:** 184-189

18 **Wang CY**, Guttridge DC, Mayo MW, Baldwin AS Jr. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999; **19:** 5923-5929

19 **Wang CY**, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998; **281:** 1680-1683

20 **Kreuz S**, Siegmund D, Rumpf JJ, Samel D, Leverkus M, Janssen O, Hacker G, Dittrich-Breiholz O, Kracht M, Scheurich P, Wajant H. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J Cell Biol* 2004; **166:** 369-380

21 **Rathore N**, Matta H, Chaudhary PM. An evolutionary conserved pathway of nuclear factor-kappaB activation involving caspase-mediated cleavage and N-end rule pathway-mediated degradation of IkappaBalpha. *J Biol Chem* 2004; **279:** 39358-39365

22 **Wu MX**, Ao Z, Prasad KV, Wu R, Schlossman SF. IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. *Science* 1998; **281:** 998-1001

23 **Islam S**, Hassan F, Mu MM, Ito H, Koide N, Mori I, Yoshida T, Yokochi T. Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-kappaB activation by inhibiting IkappaB kinase (IKK). *Microbiol Immunol* 2004; **48:** 729-736

24 **Cressman DE**, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, Taub R. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 1996; **274:** 1379-1383

25 **Legendre F**, Dudhia J, Pujol JP, Bogdanowicz P. JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of Type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression. *J Biol Chem* 2003; **278:** 2903-2912

26 **Waris G**, Huh KW, Siddiqui A. Mitochondrially associated hepatitis B virus X protein constitutively activates transcription factors STAT-3 and NF-kappa B via oxidative stress. *Mol Cell Biol* 2001; **21:** 7721-7730

27 **Chaisson ML**, Brooling JT, Ladiges W, Tsai S, Fausto N. Hepatocyte-specific inhibition of NF-kappaB leads to apoptosis after TNF treatment, but not after partial hepatectomy. *J Clin Invest* 2002; **110:** 193-202

28 **Kuhnel F**, Zender L, Paul Y, Tietze MK, Trautwein C, Manns M, Kubicka S. NFkappaB mediates apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis. *J Biol Chem* 2000; **275:** 6421-6427

29 **Gil J**, Alcami J, Esteban M. Induction of apoptosis by double-stranded-RNA-dependent protein kinase (PKR) involves the alpha subunit of eukaryotic translation initiation factor 2 and NF-kappaB. *Mol Cell Biol* 1999; **19:** 4653-4663

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• *BASIC RESEARCH* •

Effects of different ingredients of zedoary on gene expression of HSC-T6 cells

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Abstract

AIM: To investigate the effects of four different ingredients of zedoary (Curcuma aromatica oil, Curcumol, β-elemence, and Curcumin) on the gene expressions of hepatic stellate cells (HSCs), and to explore the molecular mechanism of zedoary against hepatic fibrosis at gene network level.

METHODS: We detected the mRNA sequences of 50 liver fibrosis-related genes in GenBank and designed oligonucleotide probes. We synthesized oligonucleotides with PE8909 DNA synthesizing instrument, and carried out oligonucleotide microarray with OGR-04 dropping instrument and aldehyded glass chip. Cultured HSC-T6 cells were treated with different concentrations of Colchicine, Curcuma aromatica oil, Curcumol, β-elemence, and Curcumin. According to the experiment of cell toxicity, we took the appropriate concentrations of medicines that resulted in over 50% of cell survival as experiment concentrations. We collected the cells at 1, 6, 12, and 24 h, and extracted total RNA with TRIzol reagent, then labeled cDNAs with Cy3-dUTP and Cy5-dUTP. These labeled cDNAs were hybridized to an oligonucleotide microarray which was washed several times and scanned by scanner GenePix 4000B. Different gene expressions of HSC-T6 cells were analyzed by ImaGene 4.2 software.

RESULTS: After HSC-T6 cells were cultured in a medium containing 6.25 μg/mL Colchicine for 12 h, expression of TIMP-1 decreased 2.2-folds. After HSC-T6 cells were cultured in a medium containing 78.125 μg/mL of Curcuma aromatica oil for 24 h, the expression of TIMP-2 and IL-6 decreased 2.3- and 2.2-folds, respectively. Moreover, after HSC-T6 cells were cultured in a medium containing 1.5625 μg/mL of Curcumol for 12 h, the expression of TGFβ1 and P450a decreased 2.3- and 2.1-folds, respectively.

CONCLUSION: Our results may show the possible molecular mechanism of Curcuma aromatica oil and Curcumol against hepatic fibrosis.

Key words: DNA microarray; Curcuma aromatica oil; Curcumol; Hepatic stellate cells; Hepatic fibrosis

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INTRODUCTION

In the past 10 years or more, great progress has been made in treating liver fibrosis with Chinese herbal medicines such as compound 861[1] and Fuzheng Huayu Decoction[2] and Tidu Hugan Decoction[3] as well as single herbal medicines as red sage root, zedoary, Chinese caterpillar fungus, hanfangchin A, extracts from peach seeds. Although many medicines can be used to treat liver fibrosis, effective medicines are still hard to find. Herbal medicines have the characteristics of multiple targets and poly-functioning routes, but genes in the organisms alone form a strict and complicated network. Therefore, study of herbal medicines should focus on the molecular mechanism at the level of genetic network based on the integral bio-system. Genetic chip technology is characterized by high communication, low consumption and miniaturization, thus providing a technological platform to study the mechanism of herbal medicines against liver fibrosis[4].

We found that zedoary could inhibit the proliferation of hepatic stellate cells (HSCs). Curcumin can be used to treat inflammation and tumors and Curcuma aromatica oil functions as an anti-inflammation, anti-virus, anti-tumor and anti-thrombus agent. Now more than 20 chemical ingredients such as Curcumol, Epicurzerenone, β-elemence, Camphene, Isoborneol, Borneol, Cineole, and 4-methyl-pyrazine have been identified from Curcuma

aromatica oil. It was reported that Curcumol and Elemence can function as anti-tumor and virus agents[5, 6]. Xi *et al*.[7] found that zedoary could protect hepatic cells against necrosis and degeneration as well as proliferation of fibrous tissues.

To study the molecular mechanism of zedoary against liver fibrosis, we used the genetic expression spectrum chips to represent 50 genes related to liver fibrosis and substituted HSC-T6 for original HSCs, and investigated the effects of four different ingredients of zedoary (Curcuma aromatica oil, Curcumol, β- elemence, Curcumin) on the gene expression of activated HSCs.

MATERIALS AND METHODS

Materials

Colchicine was purchased from American ALEXIS Co, Elemence injection and Curcumin were obtained from Jingang Pharmaceutic Corporation Ltd, Dalian, China. Curcumol and Curcuma aromatica oil were from Pharmaceutical University, Shenyang, China. HSC-T6 was provided by the Insititute of Hepatology, Shanghai University of Traditional Chinese Medicine and pharmacology. AXSys Probe Punctum-controlling software was purchased from Cartesian Technologies Co., and ImaGene 4.2 figure-analyzing softwares was from American Biodiscovery Co.

Preparatin of gene probes

Design of oligonucleotide probe Oligonucleotide probes were designed by the design software of oligonucleotide probe . The coding region near the 3 end was selected for BLAST analysis. One or two probes related to liver fibrosis whose homology was less than 70% were used as spare probes.

Synthesis of oligonucleotide Oligonucleotides were synthesized by the chemical method of standard subphosphorus imide using PE8909 DNA synthesizer. *N*-MMTr-6- ammonia-2-cyanogen-*N* and *N*-diisopropyl-subimide ammonia were modified by 5- or 3-amino-group. Dense ammonia was deprotected at 55 ℃ and incised for 15 h, and purified by ordinary portland cement column.

Preparation of probes In brief, 0.5 μg/μL oligonucleotide probe was resolved into 3×SSC solution, glass chip was aldehyded and stayed overnight, processed with 2 g/L SDS de-ion water for 10 min, and then dried for later use.

Preparation of medical culture medium Colchicine was dissolved in double-vaporing water to get the original solution (3.2 mg/mL). Curcumin was mixed with 950 mL/L alcohol to get the original solution (320 mg/L). Curcumol was mixed with 950 mL/L alcohol plus Tween-80 to get the original solution (3.2 mg/mL). Curcuma aromatica oil was mixed with Tween-80 (ratio, 1:10) plus 950 mL/L alcohol to get the original solution (2.5 mg/mL). Elemence injection (5 mg/mL) was used. All the medicines were filtrated through 0.45 micropores and stored at 4 ℃.

Culture of HSC-T6 cells

The ampoule was taken out of the liquid nitrogen jar (wearing protective glasses and gloves) and put into a porcelain enamel vessel containing 36-37 ℃ water with shaking. The pocket was cut and the ampoule was taken out, sterilized with 700 mL/L alcohol. The cell suspension was aspirated and put into centrifuge tube, then 10 mL culture medium was added, centrifuged for 5 min at 500-1 000 r/min and rinsed . The culture medium was changed on the next day.

Test of cytotoxicity (MTT assay[8])

The 96-well plates were incubated at 37 °C until HSC-T6 cells were grown in a single layer. The culture medium was incubated for 48 h, and then 5 mg/mL MTT was added and incubated for 48 h. The A value of the solution was tested in enzymatic marking instrument (wave length on the light-filtrating slice is 492 nm). According to the experiment of cell toxicity, we took the appropriate concentrations of medicines that resulted in over 50% of cell survival. The formula of cell survival rate: cell survival rate (%) = (medicine group/control group)×100%.

Incubation of HSC-T6 cells with medicines at different times

HSC-T6 cells were incubated with different concentrations of Colchicine, Curcuma aromatica oil, Curcumol, β-elemence, and Curcumin, followed by collecting them at 1, 6, 12, and 24 h, respectively.

Extraction and evaluation of cellular tRNA

Extraction of cellular tRNA HSC-T6 cells were washed softly with germ-free PBS. One-milliliter of TRIzol reagent was used to blow the cells and to make them dissolve completely. Then 0.2 mL of methylene trichloride was added, followed by centrifugation at 12 000 *g* for 15 min at 4 ℃. The supernatant was aspirated with 200 μL tip (dealt with DEPC) and moved to another EP tube. Then 0.5 mL of isopropylalcohol was added and put aside for 15 min, and centrifuged at 12 000 *g* for 10 min at 4 ℃. The RNA was washed with 750 mL/L alcohol (dealt with DEPC), and then 1 mL of 750 mL/L alcohol (dealt with DEPC) was added, centrifuged at 7 500 r/min for 5 min at 4 ℃, and stored at -20 ℃.

Evaluation of purity DEPC water was added to 2 μL of RNA to make a total volume of 100 μL. Ultraviolet spectrophotometer was used to measure the value of A_{260} and A_{280} as well as A_{260}/A_{280}. The formula of concentration of RNA: RNA (μg/μL) = A_{260}×40× dilution multiple/1 000.

Evaluation of integrity Five-microliter of RNA samples were put into 10 mL/L formaldehyde degeneration sepharose for cataphoresis, followed by painting with EB and examination under ultraviolet light.

Reverse transcription system and the labeling and purification of cDNA

Reverse transcription system and conditions Ten-microliter of TRNA (10 μg/μL), 0.3 μL of positive control, 4.0 μL of oligo (dT)16 (0.5 μg/μL) and 0.5 μL of

Table 1 Genetic probe matrix related to liver fibrosis

Genetic probe matrix											
TIMP1	TIMP1	TIMP2	TIMP2	TIMP3	TIMP3	MMP2	MMP2	MMP8	MMP8	TGFβ1	TGFβ1
PDGFA	PDGFA	PDGFC	PDGFC	MMP3	MMP3	IL-6	IL-6	IL-10	IL-10	IL-1	IL-1
HGF2	HGF2	HGF1	HGF1	VEGFA	VEGFA	VEGFB	VEGFB	VEGFC	VEGFC	VEGFD	VEGFD
IGF2	IGF2	IGF1	IGF1	TGFβR Ⅰ	TGFβR Ⅰ	TGFβR Ⅱ	TGFβR Ⅱ	PDGFRα	PDGFRα	PDGFRβ	PDGFRβ
N	N	P	P	N	N	P	P	N	N	P	P
C-myc	C-myc	P-450d	P-450d	P-450a	P-450a	P-450e	P-450e	P450-4A3	P450-4A	P-450-LA	P-450-LA
TNFα	TNFα	TNFR1	TNFR1	CJUNB	CJUNB	CJUND	CJUND	ETRA	ETRA	ET-1	ET-1
CYP2D4	CYP2D4	CYP1B1	CYP1B1	ESTSUL	ESTSUL	FGF1	FGF1	STAA	STAA	FGF2	FGF2
ICAM-1	ICAM-1	PAFR	PAFR	VCAM-1	VCAM-1	MIP-2	MIP-2	MCP-1	MCP-1	PAF	PAF
β-actin	β-actin	GAPDH	GAPDH								

RNase inhibitor (40 U/μL) were mixed together, incubated for 10 min and then put into ice bath. Then 5.0 μL of 5× first chain buffer, 2.0 μL of DTT (0.1 mol/L), 1.0 μL of Cy3-dUTP or Cy5-dUTP (1 mmol/L), 0.5 μL of dTTP (10 mmol/L, and 0.1 μL of each dATP, dCTP and dGTP (100 mmol/L) were mixed together and incubated for 2 min at 42 ℃. Then 1.0 μL of SuperScript II RNase H-transcriptase (10 U/μL) was added and incubated for 2 h. Then 1.0 μL of RNaseH was added and incubated for 0.5 h at 30 ℃, followed by inactivation of anti-transcriptase at 70 ℃ for 15 min. Then 5.0 μL of NaOH (1 mol/L) was added and incubated at 65 ℃ for 1 h, followed by addition of 5.0 μL of 1 mol/L HCl (pH 6.8) and 6.0 μL of 5 mol/L NaCl. The sediment was stayed overnight at –20 ℃, centrifuged at 12 000 *g* for 10 min at 4 ℃. The supernatant was abandoned, the sedimentation was washed once with 750 mL/L alcohol and then dried, followed by resolving in 2 μL of aseptic water and stored at -20 ℃.

Evaluation of cDNA One-microliter of the above product of reverse transcription was put into 10 mL/L formaldehyde degeneration sepharose for cataphoresis, and then the quality of probes was evaluated.

Hybridization and wash

Two microliters of probes labeled by fluorescence were diluted to 8 μL for hybridization, and the hybridization liquor was moved onto the cover glass chip with the density of 2 μL/cm^2. The solution was put onto the carry sheet glass chip equally by the capillarity between cover glass chip and carry sheet glass chip. Then the glass chips were put into a hybridization box and 5 μL of 3× SSC was added to keep the humidity. The probe washing temperatures varied according to the different probes, usually under room temperature. The order of washing liquor was lotion A–C.

Analysis of data from fluorescence

After hybridization, the genetic chips were scanned by Scanner Genepix 4 000B, ImaGene4.2 was used to analyze the ratio of Cy3, Cy5 and the intensity of two kinds of fluorescence signals.Housekeeping gene and positive control were taken to balance the data of fluorescence of Cy3 and Cy5. Ratio of Cy3, Cy5>2 or <0.5 was used to evaluate the differences of genetic expression.

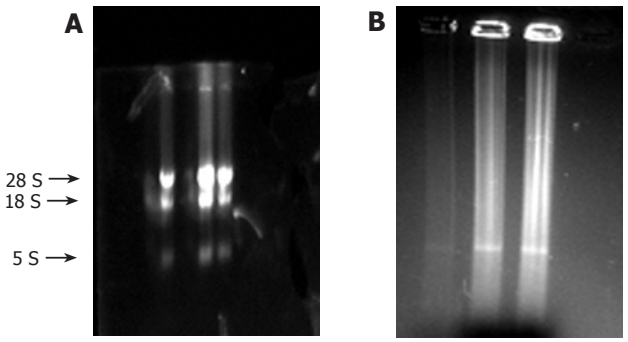

Figure 1 Influence of ingredients of zedoary on genetic expression of HSCs. **A:** tRNA formaldehyde degeneration; **B:** products of reverse transcription.

RESULTS

Effects of different medicines on the growth of HSCs

After treatment of HSC-T6 cells with different concentrations of Colchicine, Curcuma aromatica oil, Curcumol, β-elemence, and Curcumin for 48 h, along with deduction of the concentrations, the survival ratio of HSC-T6 increased.

Influence of four different ingredients of zedoary on the genetic expression of HSCs

RNA was put on 10 mL/L formaldehyde agarose gels for cataphoresis, and the results were recorded with gel photography. Three strips (28S, 18S, and 5S) can be seen in Figure 1A. After the analysis by software, the ratio of 28S and 18S was found to be between 1.5 and 2.0, showing that the RNA was integral and without degradation. Using ultraviolet spectrophotometer, the value of A_{260}/A_{280} was found to be between 1.7 and 2.0, showing that the RNA was pure and without protein pollution or phenol.

In addition, most of the cDNAs were observed between 0.5 and 2 kb (Figure 1B). Distribution of probes is shown in Table 1.

Hybridization

In the hybridization scanning figures of probes (Figures 2A, 3A, and 4A), the green spots represent the locations of downregulated genetic expression. ImaGene 4.2 software was used to analyze the intensity and ratio of Cy3 and Cy5. After correction of housekeeping gene and positive

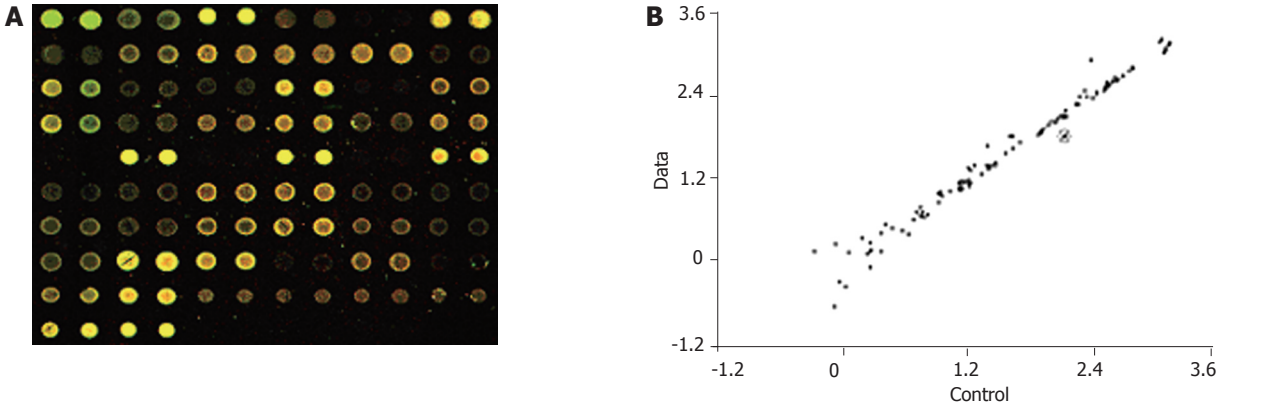

Figure 2 Effects of 6.25 μg/mL Colchicines on genetic expression of HSC-T6 cells after 12 h. **A:** Scanning diagram; **B:** scattering diagram.

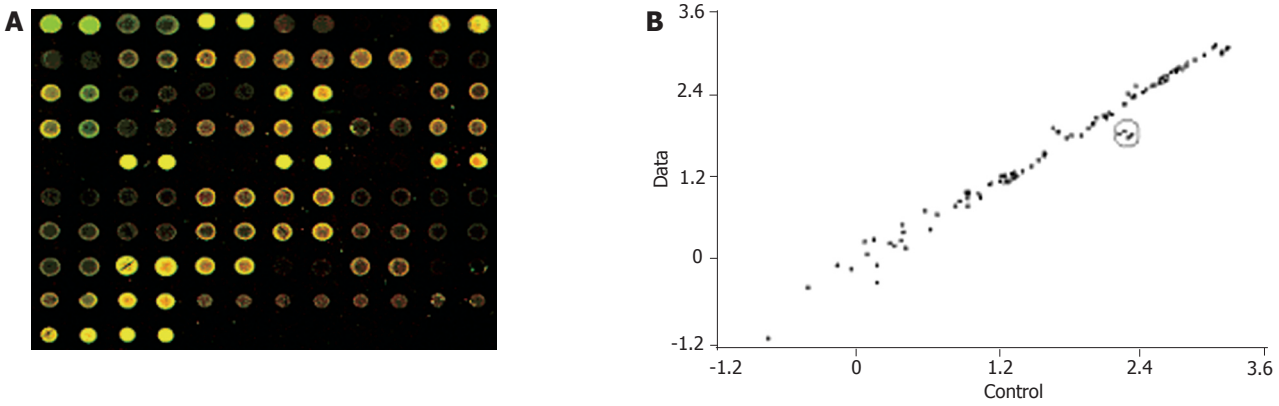

Figure 3 Effects of 78.125 μg/mL Curcuma aromatica oil on genetic expression of HSC-T6 cells after 24 h. **A:** Scanning diagram; **B:** scattering diagram.

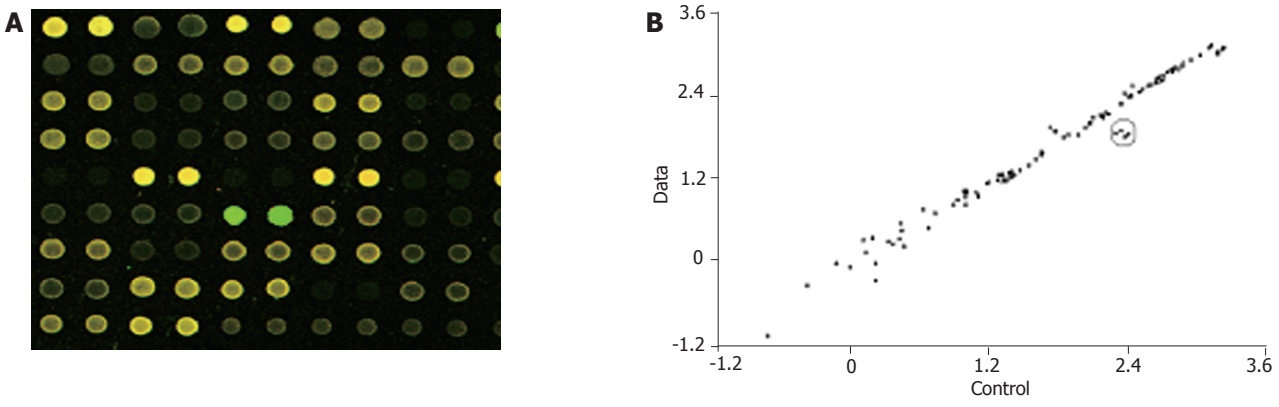

Figure 4 Effects of 1.5625 μg/mL Curcumol on genetic expression of HSC-T6 cells after12 h. **A:** Scanning diagram; **B:** scattering diagram.

control, the two conditions were taken to evaluate the difference of genetic expression as follows: (1) the ratio of Cy3 and Cy5 >2 or <0.5; and (2) one of Cy3 and Cy5 >1 000. In the scattering diagram (Figures 2B, 3B, and 4B), the spots on the opposite angles show that the intensity of two groups (treatment group and control group) was the same. The farther they depart from the opposite angles, the bigger the difference of genetic expression would be. The different effects of medicines on genetic expression are shown in Tables 2-4. After culture of HSC-T6 cells in a medium containing 6.25 μg/mL of Colchicine for 12 h, the expression of TIMP-1 decreased 2.2-folds, which was in agreement with a previous report[9], suggesting that the genetic probe testing system in the experiment was dependable. Furthermore, Figures 3 and 4 stand for the scattering diagram and the scattering diagram of Curcuma aromatica oil group and Curcumol group, respectively, and the data of analysis are shown in Tables 3 and 4.

Table 2 Genetic expression difference of HSC-T6 cells treated with Colchicines (6.25μg/mL) for 12 h

Gene	GenBank	Ratio
TIMP-1	U06179	2.20

Table 3 Genetic expression difference of HSC-T6 cells treated with Curcuma aromatica oil (78.125 μg/mL) for 24 h

Gene	GenBank	Ratio
TIMP-2	NM-021989	2.30
IL-6	M26744	2.20

Table 4 Gene expression difference of HSC-T6 cells treated with Curcumol (1.5625 μg/mL) for12 h

Gene	GenBank	Ratio
TGFβ1	NM-021578	2.30
P-450a	J02669	2.10

These results showed that after HSC-T6 cells were cultured in a medium containing 6.25 μg/mL Colchicine for 12 h, the expression of TIMP-1 decreased 2.2-folds. The expression of TIMP-2 and IL-6 decreased 2.3- and 2.2-folds, respectively, after HSC-T6 cells were cultured in a medium containing 78.125 μg/mL of Curcuma aromatica oil for 24 h. Moreover, the expression of TGFβ1 and p450a decreased 2.3- and 2.1-folds, respectively, after HSC-T6 cells were cultured in a medium containing 1.5625 μg/mL Curcumol for 12 h. Other concentrations of neither Curcuma aromatica oil nor Curcumol could bring forth gene expression differences, nor did β-elemence and Curcumin.

DISCUSSION

Genetic chip technology is characterized by high communication, low consumption and miniaturization[10-13], providing a technological platform to study the mechanism of herbal medicines against liver fibrosis. Genetic expression spectrum chips can be categorized into two kinds, namely cDNA chips and oligonucleotide chips, the probe of the former is cDNA, the probe of the latter is fragment of oligonucleotide. To study medicines with genetic expression spectrum chips can help us acknowledge the target genes of herbal medicines, western medicine and some foods with medical functions. It is also an effective way to study the toxicity of medicines, and the mechanism of causing deformity and genetic mutation of medicines.

According to the key role of HSCs in hepatic fibrosis, 50 genes related to liver fibrosis are chosen. They can be categorized into five groups as follows: (1) expressing cytokines and receptors of cytokines, such as transforming growth factor β1 (TGF β1), platelet-derived growth factor A (PDGFA), platelet-derived growth factor C (PDGFC), interleukin-1, interleukin-6, interleukin-10, hepatocyte growth factor 1 (HGF1), hepatocyte growth factor 2 (HGF2), vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor B (VEGFB), vascular endothelial growth factor C (VEGFC), vascular endothelial growth factor D (VEGFD), fibroblast growth factor 1 (FGF1), fibroblast growth factor 2 (FGF2), platelet-activating factor (PAF), tumor necrosis factor α (TNFα), insulin-like growth factor 1 (IGF1), insulin-like growth factor 2 (IGF2), endothelin 1 (ET-1), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), murine macrophage inflammatory protein 2 (MIP-2), monocyte chemotactic protein 1 (MCP-1), transforming growth factor β receptor I (TGFβRI), transforming growth factor β receptor II (TGFβRII), platelet-derived growth factor receptor α (PDGFRα), platelet-derived growth factor receptor β (PDGFRβ), tumor necrosis factor α receptor 1 (TNFαR1), platelet-activating factor receptor (PAFR), and endothelin receptor A (ETRA). TGFβ1 is the strongest factor which promotes the synthesis of extracellular matrix (ECM). PDGF can accelerate the proliferation of HSCs; (2) expressing MMPs (matrix metalloproteinases) and TIMPs (tissue inhibitor of metalloproteinases), such as MMP2, MMP3, MMP8, TIMP-1, TIMP-2, and TIMP-3, which take part in the degradation of ECM; (3) gene expression related to the preliminary activation of HSCs, such as c-myc, Ets-1, STAT1, c-jun B, c-jun D; (4) involving in gene expression related to the biological oxidation[14], such as cytochrome P450d, cytochrome P450a, cytochrome P450e, cytochrome P450-LA, and cytochrome P450 (4A3), CYP1B1, CYP2D4; and (5) house keeping gene, such as β-actin and GAPDH.

How to choose the genes related to liver fibrosis is the key of the design of oligonucleotide probes. The mRNA sequences which contain 40 amino acids are selected and then oligonucleotide probes are designed using the design software of oligonucleotide probe. By and large, the length of oligonucleotide probe is 15-80 nt, the content of GC is 45-55%. Probes of the coding region approaching end 3 are chosen for BLAST analysis. One to two probes whose homology of sequences was less than 70% are chosen as gene distinctive oligonucleotide probes.

Because some sequences of mRNA related to collagen I, III, IV are so short, the probes designed through software are easy to cause cross reactions which may result in false positive results, and such genes, therefore, cannot be chosen. However, the aforementioned 50 genes, in general, can represent approximately the changes which take place in the process of preliminary and persistent activation of HSCs in liver fibrosis.

HSC-T6 cells are SV40 transfected HSCs of Sprague-Dawley rats. The cells can be steadily cultured and their phenotype is activated HSCs which can express high-level collagen I and TIMP-1 mRNA, etc. In our study, HSC-T6 was substituted for the original HSC. Yin *et al.*[15,16] took HSC-T6 as model cells to investigate the influence of compound 861 on the gene expression of MMP-3 and TIMP-1 and they found that 0.25, 0.5, and 1.0 mg/mL of compound 861 could increase the expression of MMP3 and inhibit the expression of TIMP1.

TIMPs can prevent MMPs from degrading ECM[17-21]. Using genetic chip technology, we found that after HSC-T6 cells were cultured in a medium containing 6.25 μg/mL

Colchicine for 12 h, the expression of TIMP-1 decreased 2.2-folds. Expression of TIMP-2 decreased 2.3-folds after HSC-T6 cells were cultured in a medium containing 78.125 μg/mL Curcuma aromatica oil for 24 h. These results showed that Curcuma aromatica oil, like Colchicines, can also inhibit the expression of TIMPs and reduce the inhibition of MMPs which, in turn, help MMPs to degrade ECM. This may be one of the mechanisms for zedoary against liver fibrosis.

IL-6 is also called hepatic cell stimulating factor which can directly stimulate hepatic cells proliferation, induce the expression of IL-6 receptors in liver, and stimulate fibroblastic cells to synthesize collagens[22–26]. In our study, 78.125 μg/mL Curcuma aromatica oil could decrease the expressions of IL-6, TIMP1 and other genes related to hepatic fibrosis, thereby enhancing MMPs to degrade ECM. It might be another mechanism of zedoary against hepatic fibrosis.

TGFβ1 plays an important role in hepatic fibrosis which can activate HSCs and, as a transcription factor of collagen, accelerate its expression[27]. In our study, the expression of TGFβ1 decreased 2.3-folds after culture of HSC-T6 cells in a medium containing 1.5625 μg/L Curcumol for 12 h, suggesting that Curcumol can inhibit the synthesis of ECM through the inhibition of TGFβ1 which might be an another important mechanism of zedoary against hepatic fibrosis.

Cytochrome P450 (or Cyt P450) involves in the synthesis of steroid hormone, bile acid and bile pigments as well as the process of the bio-transformation of medicine and poison[14]. Yang *et al*.[28] have shown that lipid peroxidation takes part in the activation of HSCs. Svegliati Baroni *et al*.[29] have indicated that HCM/Fe might induce a significant increase in collagen type I accumulation in HSC culture media, and HSCs proliferation may be associated with changes in the Na^+/H^+ exchanger activity. Nieto *et al*.[30] transfected CYP2E1 to HSC-T6 cells, and found that there was an increase in the level of reactive oxygen species and type I collagen mRNA. It has been reported that when HSCs were cultured with HepG2 cells which overexpress CYP2E1 together, the level of collagen markedly increased, suggesting that the solved oxidants can activate HSCs[31]. In our study, we found that after culture of HSC-T6 cells in a medium containing 1.5625 μg/mL Curcumol for 12 h, the expression of P450a decreased 2.1-folds, suggesting that the metabolism of Curcumol in HSC-T6 cells might bring forth oxidation-conjugation reaction through the P450 enzyme system, induce a decline of oxidative stress and lipid peroxidation, and thus inhibit the activation of HSCs. This may be one of the mechanisms of zedoary against hepatic fibrosis.

In conclusion, two different ingredients of zedoary (Curcuma aromatica oil, Curcumol), when treated with HSC-T6 cells for 24 and 12 h, can decrease the expression of TIMP-2, IL-6, TGFβ1 and P450a by different degrees, indicating the molecular mechanisms of zedoary against hepatic fibrosis at gene network level. But the changes of genes and the expression of proteins might not be the same events. Along with the development of protein histology, further research needs to testify the proteins related to liver fibrosis, for example, to examine the content of type I collagen using ELISA or investigate the influence of Curcuma aromatica oil and Curcumol on protein expression of HSC-T6 cells by protein chip technology.

REFERENCES

1 **Wang L**, Wang J, Wang BE, Xiao PG, Qiao YJ, Tan XH. Effects of herbal compound 861 on human hepatic stellate cell proliferation and activation. *World J Gastroenterol* 2004; **10**: 2831-2835

2 **Liu C**, Wang X, Liu P. Serapharmacological effect of fuzheng huayu 319 Decoction on expression of type I collagen and transforming growth factor beta 1 in hepatic stellate cells. *Zhongguo Zhongxiyi Jiehe Zazhi* 1999; **19**: 412-414

3 **Zhu QJ**, Nie G, Li HW, Xiao L, Hu CQ, Yang L, Ming AP, Wang BX.Inhibitory effect of Tidu Hugan Decoction on experimental hepatic fibrosis in ducks. *Zhongxiyi Jiehe Ganbing Zazhi* 1998; **8**: 84-87

4 **Abelson PH**. *A third technological revolution.Science* 1998; **279**: 2019

5 **Wang Y**, Wang MZ. Reseach of commonly used Chinese traditional medicine: Curcuma categories. *Zhongguo Yaoxue Zazhi* 2001; **36**: 80

6 **Li GD**, Xiu F, Shen AJ. Reseach of Curcuma aramatica oil. *Zhongguo Yaoxue Zazhi* 2002; **37**: 806-809

7 **Xi ZT**, Shan CM, Jiang XL, Luan XY, Li KK.Experimental study of Sparganium stoloniferum and zedoary on immunological hepatic fibrosis in rats. *Zhongguo Zhongyao Zazhi* 2002; **27**: 929-932

8 **Bian XY**, Yin XN.The method and application of MTT assay. *Gouwai Yixue Linchuang Shengwu Huaxue Yu Jianyanxue Fence* 1998; **19**: 83-85

9 **Yang CQ**, Hu GL, Zhou WH.Effect of Colchicine on Matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 expression in rat liver fibrosis. *Zhonghua Chuanran Bing Zazhi* 2000; **18**: 176-179

10 **Schena M**, Heller RA, Theriault TP, Konrad K, Lachenmeier E, Davis RW. Microarrays: biotechnology's discovery platform for functional genomics. *Trends Biotechnol* 1998; **16**: 301-306

11 **Persidis A**. Biochips. *Nature Biotechol* 1998; **16**: 981-983

12 **Schena M**, Shalon D, Heller R, Chai A, Brown PO, Davis RW.Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc Natl Acad Sci USA* 1996; **93**: 10614-10619

13 **Debouck C**, Goodfellow PN.DNA microarrays in drug discovery and development. *Nat Genet* 1999; **21** (suppl): 48-50

14 **Zhou AR**. Biochemistry. Fifth edition. *Beijing: Renming Weisheng Chuban She* 2001: 160

15 **Yin CH**, Ma H, Wang AM, Ma XM, Jia JD, Wang BE.Effect of compound 861 on tissue inhibitor of metalloprotenase 1 gene expression of HSC-T6 cells . *Zhonghua Ganzang Bing Zazhi* 2002; **10**: 197-199

16 **Yin C**, Ma H, Wang A, Ma X, Jia J, Wang B.Effect of compound 861 on stromelysin gene expression of HSC-T6 cells. *Linchuang Gandanbing Zazhi* 2002; **18**: 168-170

17 **Iredale JP**.Tissue inhibitors of metalloproteinases in liver fibrosis. *Int J Biochem Cell Biol* 1997; **29**: 43-54

18 **McCrudden R**, Iredale JP. Liver fibrosis, the hepatic stellate cell and tissue inhibitors of metalloproteinases. *Histol Histopathol* 2000; **15**: 1159-1168

19 **Murawaki Y**, Ikuta Y, Koda M, Okamoto K, Mimura K. The pro MMP-2 activation rate in patients with chronic viral liver disease. *Clin Chim Acta* 2002; **324**: 99-103

20 **Nie QH**, Zhou YX, Xie YM, Chen YQ.The localization and

expression of TIMP-2 in experi mental hepatic fibrosis in rats. *Jiefangjun Yixue Zazhi* 2002; **27**: 208-209
21 **Luo XD**, Nie QH.The value of tissue inhibitors of metalloproteinases in the study of hepatic fibrosis. *Ganzang* 2003; **8**: 40-42
22 **Li D**, Zhang LJ, Chen ZX, Huang YH, Wang XZ. Effects of TNFα, IL-6 and IL-10 on the development of experimental rat liver fibrosis. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1242-1245
23 **Llorent L**, Richaud-Patin Y, Alcocer-Castillejos N, Ruiz-Soto R, Mercado MA, Orozco H, Gamboa-Dominguez A, Alcocer-Varela J. Cytokine gene expression in cirrhotic and non-cirrhotic human liver. *J Hepatol* 1996; **24**: 555-563
24 **Liu HL**, Li XH, Wang DY, Yang SP. Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression in fibrotic rat liver. *World J Gastroenterol* 2000; **6**: 881-884
25 **Ramadori G**, Christ B. Cytokines and the hepatic acute-phase response. *Semi Liver Dis* 1999; **19**: 141-155
26 **Moshage H**. Cytokines and the hepatic acute phase response. *J Pathol* 1997; **181**: 257-266
27 **Wang LS**, Chen YW, Li DG. Activation of TGF-β1 and liver fibrosis. *Gouwai Yixue Xiaohua Xitong Fence* 2003; **23**: 222-225
28 **Yang WF**, Chen HC, Jiang YP.Factors in hepatic stellate cells activation. *Zhonghua Ganzang Bing Zazhi* 2004; **12**: 121-123
29 **Svegliati Baroni G**, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, Ridolfi F, Saccomanno S, Jezequel AM, Benedetti A. Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998; **27**: 720-726
30 **Nieto N**, Friedman SL, Greenwel P, Cederbaum AI. CYP2E1-mediated oxidative stress induces collagen type I expression in rat hepatic stellate cells. *Hepatology* 1999; **30**: 987-996
31 **Mari M**, Cederbaum Al. Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress. *Hepatology* 2001; **33**: 652-661

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• *CLINICAL RESEARCH* •

Changes of duplex parameters and splenic size in liver transplant recipients during a long period of observation

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Abstract

AIM: To assess the changes of portal and arterial velocities, resistance index, spleen and liver size during a long observation period (13.7 years) after orthotopic liver transplantation (OLT).

METHODS: Two hundred and sixty patients were recruited retrospectively for this study and divided into groups with defined time intervals after OLT. The cross-sectional changes of portal and arterial velocities, resistance index, spleen and liver size between the defined time intervals were studied. The complications detected by ultrasound were compared to gold standard methods.

RESULTS: The mean values for liver size were all within the normal range. The splenic size decreased between the time intervals 100 and 1 000 d after OLT (*t*; $P<0.01$). While portal and arterial flow velocities decreased up to 5.5 years (*t*; portal velocity $P<0.01$, maximal systolic velocity $P = 0.05$, maximal end diastolic velocity $P<0.01$), RI increased during this interval (*t*: $P<0.01$). Higher RI values were found in older patients ($r = 0.24$, $P<0.001$).

CONCLUSION: The arterial and portal velocities show adaptation processes continuing over the course of many years after OLT and are reported for the first time. The vascular complications detected by ultrasound occur mostly up to 100 d after OLT.

Key words: Ultrasound; Color Doppler; Liver transplantation; Monitoring

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INTRODUCTION

Since the 1980s, OLT has become a standard therapy for patients with end-stage liver disease[1] and till date, the outcome of this therapy depends on the early diagnosis and appropriate treatment of complications[2]. Vascular complications are a frequent cause of early graft failure[3]. Graft damage can be either directly caused by hypoxemia or indirectly by biliary ischemia during graft handling which equally leads to chronic biliary damage[4,5]. Time-dependent changes of splanchnic hemodynamics in liver graft recipients have been reported[6]. Based on these data, it is likely that an understanding and interpretation of splanchnic hemodynamics may lead to the prevention and diagnosis of vascular complications[6].

Apart from physical examination and biochemical work-up, routine and diagnostic imaging procedures are recommended to detect post-OLT vascular complications[7-9]. B-mode and especially color Doppler sonography have played a central role in the monitoring of post-transplantation patients. The use of ultrasound allows for the early diagnosis of hepatic arterial as well as biliary complications[3,9,10] and contributes to a better understanding of splanchnic hemodynamic changes[6].

Long-term follow-up studies of splanchnic hemodynamic changes after OLT are time consuming and therefore not applicable. The aim of this study was to assess the post-transplantation changes of duplex parameter as well as liver and splenic size in liver graft recipients for a long period of observation. This study was performed retrospectively as a cross-sectional analysis in a large cohort of OLT patients. The secondary objective of this study was to assess the post-transplantation complications detected by ultrasound during this observation time.

MATERIALS AND METHODS

All the liver graft recipients received routine ultrasound

examinations in our sonography department. For this study, we chose a sample of all OLT patients (with or without clinical problems) who received ultrasound examinations between February 2000 and December 2001. Five hundred and eighteen ultrasound examinations on 282 patients were performed during this period. Patients who received at least one ultrasound examination after liver transplantation were included in this study. In the case of multiple ultrasound examinations, one examination was chosen randomly in order to prevent dependencies, due to multiple measurements of a single case. Patients who had acute cardiac or renal failure were excluded (n = 2). Two hundred and sixty consecutive patients were included, and their clinical characteristics are shown in Tables 1 and 2.

All patients were examined with high-end ultrasound equipment Power Vision 8000 (Toshiba, Japan) and Elegra Sonoline Advanced (Siemens, Germany) using convex arrays 3.5C40H (Siemens, Germany) and C 3-6 MHz (Toshiba, Japan) as well as sector array 3-6 MHz (Toshiba, Japan). The patients were examined by two gastroenterologists with more than 20 years of experience in the field of ultrasound.

Systematic B-mode examination of all abdominal organs including the retroperitoneum was performed following the recommendations of the German Association of Ultrasound in Medicine (DEGUM). Liver size was measured by the diameter of the right lobe in the mid clavicular line (MCL). The spleen length was measured from upper to lower pole in an oblique intercostal array position. The normal liver size was defined as 13±0.5 cm[11]. The normal spleen size was defined as 11±0.5 cm[11]. In addition, the portal vein and the bile duct anastomosis were examined in the proximal and distal portion.

The maximum velocity of the portal vein [(P)V_{max}], the maximum systolic velocity [(A)V_{max}], the maximum end diastolic velocity [(A)V_{min}] and the resistance index of the hepatic artery (RI) were measured before and after the anastomosis in each case after an overnight fasting. Settings such as gain, filter, and pulse-repetition frequencies were adjusted as needed for optimal signal detection to prevent artifacts. Segmental arterial stenosis was considered to be present when circumscript aliasing was visualized by color Doppler sonography using the maximal pulse repetition frequency (PRF) as well as the presence of a V_{max} exceeding 170 cm/s. Alternatively, in case of the absence of a segmental stenosis, the detection of a tardus-parvus duplex spectrum with a cut off RI below 0.5 in addition to a V_{max}>170 cm/s was required for the definition of a stenosis. Stenosis of the portal vein required a two-fold increase of flow velocity in the stenosis. Dilated intrahepatic and extrahepatic bile ducts were defined as exceeding 3 and 10 mm in diameter (DEGUM guidelines), respectively.

The complications detected by ultrasound were confirmed by following the gold standard methods. In all patients with suspected stenosis or thrombosis of the hepatic artery by ultrasound examination, a CT-angiography was performed to confirm the diagnosis. Most of the biliary complications such as bile duct dilatation, thickening, stricture, and calculi in the bile duct system were confirmed by endoscopic retrograde cholangiopancreatic ography (ERCP) in patients with chocholedochostomy[12] and by percutaneous transhepatic cholangiography (PTC) in patients with status after hepaticojejunostomy. The occurrence of intrahepatic abscesses was confirmed by biopsy. Liver biopsies after OLT were available from 94 patients (n = 45 acute rejections, n = 2 chronic rejections, n = 2 ischemia, n = 21 viral re-infection, n = 21 fibrosis, n = 7 cirrhosis, n = 33 cholangitis).

Statistical evaluation was performed using the SPSS 11.5 software package for Windows™. Mean values and standard errors of the means (mean±SE) as well as frequencies were calculated. Correlations were done using Spearman's (S) rank correlation coefficient. We also analyzed the changes of the velocity values as well as organ sizes in the course of time. Due to different time points of ultrasound examinations after OLT, we divided the patients in groups with defined time intervals after OLT. The time points were days 100 (1), 1 000 (2), 2 000 (3), 3 000 (4), 4 000 (5) and 5 000 (6) after OLT. The number of patients at the defined time intervals was as follows: n = 20 (1), n = 120 (2), n = 81 (3), n = 48 (4), n = 25 (5), n = 11 (6). At these time points, the data of patients before the defined

Table 1 Clinical characteristics of the patients included in this study

Parameter	Value
Gender (M/F)	165/95
Age (yr)	49.8±0.8
Age (yr)	18.5–74.5
Mean time after OLT (d)	1 523.3±97.6
Mean US evaluation time after OLT (d)	2–8 912
Full size OLT (n)	244
Split liver OLT (n)	16

Table 2 Etiologies of liver diseases leading to OLT

Etiology	Cases (n)
Chronic hepatitis B	37
Primary sclerosing cholangitis	36
Chronic hepatitis C	33
Chronic hepatitis and hepatocellular carcinoma	24
Primary biliary cirrhosis	18
Cryptogenic liver disease	18
Cystic liver degeneration	12
Autoimmune hepatitis	12
Budd-Chiari syndrome	12
Alcoholic cirrhosis	10
Primary hepatocellular carcinoma	8
Wilson's disease	8
Others[1]	32

[1]Includes: Oxalosis n = 1 (0.4%), acute hepatitis A, n = 1 (0.4%), CMV infection, n = 1 (0.4%), veno occlusive disease, n = 1 (0.4%), Halothane induced hepatic failure, n = 1 (0.4%), cystic fibrosis, n = 1 (0.4%), tyrosinemia, n = 1 (0.4%), amyloidosis, n = 2 (0.8%), hemochromatosis, n = 2 (0.8%), toxic liver failure, n = 2 (0.8%), secondary sclerosing cholangitis, n = 3 (1.2%), alpha 1 antitrypsin deficiency, n = 3 (1.2%), Caroli's syndrome, n = 3 (1.2%), adenomatosis of the liver, n = 3 (1.2%), carcinoid disease, n = 3 (1.2%), biliary atresia, n = 4 (1.5%).

time point (days after transplantation) were compared with the data of patients after this time point. Mean values of the parameters were calculated and compared for significance using the *t*-test. Therefore, our data reflected only the changes of the studied parameters at these time points. The changes of the parameters in the group of patients before the defined time points were graphically demonstrated. The graphs were assembled using the Prism 3.0 software package.

Correlations as well as the mean values were calculated with and without extremes. The extremes were defined as stenosis or thrombosis of the portal vein and/or the hepatic artery ($n = 8$).

RESULTS

Frequencies of ultrasound-detected B-mode findings/ complications after OLT

Vascular complications over a 13.7-year observation period were detected in one case (0.4%) of thrombosis and five cases (1.9%) of stenosis of the hepatic artery and in seven cases (2.7%) of stenosis of the portal vein by ultrasound (Table 3). All cases were confirmed by CT angiography.

In a 13.7-year observation period by ultrasound examination, 58 (22.3%) patients showed biliary complications, of them 33 (56.9%) had either ERCP or PTC. In 29 patients (87.9%), ultrasound diagnosis was confirmed either by ERCP or PTC. Nine patients (3.5%) had extrahepatic dilatation and 37 cases (14.2%) had intrahepatic dilatation of the bile ducts. One patient (0.4%) had calculi, 14 (5.4%) patients had sludge in the intra- and extra-hepatic bile ducts. Eighteen patients (6.9%) showed a thickening of intra- and extra-hepatic bile duct walls, two cases (0.8%) had intrahepatic abscesses, one patient (0.4%) had stricture of the main hepatic bile duct, 114 patients (43.8%) had splenomegaly after OLT, and 14 patients (5.4%) had detectable ascites (Table 3).

Changes in organ size after liver transplantation

The mean liver size after OLT was 12.2±0.2 cm in MCL. The mean splenic size after liver transplantation was 12.9 ±0.2 cm (Table 4). Women had a smaller spleen than men (12.2±0.3 cm *vs* 13.4±0.2 cm, *t*; $P<0.01$).

Table 3 Ultrasound findings and complications in liver graft recipients

US findings	US frequency in this study (%)	Reported frequency (%)
Thrombosis of the hepatic artery	0.4	12 [21]
Stenosis of the hepatic artery	1.9	3–5 [8]
Stenosis of the portal vein	2.7	1–6.2 [3,17]
Biliary complications generally	22.3	18 [22]
Bile duct dilatations	17.7	7.3–48.8 [20]
Thickening of bile ducts	6.9	
Abscess	0.8	
Calculi of biliary system	6.2	36.6 [20]
Bile duct stricture	0.4	5–14 [21]
Ascites	5.4	
Splenomegaly	43.8	

US, ultrasound.

Table 4 Ultrasound data (mean±SE)

Parameter	Value	Unit
(P) V_{max}	30.0±1.5	cm/s
(A) V_{max}	67.1±4.2	cm/s
(A) V_{min}	20.4±1.6	cm/s
RI	0.69±0.01	
Spleen size	12.9±0.2	cm
Liver size in MCL	12.2±0.2	cm

The changes of mean values for liver and splenic size 100, 1 000, 2 000, 3 000, 4 000, and 5 000 d after OLT were calculated. The mean values for liver size after OLT were all within the normal range and did not change significantly between the studied time intervals. In contrast, the splenic size decreased between the intervals 100 and 1 000 d after OLT. After this interval, the splenic size increased. Consequently, the spleens in the patients more than 1 000 d after OLT were significantly larger than those in the patients less than 1 000 d after OLT (13.6±0.3 cm *vs* 12.2±0.3 cm, *t*; $P<0.01$). The mean splenic size remained higher than the normal range throughout the observed time in this study.

Dynamics of color Doppler parameters in patients after liver transplantation

The mean value of color Doppler data for all patients is shown in Table 4. There was an inverse correlation between (P) V_{max} and time after OLT ($r = -0.41$, $P<0.001$). After exclusion of patients with stenosis and thrombosis of the arterial and portal vein anastomosis in order to eliminate the velocity extremes, the correlation coefficients still remained high ($r = 0.38$, $P<0.001$). There was also an inverse correlation between (A) V_{max} and (A) V_{min} of the hepatic artery and time after OLT [for (A) V_{max} $r = 0.21$, $P<0.01$ and for (A) V_{min} $r = 0.23$, $P<0.01$]. After exclusion of extremes, the correlation persisted [for (A) V_{max} $r = 0.21$, $P<0.01$ and for (A) V_{min} $r = 0.22$, $P<0.01$]. We could not observe a correlation between RI and time after OLT, but we confirmed a significant correlation between RI and age of the patients ($r = 0.24$, $P<0.001$).

The changes of mean values of all color Doppler parameters were calculated 100, 1 000, 2 000, 3 000, 4 000, and 5 000 d post OLT after exclusion of extremes. (P) V_{max} decreased between the time points 100 and 3 000 d after OLT. It was stabilized at a level of 23 cm/s between the time points 3 000 and 4 000 d after OLT and dropped again from day 4 000 (Figure 1A). (A) V_{max} decreased from 100 up to 4 000 d after OLT (Figure 1B). (A) V_{min} decreased from 2 000 d after OLT (Figure 1B). RI increased between 100 and 2 000 d after OLT. From this time point on, we observed a decrease of RI which was stabilized at a level of 0.70 from time point 4 000 d after OLT to the end of the observation time (Figure 1A).

DISCUSSION

All vascular complications detected by duplex measurements in this study occurred within the first 100 d after

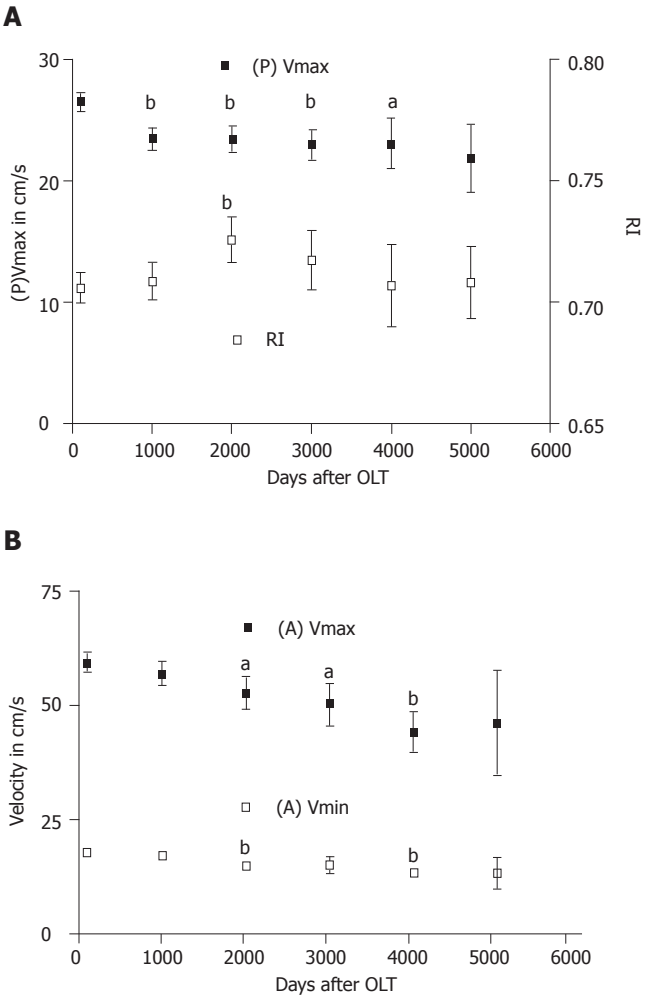

Figure 1 Changes in portal flow velocity and resistance index **(A)** and arterial velocities (A)V_{max} and (A)V_{min} after OLT **(B)** during the course of time after liver transplantation in 260 patients. ($^{a}P<0.05$; $^{b}P<0.01$ *vs* others).

Table 5 Reasons of vascular complications after liver transplantation

Arterial system	
Reperfusion damage	(n = 2)
Thrombosis of the celiac trunk	(n = 1)
Thrombosis of the hepatic artery[1]	(n = 1)
Dissection of the common hepatic artery	(n = 1)
Unknown	(n = 1)
Portal system	
Over average length of the portal anastomosis	(n = 3)
Intraoperative thrombectomy[2]	(n = 2)
Intraoperative thrombectomy of malign thrombus[3]	(n = 1)
Leakage of the biliary anastomosis and consequently	(n = 1)
Systemic infection leading to portal thrombosis	

[1]The patient suffered from fulminant acute hepatitis A with thrombosis of the arterial and portal vascular system at the time of OLT. [2]The patients had Budd-Chiari syndrome as an underlying liver disease. [3]In this particular case, infiltration of a tumor thrombus in the portal venous trunk was observed which led to portal vein stenosis after OLT.

liver transplantation (Table 5). Due to this information as well as the fact that complications such as rejection appear mostly during the early phase after OLT, drastic long-term hemodynamic changes in these patients are not expected. All vascular complications were initially diagnosed by ultrasound examination. Interestingly, a satisfactory blood flow was observed intraoperatively, but these patients developed stenosis after OLT. Routine and protocol ultrasound examinations during this interval are therefore recommended[13,14]. The flow characteristics of patients early after OLT have been reported in prospective studies[6,15]. We therefore focused our study on the long-term changes of these parameters.

The long-term hemodynamic changes in these patients seem to be influenced by factors in the graft itself or alteration of the vascular track. The prospective study of Bolognesi *et al.*[6] is the largest follow-up study investigating the hemodynamic changes in patients after OLT. Compared to this study, our study showed a more heterogeneous spectrum of the underlying liver diseases. Our findings showed that the mean liver size remained within the normal range, independent of the complications, even many years after OLT. Based on the results of liver biopsies after OLT, at least 20% of the patients included in this study had parenchymal changes such as cirrhosis or fibrosis which consequently leads to a smaller liver size. Interestingly, the mean values of liver size still remained stable in the course of studied time points. The mean splenic size was smaller than that of previously reported[6] which may be explained by the heterogeneity of liver diseases with a considerable amount of transplantations without portal hypertension.

Depending on the evaluation time after OLT, an initial increase in portal blood flow in patients with cirrhosis has been reported, which was normalized within 2 years[6]. We observed a decrease of portal blood flow from 100 d to 8.2 years after OLT. This stable decrease may also be influenced by the low prevalence of portal stenosis and thrombosis in our patients. We also detected a decrease of arterial velocities over a long period of time after OLT. This could be caused by the normalization of hyperdynamic circulatory syndromes of patients with cirrhosis at the time of OLT. While the role of arterial RI in patients after kidney transplantation has been extensively studied, the RI changes in liver graft recipients are still unclear. It was reported that a high arterial RI after kidney transplantation is associated with poor subsequent allograft performance and death[16]. In liver graft recipients, an early increase in hepatic arterial resistance has been reported[6,17], which is related to older donor age and prolonged period of ischemia. Higher RI values were found in older patients in our study. RI increase is also attributed to the elevation of portal blood flow early after OLT[6]. In our study, RI did not correlate with portal flow.

Our data have confirmed the reported rate of ultrasound detected complications in patients after liver transplantation. However, the sensitivity of ultrasound for detection of biliary complications is better than that of previously reported[12]. Arterial thrombosis after transplantation has an estimated incidence of 12%[8]. In our patients, thrombosis of the hepatic artery was detected by ultrasound in 0.4% of cases. Since the majority of arterial thromboses occur during the early post-transplantation

period[3] and most of our examinations were performed 100 or more days after OLT, the low prevalence of arterial thrombosis in this study may be related to the time point of investigation. The detection rate of hepatic artery stenosis as well as portal vein thrombosis in our study was comparable to the reported incidence[8,18,7]. The detection rate of biliary tract complications in this study (22.3%) is higher than the reported incidence approaching 18%[19]. Regarding the cases, which were confirmed by ERCP or PTC in our study, ultrasound has a sensitivity of 88%[12].

This study provides an insight into time point of vascular complications as well as hemodynamic and parenchymal adaptive processes present in liver graft recipients. The cross sectional characteristic of our study did not allow a continuous assessment of hemodynamic changes in the course of time, but it enables the analysis of a very long observation period. These data can serve as an orientation for the examining physicians to have a better understanding and interpretation of duplex changes in these patients.

REFERENCES

1 **Nashan B**, Luck R, Becker T, Grannas G, Strassburg C, Schneider A, Melter M, Strassburg A, Klempnauer J. **Expansion** of the donor pool in liver transplantation: the Hannover experience 1996-2002. *Clin Transpl* 2002**; : 221-228**

2 **Brems JJ**, Hiatt JR, Colonna JO 2nd, el-Khoury G, Quinones WJ, Ramming KP, Ziomek S, Busuttil RW. **Variables** influencing the outcome following orthotopic liver transplantation. *Arch Surg* 1987; **122**: 1109-1111

3 **De Gaetano AM**, Cotroneo AR, Maresca G, Di Stasi C, Evangelisti R, Gui B, Agnes S. **Color Doppler sonography in the** diagnosis and monitoring of arterial complications after liver transplantation. *J Clin Ultrasound* 2000; **28:** 373-380

4 **Langnas AN**, Marujo W, Stratta RJ, Wood RP, Shaw BW Jr. Vascular complications after orthotopic liver transplantation. *Am J Surg* 1991**; 161:** 76-82; discussion 82-83

5 **Greif F**, Bronsther OL, Van Thiel DH, Casavilla A, Iwatsuki S, Tzakis A, Todo S, Fung JJ, Starzl TE. **The incidence, timing,** and management of biliary tract complications after orthotopic liver transplantation. *Ann Surg* 1994**; 219:** 40-**45**

6 **Bolognesi M**, Sacerdoti D, Bombonato G, Merkel C, Sartori G, Merenda R, Nava V, Angeli P, Feltracco P, Gatta A.**Change in** portal flow after liver transplantation: effect on hepatic arterial resistance indices and role of spleen size. *Hepatology* 2002; **35**: 601-608

7 **Kok T**, Boeve WJ, Prins TR, Baarslag HJ, Woesthuis M, Slooff MJ, Haagsma EB, Bijleveld CM, van der Jagt EJ. **Arteriography** and portal venography on routine follow-up after orthotopic liver transplantation. *Invest Radiol* 2000; **35:** 653-660

8 **Pinna AD**, Smith CV, Furukawa H, Starzl TE, Fung JJ. **Urgent** revascularization of liver allografts after early hepatic artery thrombosis. *Transplantation* 1996; **62:** 1584-1587

9 **Dalgic A**, Dalgic B, Demirogullari B, Ozbay F, Latifoglu O, Ersoy E, Mahli A, Ilgit E, Ozdemir H, Arac M, Akyol G, Tatlicioglu E.**Clinical approach to graft hepatic artery thrombosis** following living related liver transplantation. *Pediatr Transplant* 2003; **7:** 149-152

10 **Shaw AS**, Ryan SM, Beese RC, Sidhu PS. **Ultrasound of non-**vascular complications in the post liver transplant patient. *Clin Radiol* 2003**; 58:** 672-**680**

11 **Schmidt G**. Ultraschall Kursbuch. 4 ed. Stuttgart: Thieme Verlag, 2003

12 **Hussaini SH**, Sheridan MB, Davies M. **The predictive value** of transabdominal ultrasonography in the diagnosis of biliary tract complications after orthotopic liver transplantation. *Gut* 1999; **45:** 900-903

13 **Heffron TG**, Emond JC, Whitington PF, Thistlethwaite JR Jr, Stevens L, Piper J, Whitington S, Broelsch CE.**Biliary** complications in pediatric liver transplantation. A comparison of reduced-size and whole grafts. *Transplantation* 1992; **53:** 391-395

14 **Nishida S**, Kato T, Levi D, Naveen M, Thierry B, Vianna R, Selvaggi G, Buitorago E, Al-Niami A, Nakamura N, Vaidya A, Nery J, Tzakis A. **Effect of protocol Doppler ultrasonography** and urgent revascularization on early hepatic artery thrombosis after pediatric liver transplantation. *Arch Surg* 2002 137; **11**: 1279-**1283**

15 **Piscaglia F**, Zironi G, Gaiani S, Mazziotti A, Cavallari A, Gramantieri L, Valgimigli M, Bolondi L.**Systemic and splanchnic** hemodynamic changes after liver transplantation for cirrhosis: a long-term prospective study. *Hepatology* 1999; **30;** 58-64

16 **Radermacher J**, Mengel M, Ellis S, Stuht S, Hiss M, Schwarz A, Eisenberger U, Burg M, Luft FC, Gwinner W, Haller H. **The** renal arterial resistance index and renal allograft survival. *N Engl J Med* 2003; **349**: 115-124

17 **Garcia-Criado A**, Gilabert R, Salmeron JM, Nicolau C, Vilana R, Bianchi L, Bunesch L, Garcia-Valdecasas JC, Rimola A, Bru C.**Significance of and contributing factors for a high** resistive index on Doppler sonography of the hepatic artery immediately after surgery: prognostic implications for liver transplant recipients. *AJR Am J Roentgenol* 2003; **181**: 831-838

18 **Molmenti EP**, Levy MF, Molmenti H, Casey D, Fasola CG, Hamilton WM, Jung G, Marubashi S, Gogel BM, Goldstein RM, Gonwa TA, Klintmalm GB.**Correlation between** intraoperative blood flows and hepatic artery strictures in liver transplantation. *Liver Transpl* 2002; **8**: 160-163

19 **Gomez R**, Moreno E, Castellon C, Gonzalez-Pinto I, Loinaz C, Garcia I.**Choledochocholedochostomy conversion to** hepaticojejunostomy due to biliary obstruction in liver transplantation. *World J Surg* 2001**; 25**: 1308-**1312**

20 **Huang DZ**, Le GR, Zhang QP, Li KY, Ye QF, Zhu W, Chen YC.**The value of color Doppler ultrasonography in monitoring** normal orthotopic liver transplantation and postoperative complications. *Hepatobiliary Pancreat Dis Int* 2003; **2**: 54-58

21 **Sanchez-Urdazpal L**, Gores GJ, Ward EM, Maus TP, Wahlstrom HE, Moore SB, Wiesner RH, Krom RA.Ischemic-type biliary complications after orthotopic liver transplantation. *Hepatology* 1992; **16**: 49-53

22 **Busuttil RW**, Goldstein LI, Danovitch GM, Ament ME, Memsic LD. **Liver transplantation today.** *Ann Intern Med* 1986; **104**: 377-389

23 **Wolfsen HC**, Porayko MK, Hughes RH, Gostout CJ, Krom RA, Wiesner RH. **Role of endoscopic retrograde cholangiop-**ancreatography after orthotopic liver transplantation. *Am J Gastroenterol* 1992; **87:** 955-960

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• *CLINICAL RESEARCH* •

Improvement of regional cerebral blood flow after oral intake of branched-chain amino acids in patients with cirrhosis

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Abstract

AIM: To evaluate the effect of oral intake of branched-chain amino acids (BCAA) on brain perfusion in patients with liver cirrhosis.

METHODS: Single photon emission computed tomography scans were performed in 43 patients with cirrhosis and in 15 age-matched healthy subjects. Twenty-nine out of forty-three patients were randomly treated with either BCAA granules or placebo, and single photon emission computed tomography was performed before and after the treatment. We measured the regional cerebral blood flow values using a three-dimensional stereotaxic region of interest template.

RESULTS: Cirrhotic patients had regions of significant hypoperfusion in the bilateral central (right $P = 0.039$, $P<0.05$; left $P = 0.006$ $P<0.01$), parietal (right $P = 0.018$, $P<0.05$; left $P = 0.009$, $P<0.01$), angular (right $P = 0.039$, $P<0.05$; left $P = 0.008$, $P<0.01$), and left pericallosal segments ($P = 0.038$ P<0.05) as compared with healthy subjects. A significant increase in cerebral perfusion was observed 70 min after the oral intake of BCAA in the angular (right $P = 0.012$, $P<0.05$; left $P = 0.049$, $P<0.05$), temporal (right $P = 0.012$, $P<0.05$; left $P=0.038$, $P<0.05$), pericallosal segments (right $P = 0.025$, $P<0.05$; left $P = 0.049$, $P<0.05$) and left precentral ($P = 0.044$, $P<0.05$), parietal ($P = 0.040$, $P<0.05$) and thalamus ($P = 0.033$, $P<0.05$). No significant change in perfusion was observed in the placebo group.

CONCLUSION: Administration of BCAA rapidly improves cerebral perfusion.

INTRODUCTION

Beneficial effects of branched-chain amino acids (BCAA) supplementation on hepatic encephalopathy (HE) have been previously reported[1-3]. Recently, Marchestini *et al.* carried out a large multicenter, randomized controlled trial with BCAA-enriched dietary supplements in comparison with lactoalbumin or maltodextrin dietary supplementation[4]. The results of this study showed reduced hospital admission rate in patients treated with BCAA compared with the control group[4]. Thus, BCAA therapy may be effective for the treatment of HE, which is a common cause of hospital admission.

Some cirrhotic patients with apparently normal mental status may have abnormalities in cognitive function when they are examined with sensitive and quantitative neuropsychological tests[5,6]. This group of patients is considered to have minimal HE[7]. This HE-associated cognitive impairment may be sometimes associated with a poor quality of life[8-10] and thus early diagnosis and treatment of this condition is important[8]. It has been reported that therapy with lactulose improves neuropsychological functions[11]. However, there are no data on whether BCAA supplementation ameliorates cerebral disturbance in cirrhotic patients.

Single photon emission computed tomography (SPECT) and positron emission tomography studies can demonstrate alterations in regional cerebral blood flow (CBF) and cerebral glucose metabolism in cirrhotic patients[12-19]. We previously reported that administration of solutions enriched with BCAA improves cerebral perfusion in patients with cirrhosis[20]. However, the BCAA-rich solutions used in this previous study in cirrhotic patients contained L-arginine, which is a precursor of nitric oxide, a potent vasodilator[21]. The dose of L-arginine used in previous study might have increased blood flow in the brain[21].

The main aim of the present study was to confirm regional differences in CBF in patients with liver cirrhosis and to evaluate whether alterations in CBF is reversed by the oral administration of BCAA. In this study, we used granules containing only BCAA and a three-dimensional stereotaxic region of interest template (SRT) for objective estimation of anatomically standardized CBF SPECT images[22,23].

MATERIALS AND METHODS

Subjects

Forty-three Japanese patients with liver cirrhosis (30 men and 13 women, mean age 63±8 years) were enrolled in this study. The diagnosis of cirrhosis was based on the results of liver function tests, ultrasonography, computed tomography imaging, laparoscopy and liver biopsy. The cause of liver cirrhosis was viral infection in the majority of patients (n = 37). No patient with alcoholic liver disease was included in the study. In six patients, the cause of liver cirrhosis was unclear. None of the patients had overt HE (grade I or more) at the time of the examination, and none of them exhibited neuropsychiatric signs or symptoms on standard bedside clinical assessment. Patients with focal brain lesions, severe brain atrophy, abnormalities on computed tomography or magnetic resonance images, or neurological or psychiatric disorders were excluded from the study. None of the patients were receiving psychoactive drugs. Twenty-nine out of forty-three patients were randomized into two groups: one group received BCAA granules orally (16 patients) and another group received placebo (13 patients). We have previously reported that the ratio of serum BCAA to tyrosine increases nearly twofold, 1 h after the administration of oral BCAA and that it decreases to basal values after 10 h[24]. The clinical and biochemical characteristics of the patients are summarized in Table 1.

Control SPECT images were obtained from 15 subjects (11 men and 4 women; mean age, 62±9 years) referred to our neurology department for minor subjective symptoms. These subjects were free of liver disease, neurological disorder or dementia and had normal brain magnetic resonance images. The control subjects were not taking any medication.

Informed consent was obtained from all subjects, and the study was performed in accordance with the Helsinki Declaration. The Ethics Committee of Mie University School of Medicine approved the protocol of this study.

Table 1 Patients' clinical characteristics

Age (yr)	63 ± 8	(48 - 74)
Sex ratio, M/F	30/13	
Etiology of cirrhosis, HBV/HCV/unknown	5/32/6	
Previous history of overt hepatic encephalopathy,		
None/Chronic	39/4	
Child-Pugh score	8.0 ± 2.2	(5 -13)
Laboratory examinations		
Platelet (10^4 μL)	7.5 ± 5.1	(8 - 31.4)
Albumin (g/dL)	2.9 ± 0.5	(1.9 - 3.9)
Total bilirubin (mg/dL)	2.0 ± 2.3	(0.5 - 13.8)
Cholinesterase (ΔpH)	0.36 ± 0.19	(0.11 - 0.97)
Plasma ammonia (μmol/L)	38 ± 23	(4 - 104)
Prothrombin time (%)	69.1 ± 12.8	(31.3 - 93.5)
BCAA to tyrosine ratio	3.3 ± 1.3	(1.32 - 6.95)
Neuropsychological test		
Trail making test (s)	54 ± 26	(28 - 160)
Digit symbol test (gross point)	35 ± 10	(12 - 54)

BCAA, branched-chain amino acids.

Scan acquisition and image processing

Each subject received 278 MBq of technetium-99 m L,L-ethyl cysteinate dimer (ECD) by intravenous injection in the morning after an overnight fast. Ten minutes after the injection of ECD, brain SPECT images were acquired using a three-head gammacamera system (GCA-9300A/DI, Toshiba, Tokyo, Japan) equipped with low-energy, high-resolution fanbeam collimators. The projection data were obtained using a matrix size of 128×128. SPECT images were reconstructed by filtered back-projection using a ramp filter follower by postprocessing with a Butterworth filter. Attenuation correction was performed using Chang's method[25]. The triple-energy window technique was employed for scatter correction. After baseline SPECT, the patients were orally treated with BCAA granules (8 g BCAA, 8 g protein, 32 kcal, Ajinomoto, Tokyo, Japan: L-isoleucine 1 904 mg, L-leucine 3 808 mg, L-valine 2 288 mg), or placebo (cornstarch, 8 g protein, 32 kcal). After 60 min, 278 MBq of ECD was intravenously administered, and 10 min later a second SPECT acquisition was performed. The methods are briefly summarized in Figure 1.

Image analysis

The spatial normalization was performed using linear and non-linear transformation and SPECT template in the statistical parametric mapping (SPM) 99 (Wellcome Department of Cognitive Neurology, London, UK)

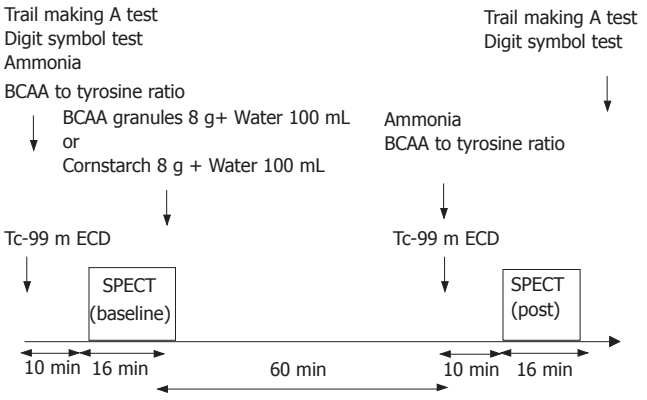

Figure 1 Study protocol. Baseline SPECT imaging was taken 10 min after the injection of 278 MBq ECD. After baseline SPECT, BCAA granules or placebo were orally administered. Sixty minutes later, another 278 MBq of technetium-99 m ECD was intravenously administered and a second SPECT acquisition was performed 10 min after the injection of ECD. Laboratory and neuropsychological tests were taken before and after the administration of BCAA or placebo. SPECT, single photon emission computed tomography; ECD, ethyl cysteinate dimer; BCAA, branched-chain amino acid

program. Smoothing was performed using 12 mm full width at half maximum Gaussian filter in SPM99. To obtain post-BCAA counts, baseline mean SPECT counts were subtracted from the second SPECT counts, multiplied by a correction factor, which is the coefficient of the decay of technetium-99 m between the baseline and post-treatment measurement. In each hemisphere, we estimated the regional CBF values of 270 constant regions of interest (ROI, three-dimensional SRT) grouped into 12 segments as follows: callosomarginal, 48 ROI; precentral, 45 ROI; central, 28 ROI; parietal, 14 ROI; angular, 8 ROI; temporal, 27 ROI; posterior cerebral, 33 ROI; pericallosal, 16 ROI; lenticular nucleus, 12 ROI; thalamus, 11 ROI; hippocampus, 17 ROI; and cerebellum, 11 ROI; and the segmental CBF was calculated as the area-weighed mean value for each of the 12 segments based on the regional CBF of each ROI[22,23].

Semiquantitative analysis was performed to obtain region-to-reference ratios for each segmental CBF value. The CBF value in cerebellum was selected as the reference region, because cerebellar abnormalities were not detected in SPECT images, computed tomography or magnetic resonance images.

Neuropsychological tests and laboratory examinations

The trail making A test (number connection test) and digit symbol test (revised Wechsler adult intelligence scale) were performed as neuropsychological tests. Laboratory examinations included plasma ammonia and BCAA to tyrosine ratio. These data were taken before and after the administration of BCAA or placebo (Figure 1).

Statistical analysis

Results were expressed as the mean±SD of the mean and range. The Mann–Whitney *U* test was used to evaluate the statistical difference in clinical or laboratory variables between BCAA and placebo groups and in CBF between patients and healthy subjects. The analysis of variance with Bonferroni's correction for multiple comparisons in the three groups was analyzed. The Wilcoxon was used to compare pre-, post-BCAA or -placebo values in the same group of patients. A $P<0.05$ was considered as statistical significance.

RESULTS

Baseline study

Cirrhotic patients ($n = 43$) had regions of significant hypoperfusion in the bilateral central (right, $P<0.05$; left, $P<0.01$), parietal (both, $P<0.05$), angular (right, $P<0.05$; left, $P<0.01$), and left pericallosal segments ($P<0.05$) as compared with healthy subjects ($n = 15$, Figure 2).

The influence of the clinical profile on regional CBF was also evaluated. There were no significant differences in cerebral perfusion between the mild (Child–Pugh A, $n = 13$), moderate/severe (Child–Pugh B+C, $n = 30$) liver dysfunction groups and healthy subjects (data not shown). In cirrhotic patients with hyperammonemia (i.e., more than 50 μm/L, $n = 15$), SRT showed regions of significant hypoperfusion in the right parietal ($P<0.05$) and left callosomarginal ($P<0.05$), central ($P<0.01$), angular ($P<0.01$) and pericallosal segments ($P<0.01$) as compared to healthy subjects (Figure 3A, right hemisphere is not shown). Likewise, in cirrhotic patients with severe decrease of serum BCAA to tyrosine ratio (i.e., <3, $n = 19$), SRT showed regions of significant hypoperfusion in the angular (right, $P<0.05$; left, $P<0.01$) and right parietal ($P<0.05$) and left callosomarginal ($P<0.01$), central ($P<001$) and pericallosal segments ($P<0.01$) as compared to healthy subjects (Figure 3B, right hemisphere is not shown).

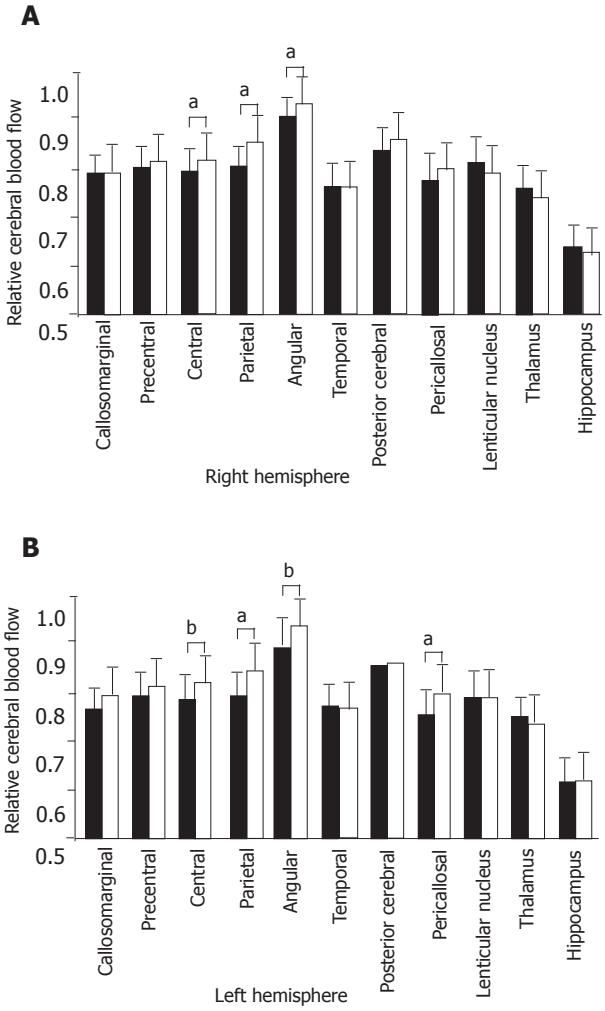

Figure 2 Relative CBF of different brain regions. Comparison of patients with liver cirrhosis (closed bars) and healthy subjects (open bars). Data were expressed as the mean±SD. [a]$P<0.05$, [b]$P<0.01$, [d]$P<0.001$. CBF, cerebral blood flow.

Abnormalities in neuropsychological tests [values more than two SD from the mean values for the age-matched healthy subjects at our hospital (i.e., more than 50 s on the trail making A test and less than 30 points on digit symbol test)] were considered to be indicative of minimal HE. Among cirrhotic patients, 10 showed abnormalities in both neurological tests and thus they were considered to have minimal HE. In patients with abnormalities in neurological tests ($n = 10$), SRT showed significant hypoperfusion in the left parietal ($P<0.01$), pericallosal ($P<0.01$), lenticular nucleus ($P<0.01$) and hippocampus ($P<0.05$) regions as compared to patients with grade-0 HE ($n = 14$, Figure 3C,

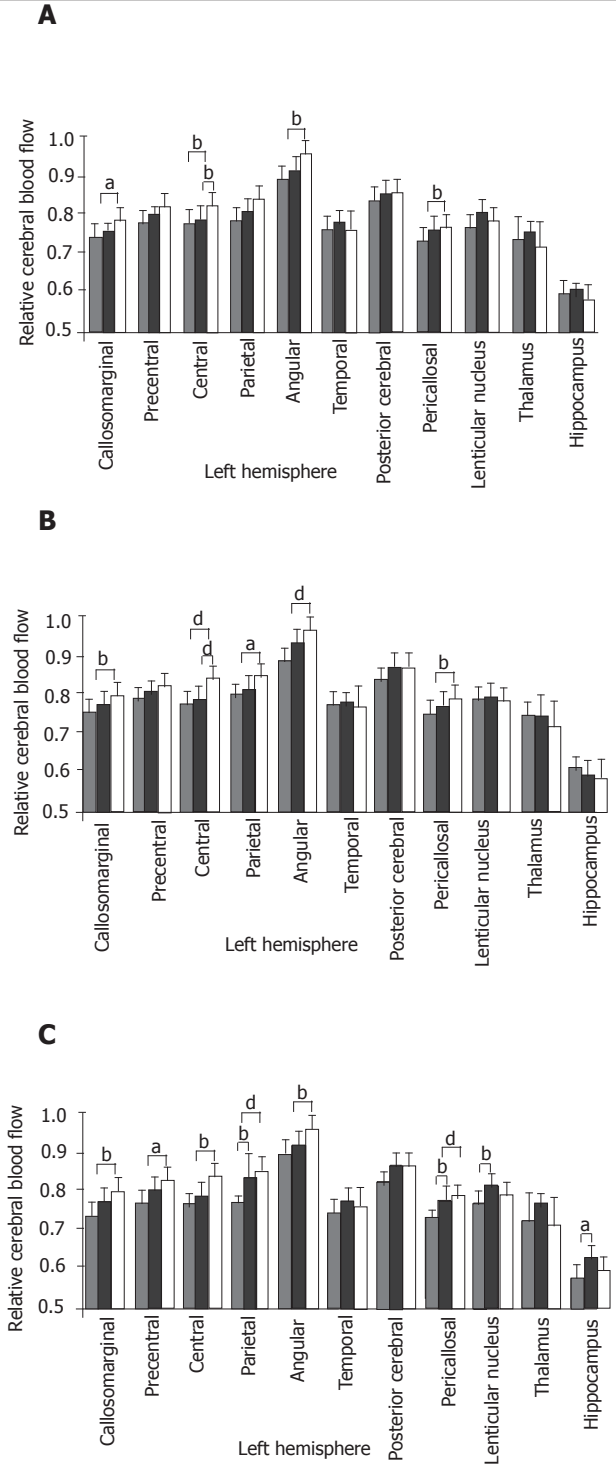

Figure 3 Relative CBF of different brain regions. **A:** Comparison of cirrhotic patients with hyperammonemia (gray bars), normal ammonemia (dashed bars) and healthy subjects (open bars); **B:** Comparison of cirrhotic patients with severe decrease in the levels of serum BCAA to tyrosine ratio (gray bars), with mild decrease in serum BCAA to tyrosine ratio (dashed bars) and healthy subjects (open bars); **C:** Comparison of patients with minimal encephalopathy (gray bars), with grade-0 encephalopathy (dashed bars) and healthy subjects (open bars).

right hemisphere is not shown).

Effect of BCAA

At entry, there was no difference in clinical or laboratory variables between BCAA (n = 16) and placebo (n = 13) groups (Table 2). A significant increase in cerebral perfusion was observed 70 min after oral intake of BCAA in the angular (both regions, P<0.05, Figure 4C), temporal (both regions, P<0.05, Figure 4D), pericallosal segments (both regions, P<0.05, Figure 4E) and left precentral (P<0.05, Figure 4A), parietal (P<0.05, Figure 4B) and thalamus (P<0.05, Figure 4F). In addition, after the administration of oral BCAA, the values of relative CBF improved in almost all segments, reaching values observed in healthy subjects. There were no significant differences in cerebral perfusion between cirrhotic patients after oral intake of BCAA and healthy subjects. No significant change in relative CBF values was observed in the placebo group (Figure 5). In the BCAA group, the serum BCAA to tyrosine ratio increased fourfold, 86 min after the administration of oral BCAA (P<0.01). No significant change in plasma ammonia levels or in neuropsychological tests was observed in BCAA and placebo groups (Table 3).

DISCUSSION

Functional imaging techniques such as CBF SPECT and positron emission tomography can demonstrate abnormalities in patients with cirrhosis[12-19]. In the present study, cirrhotic patients had regions of significant hypoperfusion in central, parietal, angular and pericallosal segments as compared to healthy subjects. These areas included parts of the frontal and parietal associated areas of the cortex and cingulum. Impaired flow and oxygen metabolism in the frontal, parietal and cingulate cortices in cirrhotic patients have also been reported[12-14,16-18]. Cognitive impairment, especially defect in attention is an important feature of HE[26,27]. The anterior cingulate gyri may provide an important connection between widely divergent aspects of attention and visual location[28]. The internal organization of the anterior cingulate gyri shows alternating bands of cells with close connections to the dorsolateral frontal cortex and the posterior parietal lobe[29]. The results of studies using N-13 ammonia positron emission tomography of cerebral ammonia metabolism in patients with cirrhosis and minimal encephalopathy coincide well with these regional differences[30]. This regional hypoperfusion may be the pathophysiological basis for the minimal cerebral dysfunction that is often detected by neuropsychological tests in patients with cirrhosis.

We found no significant relationship between cerebral perfusion and severity of liver disease, as assessed by the Child–Pugh scores. This observation may be due to the fact that non-hepatic factors, such as neurotoxins produced in the gut or toxic agents that cross the blood-brain barrier such as ammonia, contribute to impairment of cerebral function. Although no single metabolic derangement can account for the occurrence of HE, the plasma level of ammonia is believed to be an important causative factor of HE. Lockwood *et al*. reported regional metabolic abnormalities in cirrhotic patients with hyperammonemia[16]. They also reported increased permeability of the blood-brain barrier to ammonia and suggested that ammonia might be responsible for cerebral dysfunction in HE[31]. In

Table 2 Clinical characteristics of BCAA and placebo groups

	BCAA group		Placebo group	
Age (yr)	64 ± 9	(50 - 72)	65 ± 8	(51 - 74)
Sex ratio, M/F	10/6		9/4	
Etiology of cirrhosis, HBV/HCV/unknown	1/11/4		0/12/1	
Previous history of overt hepatic encephalopathy, None/Chronic	14/2		12/1	
Child-Pugh score	8.1 ± 2.5	(5 - 13)	7.8 ± 2	(5 - 11)
Laboratory examinations				
Platelet (10^4 μL)	8.3 ± 6.8	(3.4 - 31.4)	7.6 ± 3.1	(2.8 – 12.3)
Albumin (g/dL)	2.8 ± 0.5	(2.1 - 3.9)	2.9 ± 0.4	(2.4 - 3.9)
Total bilirubin (mg/dL)	2.2 ± 3.2	(0.8 - 13.8)	1.9 ± 1.8	(0.5 – 6.9)
Cholinesterase (ΔpH)	0.32 ± 0.16	(0.12 - 0.64)	0.38 ± 0.12	(0.17 - 0.55)
Plasma ammonia (μmol/L)	35 ± 20	(9 - 79)	34 ± 18	(7 - 74)
Prothrombin time (%)	68.9 ± 14.4	(31.3 - 93.5)	71.1 ± 13.4	(51.8 – 89.2)
BCAA to tyrosine ratio	3.7 ± 1.2	(2.3 - 7)	3 ± 1.3	(1.3 – 5.6)
Neuropsychological test				
Trail making test (s)	49 ± 16	(34 - 77)	50 ± 16	(25 - 72)
Digit symbol test (gross point)	38 ± 10	(21 - 53)	37 ± 15	(19 - 63)

BCAA, branched-chain amino acids.

Table 3 Changes in laboratory and neuropsychological tests after BCAA and placebo administration

	BCAA group				Placebo group			
	Before		After		Before		After	
Laboratory variables								
Plasma ammonia (μM/L)	35 ± 20	(9 - 79)	31 ± 14	(8 - 54)	36 ± 20	(7 - 74)	37 ± 14	(17 - 58)
BCAA to tyrosine ratio	3.7 ± 1.2	(2.3 - 7)	16.7 ± 3.4[b]	(13.1 - 24.6)	3 ± 1.3	(1.3 - 5.6)	3.1 ± 1.3	(1.6 - 5.7)
Neuropsychological test								
Trail making test (s)	49 ± 16	(34 - 77)	47 ± 13	(30 - 72)	50 ± 16	(25 - 72)	51 ± 15	(29 - 75)
Digit symbol test (goss point)	38 ± 10	(21 - 53)	45 ± 14	(21 - 71)	37 ± 15	(19 - 63)	40 ± 14	(23 - 61)

[b]$P < 0.01$ BCAA, branched-chain amino acids

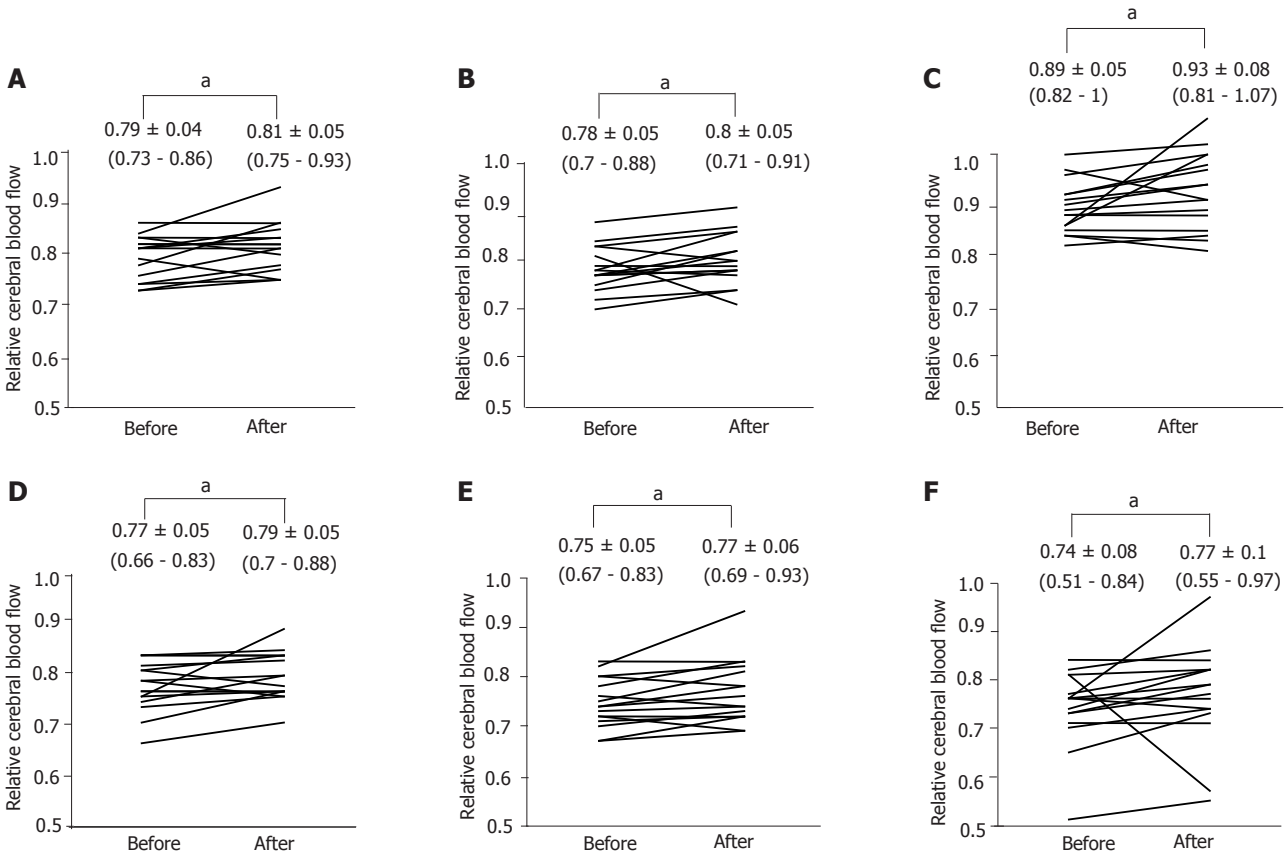

Figure 4 Changes in relative CBF of different brain regions. CBF was compared before and after BCAA. **A:** Left precentral; **B:** left parietal; **C:** left angular; **D:** left temporal; **E:** left pericallosal; **F:** left thalamus. Data were expressed as the mean±SD and range. [a]$P<0.05$V *vs* CBF, cerebral blood flow; BCAA, branched-chain amino acid.

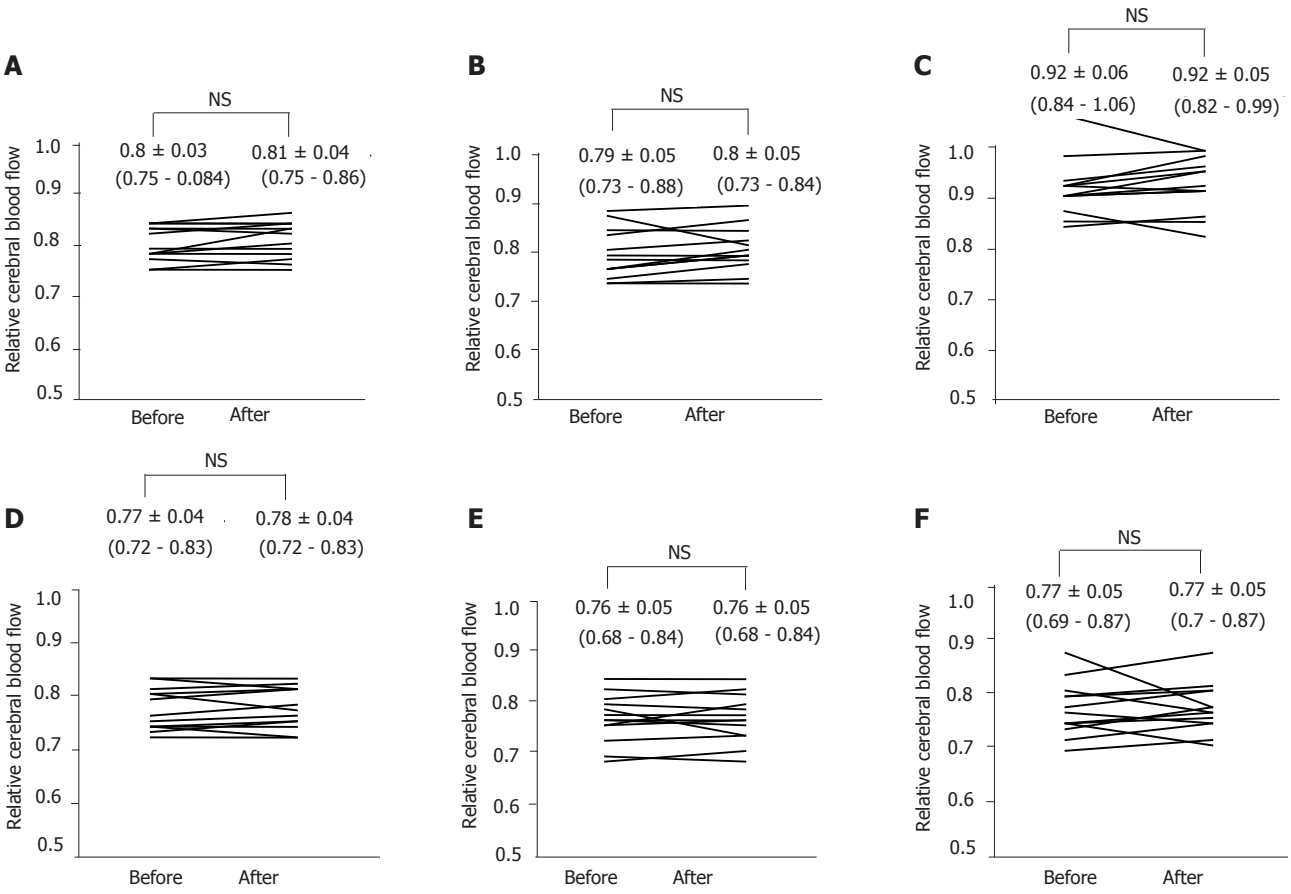

Figure 5 Changes in relative CBF of different brain regions. CBF was compared before and after placebo. **A:** Left precentral; **B:** left parietal; **C:** left angular; **D:** left temporal; **E:** left pericallosal; **F:** left thalamus. Data were expressed as the mean±SD and range. CBF, cerebral blood flow.

our patients with hyperammonemia, SRT analysis showed several segments, including the cingulum, with significant decreased perfusion as compared to healthy subjects. This finding supports the hypothesis of Lockwood *et al*. In addition, SPECT with SRT analysis showed a significant reduction in relative CBF values in cirrhotic patients with minimal HE as compared to patients with grade-0 HE and normal subjects. This result is also consistent with the observation of Lockwood *et al*.[18]. Moreover, we found that the relative CBF values are significantly correlated with the baseline venous BCAA to tyrosine ratio. Relationship between CBF values and the serum BCAA to tyrosine ratio has not been previously assessed.

There are several studies in which stereotaxic ROI analysis was used[32,33], but in all of them the ROI values were transformed to fit the subjects' individual anatomical arrangements. Inter-individual anatomical variations may exist giving non-consistent relationship between ROI location and anatomy. In the present study, we used a fully automated regional CBF quantification software, SRT. This incorporates an anatomical standardization engine transplant into SPM99 and ROI for quantification on the Montreal Neurological Institute space of the magnetic resonance image anatomically standardized by SPM99. We believe that our results are valid due to the accuracy of the SRT analysis.

We took the cerebellum as reference for the semiquantitative analysis, because brain segments of cirrhotic patients exhibit variable perfusion values whose distribution may affect the validity of the uptake ratios. We previously reported that there is no alteration in cerebellar perfusion in cirrhotic patients as analyzed by SPM with ECD SPECT[13]. In addition, the absence of symptoms and the normal appearance of cerebellar perfusion in our patients led us to consider the cerebellum as the best reference to evaluate regional CBF. However, cerebellar hypermetabolism has been observed in cirrhotic patients with cerebellar degeneration[30], and thus it may be difficult to deal with methodological limitations in such region-to-cerebellar ratios as it was in this study. To evaluate the effect of BCAA, repeated SPECT studies are necessary. In the pre/post studies, the assumption is that the original distribution pattern is still the same during the second SPECT image. Moretti *et al*. reported that during the 50-120 min postinjection period, the regional structures are washing out at the same rate[3]; however, there might be differences between cirrhotic and normal brain. This may be a potential pitfall in split-dose and sequential SPECT method with ECD.

The mechanism by which BCAA granules rapidly improve relative CBF in cirrhotic patients without overt HE is unknown. The rationale for BCAA therapy is based

on the results of studies showing that the use of solutions rich in BCAA reversed the abnormal blood levels of amino acids and led to mental recovery from acute HE in patients with cirrhosis[35,36]. Mental recovery is observed immediately after the treatment with BCAA solution. One of the proposed mechanisms of HE is the interference of cerebral metabolism by ammonia, including depletion of operational rates of tricarboxylic acid cycle by removing α-ketoglutarate for ammonia detoxification[37]. Decreased brain levels of glutamate have been reported in various models of HE. Glutamate is an integral component of malate-aspartate shuttle. It is therefore possible that reduced glutamate levels contribute to impaired cerebral energy metabolism in HE[38]. BCAA is known to cross rapidly the blood-brain barrier and to serve as an energy source in the brain[39,40]. It has been previously shown in animal models of chronic HE that decreased brain BCAA concentrations is normalized and that acceleration of ammonia metabolism occurs by stimulated glutamine synthesis, after intravenous infusion of BCAA[41]. Thus, it is possible that administration of BCAA blocks the vicious cycle of cerebral energy metabolism in HE by providing the amino group for glutamate synthesis from a-ketoglutarate in astrocytes.

In the present study, we found that cirrhotic patients increased thalamic perfusion after the administration of oral BCAA. According to current knowledge, information coming from the cortex passes through the striatopallidal system to the thalamus and then returns to the cortex. Alterations of neurotransmission within the pallidum and thalamus therefore may lead to impairment of cortical function, as in HE[42]. Catafau *et al.* reported that thalamic CBF increases in proportion to neuropsychological deficits as a compensatory effect[12]. Supplementation with BCAA may be effective in the treatment of cirrhotic patients for improving thalamic CBF.

A number of studies have reported association between minimal HE and impairment of quality of life[5,6,8-10]. It has also been suggested that the ability to drive a car is impaired in patients with cirrhosis and minimal HE[43]. Therefore, it is important to initiate therapy to improve neuropsychological function. Protein restriction may not be beneficial for long-term therapy in patients with protein malnutrition. Oral BCAA supplementation may be one of the candidates for the initial treatment of minimal HE. In the present study, there was no significant change in the results of neuropsychological test after the administration of BCAA. Additional study is necessary to clarify the time course of regional CBF changes after oral administration of BCAA. Neuropsychological performance and cerebral perfusion after long-term oral administration of BCAA also needs further evaluation.

In conclusion, this study shows that patients with cirrhosis and no neurologic symptoms have widespread reduction in relative CBF and that this is restored after oral intake of BCAA. These findings suggest that oral supplementation with BCAA may be a therapeutic adjunct of conventional therapy for the treatment of cirrhotic patients for its beneficial action on regional CBF.

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REFERENCES

1 **Plauth M**, Egberts EH, Hamster W, Torok M, Muller PH, Brand O, Furst P, Dolle W. Long-term treatment of latent portosystemic encephalopathy with branched-chain amino acids. A double-blind placebo-controlled crossover study. *J Hepatol* 1993; **17:** 308-314

2 **Marchesini G**, Dioguardi FS, Bianchi GP, Zoli M, Bellati G,Roffi L, Martines D, Abbiati R. Long-term oral branched-chain amino acid treatment in chronic hepatic encephalopathy. A randomized double-blind casein-controlled trial. The Italian Multicenter Study Group. *J Hepatol*; **11**: 92-101

3 **Egberts EH**, Schomerus H, Hamster W, Jurgens P. Branched chain amino acids in the treatment of latent portosystemic encephalopathy. A double-blind placebo-controlled crossover study. *Gastroenterology* 1985; **88:** 887-895

4 **Marchesini G**, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, Rossi Fanelli F, Abbiati R. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology* 2003; **124:** 1792-1801

5 **Li YY**, Nie YQ, Sha WH, Zeng Z, Yang FY, Ping L, Jia L. Prevalence of subclinical hepatic encephalopathy in cirrhotic patients in China. *World J Gastroenterol* 2004; **10:** 2397-2401

6 **vom Dahl S**, Kircheis G, Haussinger D. Hepatic encephalopathy as a complication of liver disease. *World J Gastroenterol* 2001; **7:** 152-156

7 **Ferenci P**, Lockwood A, Mullen K, Tarter R, Weissenborn K, Blei AT. Hepatic encephalopathy--definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. *Hepatology* 2002; **35:** 716-721

8 **Gu S**, Li L, Li Z, He D. Analysis on clinical features of hepatic encephalopathy of 108 cases. *World J Gastroenterol* 1998; **4:** tk 101abstract

9 **Groeneweg M**, Quero JC, De Bruijn I, Hartmann IJ, Essink-bot ML, Hop WC, Schalm SW. Subclinical hepatic encephalopathy impairs daily functioning. *Hepatology* 1998; **28:** 45-49

10 **Marchesini G**, Bianchi G, Amodio P, Salerno F, Merli M, Panella C, Loguercio C, Apolone G, Niero M, Abbiati R. Factors associated with poor health-related quality of life of patients with cirrhosis. *Gastroenterology* 2001; **120:** 170-178

11 **Horsmans Y**, Solbreux PM, Daenens C, Desager JP, Geubel AP. Lactulose improves psychometric testing in cirrhotic patients with subclinical encephalopathy. *Aliment Pharmacol Ther* 1997; **11:** 165-170

12 **Catafau AM**, Kulisevsky J, Berna L, Pujol J, Martin JC, Otermin P, Balanzo J, Carrio I. Relationship between cerebral perfusion in frontal-limbic-basal ganglia circuits and neuropsychologic impairment in patients with subclinical hepatic encephalopathy. *J Nucl Med* 2000; **41:** 405-410

13 **Nakagawa Y**, Matsumura K, Iwasa M, Kaito M, Adachi Y, Takeda K. Single photon emission computed tomography and statistical parametric mapping analysis in cirrhotic patients with and without minimal hepatic encephalopathy. *Ann Nucl Med* 2004; **18:** 123-129

14 **O'Carroll RE**, Hayes PC, Ebmeier KP, Dougall N, Murray C, Best JJ, Bouchier IA, Goodwin GM. Regional cerebral blood flow and cognitive function in patients with chronic liver disease. *Lancet* 1991; **337**: 1250-1253

15 **Iwasa M**, Matsumura K, Kaito M, Ikoma J, Kobayashi Y, Nakagawa N, Watanabe S, Takeda K, Adachi Y. Decrease of regional cerebral blood flow in liver cirrhosis. *Eur J Gastroenterol Hepatol* 2000; **12:** 1001-1006

16 **Lockwood AH**, Murphy BW, Donnelly KZ, Mahl TC, Perini S.

Positron-emission tomographic localization of abnormalities of brain metabolism in patients with minimal hepatic encephalopathy. *Hepatology* 1993; **18:** 1061-1068
17 **Trzepacz PT**, Tarter RE, Shah A, Tringali R, Faett DG, Van Thiel DH. SPECT scan and cognitive findings in subclinical hepatic encephalopathy. *J Neuropsychiatry Clin Neurosci* 1994; **6:** 170-175
18 **Lockwood AH**, Weissenborn K, Bokemeyer M, Tietge U, Burchert W. Correlations between cerebral glucose metabolism and neuropsychological test performance in nonalcoholic cirrhotics. *Metab Brain Dis* 2002; **17:** 29-40
19 **Dam M**, Burra P, Tedeschi U, Cagnin A, Chierichetti F, Ermani M, Ferlin G, Naccarato R, Pizzolato G. Regional cerebral blood flow changes in patients with cirrhosis assessed with 99mTc-HM-PAO single-photon emission computed tomography: effect of liver transplantation. *J Hepatol* 1998; **29:** 78-84
20 **Iwasa M**, Matsumura K, Watanabe Y, Yamamoto M, Kaito M, Ikoma J, Gabazza EC, Takeda K, Adachi Y. Improvement of regional cerebral blood flow after treatment with branched-chain amino acid solutions in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2003; **15:** 733-737
21 **Reutens DC**, McHugh MD, Toussaint PJ, Evans AC, Gjedde A, Meyer E, Stewart DJ. L-arginine infusion increases basal but not activated cerebral blood flow in humans. *J Cereb Blood Flow Metab* 1997; **17:** 309-315
22 **Takeuchi R**, Matsuda H, Yoshioka K, Yonekura Y. Cerebral blood flow SPET in transient global amnesia with automated ROI analysis by 3DSRT. *Eur J Nucl Med Mol Imaging* 2004; **31:** 578-589
23 **Takeuchi R**, Yonekura Y, Matsuda H, Konishi J. Usefulness of a three-dimensional stereotaxic ROI template on anatomically standardised 99mTc-ECD SPET. *Eur J Nucl Med Mol Imaging* 2002; **29:** 331-341
24 **Kawamura-Yasui N**, Kaito M, Nakagawa N, Fujita N, Ikoma J, Gabazza EC, Watanabe S, Adachi Y. Evaluating response to nutritional therapy using the branched-chain amino acid/tyrosine ratio in patients with chronic liver disease. *J Clin Lab Anal* 1999; **13:** 31-34
25 **Chang LT**. A method for attenuation correction in radionuclide computed tomography. *IEEE Trans Nucl Sci Soc* 1979; **25:** 638-643
26 **Gilberstadt SJ**, Gilberstadt H, Zieve L, Buegel B, Collier RO Jr, McClain CJ. Psychomotor performance defects in cirrhotic patients without overt encephalopathy. *Arch Intern Med* 1980; **140:** 519-521
27 **Tarter RE**, Hegedus AM, Van Thiel DH, Schade RR, Gavaler JS, Starzl TE. Nonalcoholic cirrhosis associated with neuropsychological dysfunction in the absence of overt evidence of hepatic encephalopathy. *Gastroenterology* 1984; **86:** 1421-1427
28 **Posner MI**. Attention in cognitive neuroscience: An overview. The cognitive neurosciences. Gazzangia MS. Cambridge Massachusetts; MIT Press, 1995: 615-624
29 **Goldman-Rakic PS**. Topography of cognition: parallel distributed networks in primate association cortex. *Annu Rev Neurosci* 1988; **11:** 137-156
30 **Ahl B**, Weissenborn K, van den Hoff J, Fischer-Wasels D, Kostler H, Hecker H, Burchert W. Regional differences in cerebral blood flow and cerebral ammonia metabolism in patients with cirrhosis. *Hepatology* 2004; **40:** 73-79
31 **Lockwood AH**, Yap EW, Wong WH. Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. *J Cereb Blood Flow Metab* 1991; **11:** 337-341
32 **Hooper HR**, McEwan AJ, Lentle BC, Kotchon TL, Hooper PM. Interactive three-dimensional region of interest analysis of HMPAO SPECT brain studies. *J Nucl Med* 1990; **31:** 2046-2051
33 **San Pedro EC**, Deutsch G, Liu HG, Mountz JM. Frontotemporal decreases in rCBF correlate with degree of dysnomia in primary progressive aphasia. *J Nucl Med* 2000; **41:** 228-233
34 **Moretti JL**, Tamgac F, Weinmann P, Caillat-Vigneron N, Belin CA, Cesaro P, Holman BL, Defer G. Early and delayed brain SPECT with technetium-99m-ECD and iodine-123-IMP in subacute strokes. *J Nucl Med* 1994; **35:** 1444-1449
35 **Naylor CD**, O'Rourke K, Detsky AS, Baker JP. Parenteral nutrition with branched-chain amino acids in hepatic encephalopathy. A meta-analysis. *Gastroenterology* 1989; **97:** 1033-1042
36 **Rossi Fanelli F**, Cangiano C, Capocaccia L, Cascino A, Ceci F, Muscaritoli M, Giunchi G. Use of branched chain amino acids for treating hepatic encephalopathy: clinical experiences. *Gut* 1986; **27 Suppl 1:** 111-115
37 **Bessman SP**, Paul N. The Krebs's cycle depletion theory of hepatic coma. Urea Cycle, New York, Wiley; 1976: 83-89.
38 **Hindfelt B**, Plum F, Duffy TE. Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. *J Clin Invest* 1977; **59:** 386-396
39 **Chaplin ER**, Goldberg AL, Diamond I. Leucine oxidation in brain slices and nerve endings. J Neurochem 1976; 26: 710-717
40 Oldendorf WH. Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am J Physiol* 1971; **221:** 1629-1639
41 ***Fraser CL***, Arieff AI. Hepatic encephalopathy. *N Engl J Med* 1985; **313:** 865-873
42 **Weissenborn K**, Kolbe H. The basal ganglia and portal-systemic encephalopathy. *Metab Brain Dis* 1998; **13:** 261-272
43 **Wein C**, Koch H, Popp B, Oehler G, Schauder P. Minimal hepatic encephalopathy impairs fitness to drive. *Hepatology* 2004; **39:** 739-745

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• *CLINICAL RESEARCH* •

Pregnancy is not a risk factor for gallstone disease: Results of a randomly selected population sample

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Abstract

AIM: To investigate the prevalence, risk factors, and selection of the study population for cholecystolithiasis in an urban population in Germany, in relation to our own findings and to the results in the international literature.

METHODS: A total of 2 147 persons (1 111 females, age 42.8 ± 12.7 years; 1 036 males, age 42.3 ± 13.1 years) participating in an investigation on the prevalence of *Echinococcus multilocularis* were studied for risk factors and prevalence of gallbladder stone disease. Risk factors were assessed by means of a standardized interview and calculation of body mass index (BMI). A diagnostic ultrasound examination of the gallbladder was performed. Data were analyzed by multiple logistic regression, using the SAS statistical software package.

RESULTS: Gallbladder stones were detected in 171 study participants (8.0%, *n* = 2 147). Risk factors for the development of gallbladder stone disease included age, sex, BMI, and positive family history. In a separate analysis of female study participants, pregnancy (yes/no) and number of pregnancies did not exert any influence.

CONCLUSION: Findings of the present study confirm that age, female sex, BMI, and positive family history are risk factors for the development of gallbladder stone disease. Pregnancy and the number of pregnancies, however, could not be shown to be risk factors. There seem to be no differences in the respective prevalence for gallbladder stone disease in urban and rural populations.

Key words: Cholecystolithiasis; Pregnancy; Risk factors; Selection bias; Ultrasonography

Walcher T, Haenle MM, Kron M, Hay B, Mason RA, von Schmiesing AFA, Imhof A, Koenig W, Kern P, Boehm BO, Kratzer W. Pregnancy is not a risk factor for gallstone disease: Results of a randomly selected population sample. *World J Gastroenterol* 2005; 11(43): 6800-6806
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INTRODUCTION

Disorders of the gallbladder are a major cause of morbidity and a leading indication for hospital admissions in the United States[1-4] and in Europe[5,6]. In these developed nations, the economic impact of gallstone disease is high[1-5]. In the United States, more than 500 000 cholecystectomies are performed annually and direct costs for the diagnosis and treatment of gallbladder stones are estimated at 5 billion US Dollar per year[7,8]. For the treatment of gallstone disease in Germany, 200 inpatient hospital days per 10 000 health insured persons accumulate every year[9]. This creates costs of more than ½ billion[10]. Gallstone disease is not only an unsolved problem in Western industrialized nations but also in African nations[11,12] as well as in Asian countries like China, India, Bangladesh, and Japan[13-17]. Cholelithiasis is one of the commonest surgical diseases in China and accounted for 11.5% of overall hospitalized patients during the period from 1985 to 1995[18].

The most important risk factors for the development of gallstone disease currently being discussed in the literature include age[19-23], female gender[14,20-22,24], obesity[6,25-28] and heredity[19,20,29-31]. Other factors like pregnancy or number of pregnancies are still discussed are contradictory[12,21,32-34].

To our knowledge, there are no publications that assess the influence of the selection of study population on gallstone disease prevalence.

The present prospective ultrasound-based survey investigates the prevalence and risk factors for cholecystolithiasis in an urban population and also addresses the effect of selection of study population on the different risk factors.

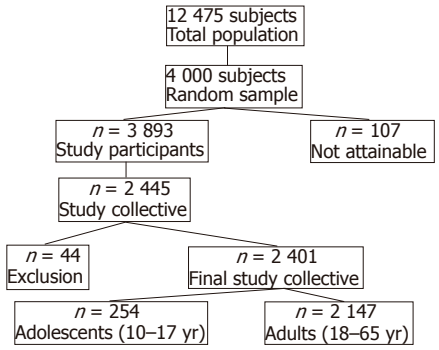

Figure 1 Study collective and participation.

METHODS

Study collective and participation

A random sample of 4 000 subjects was selected from the population of a city in southwestern Germany (total population: 12 475) for participation in a seroprevalence study for *Echinococcus multilocularis*. Of the 4 000 randomly selected and invited subjects, 107 could not be included in the final evaluation due to factors such as non-response to repeated invitations or incompetent legal status (n = 39), or moved away with no forwarding address (n = 68), resulting in a total random sample size of 3 893 subjects. Out of this pool, a total of 2 445 persons actually participated in the study (response rate: 62.8%). The following inclusion and exclusion criteria determined the composition of the collective studied for gallbladder stone disease (Figure 1):

Only persons in the age of 10-65 years were included into the study. Written consent for the examination and collection of personal health information was required.

Failure to visualize and assess the gallbladder or poor or restricted examination conditions lead to the exclusion from the study collective (n = 26 subjects). Significant contraction of the gallbladder following an inadequate fasting period (when no clinical signs of cholecystitis were identified) (n = 9 subjects), a history of cholecystectomy for gallbladder polyps or cholecystectomy of unknown reason (n = 4 subjects) or subject's refusal to undergo examination (n = 1 subject) also constituted exclusion criteria. Missing or invalid data acquisition (n = 4 subjects). Patients with prior cholecystectomy for gallbladder stones were added in the calculations of the gallbladder stone prevalence.

The total collective of subjects undergoing ultrasound examination of the gallbladder was 2 401 persons. In order to enhance comparability with published studies, we explicitly examined adult subjects aged 18-65 years. This non-selected adult collective consisted of 2 147 subjects (1 036 males, 48.3%; 1 111 females, 51.7%).

Subjects' informed written consent was obtained for examination and collection of personal health information. The study met the international agreements of the Helsinki Declaration from 1996 and was approved by the research Ethics Committee of the Baden-Württemberg General Medical Council (Landesärztekammer Baden-Württemberg).

Questionnaire and physical examination

Under the guidance of a trained interviewer, each subject completed a comprehensive questionnaire covering the following parameters: Demographic information (age, sex, nationality, marital status, education, occupation), recreational activities (sports, exercise), medical history (gallbladder stones, gastrointestinal, hepatic, cardiovascular, respiratory, endocrine, renal, rheumatic, or malignant diseases), dietary behavior (meal patterns including intake of certain foods; fluid intake including alcohol, use of tobacco products), family history (gall bladder stone disease, diabetes mellitus, overweight, history of cancer) and medication history.

Based on the recommendations of the WHO[35] for anthropometric measurements, patients then underwent determination of body height and weight and waist and hip circumference. BMI was calculated according to the common formula[35].

Ultrasound examination

Study participants were asked to present for the examination following a 4-h fasting period. All subjects underwent ultrasound examination of the upper abdomen under standard conditions to assure exact evaluation of the gallbladder. In order to enhance visualization of the gallbladder, subjects were asked to raise their right arm over their head, which increases both the intercostal spaces and the distance between the lower margin of the rib cage and the iliac crest. Examination was performed upon deep inspiration and with outward pressure on the abdominal wall.

The gallbladder was examined in three planes (longitudinal, cross-sectional and diagonal), providing the examiner with a three-dimensional impression of the organ. In cases in which cholecystolithiasis was present, the mobility of the stone(s) was assessed. Subjects, in whom differentiation between mobile stones and wall-adhering polyps was difficult, were examined again in standing position in order to reliably distinguish between stone and polyps on the basis of their mobility. The thickness of the gallbladder wall was measured and, in subjects with gallbladder stones, the number, size, and localization of stones before mobilization were determined. Ultrasound examinations were performed by a group of six examiners trained in gallbladder sonography. These examiners worked under supervision of an experienced specialist (>4 000 examinations per year), who also reviewed all questionable findings. Examinations were performed using four identical, state-of-the-art HDI-5000 ultrasound scanners (Advanced Technology Laboratories Ultrasound, Philips Medical Systems, Bothell, WA, USA).

Criteria for the diagnosis of gallstones were as follows: one or more hyperechoic structure(s) in the gallbladder with dorsal shadow; one or more hyperechoic structure(s) in the gallbladder without dorsal shadow but which by means of examination in multiple planes and/or attempt at mobilization can be certainly distinguished from a gallbladder septum, Heister's valve or a gallbladder polyp; a strongly hyperechoic structure with dorsal shadow in

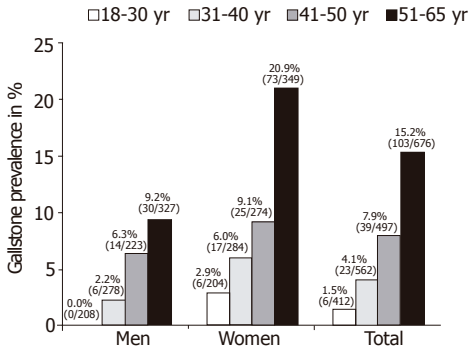

Figure 2 Distribution of gallbladder stone prevalence in relation to sex and age.

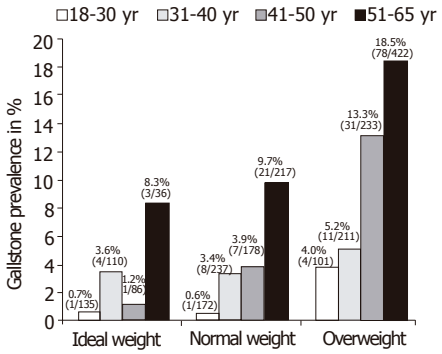

Figure 3 Prevalence of gallbladder stones in relation to BMI and age.

the anatomic location of gallbladder, with no or only slight visualization of residual gallbladder lumen; failure to delineate the gallbladder lumen in patients who have undergone prior cholecystectomy and who demonstrate corresponding surgical incisions in the right upper abdominal quadrant; presence of a significant amount of gallbladder sludge filling at least one-quarter of the gall bladder lumen with corresponding dorsal shadow.

Subjects, who because of recent food intake or other reasons, such as overlying intestinal gas, presented unfavorable examination conditions, were excluded from the study.

Statistical analysis

Multiple logistic regression[36] was performed to assess the impact of the known risk factors age, sex, BMI, and positive family history on the development of gallbladder stones. Two further multiple logistic regression models were fitted for female study participants in order to assess the impact of pregnancy and number of pregnancy, whereby in both models odds ratios were adjusted for the known risk factors like age, BMI, and positive family history for gallbladder stones. Odds ratios with 95% confidence interval and corresponding *P*-value are given. Statistical analyses were performed using the SAS statistical software package (version 8.02).

RESULTS

Prevalence in relation to age and sex

Gallbladder stones were detected at upper abdominal ultrasound examination in 87 of 2 147 subjects examined (4.1%), while gallbladder sludge was identified in two subjects (0.1%). A further 84 subjects (3.9%) had undergone prior cholecystectomy for the treatment of gallbladder stone disease. Thus, 171 subjects satisfied the inclusion criteria for cholecystolithiasis, representing an overall prevalence of cholecystolithiasis of 8.0% in the study population.

Among females, the proportion of subjects with current or prior gallbladder stone disease stood at 10.9% (121 of 1 111 subjects), while 4.8% of males (50 of 1 036 subjects) fulfilled the criteria for the diagnosis of cholecystolithiasis. The prevalence of gallbladder stone disease was higher for females than for males in all age classes. The highest prevalence was found in the group of females aged 51-65 years (20.9%; 73 of 349 subjects; Figure 2). Overall, the prevalence of gallbladder stones (defined as current and past cholecystolithiasis) increases with advancing age from 1.5% among subjects aged 18-30 years to 15.2% in the 51-65 years age group.

Prevalence in relation to BMI

BMI was calculated in 99.6% of study participants (n = 2 138). Mean BMI for the subcollective of subjects without gallbladder stone disease was 25.8±4.9 kg/m^2 (median 25.1 kg/m^2; range: 14.1-52.6 kg/m^2). Corresponding value for subjects with current or prior cholecystolithiasis was 29.2±5.9 kg/m^2 (median 28.7 kg/m^2; range: 17.6-51.5 kg/m^2). For description, BMI results were assigned to one of three classes defined according to the recommendations of the World Health Organization (WHO; Figure 3).

Class I, defined as at or below a subject's respective ideal weight (BMI <21 kg/m^2 in females and <22 kg/m^2 in males), included 367 subjects (17.2%). Class II (BMI 21-25 kg/m^2 in females and 22-26 kg/m^2 in males) included 804 subjects (37.6%), while 967 subjects (45.2%) met the criteria for Class III (BMI >25 kg/m^2 in females and BMI >26 kg/m^2 in males), and thus were considered as overweight. Only 9 subjects (2.5%) in BMI Class I exhibited evidence of gallbladder stone disease compared to 37 subjects (4.6%) in Class II and 124 subjects (12.8%) in Class III (Figure 3).

Prevalence in relation to positive family history of gallbladder stones

Of 2 147 subjects, 105 (4.9%) were unable to provide information on their biological parents; thus, evaluation of the influence of hereditary predisposition was limited to a subcollective of 2 042 subjects. Gallbladder stones were diagnosed more frequently in subjects with a positive family history of cholecystolithiasis. In subjects with a positive family history involving one biological parent, the prevalence of gallbladder stones stood at 12.6% (51 of 405 subjects) and at 14.3% (3 of 21 subjects) in subjects, both of whose biological parents suffered from gallbladder stone disease. In the remaining 1 616 subjects with negative family history of gallbladder stone disease, prevalence of cholecystolithiasis stood at only 6.3% (n = 102).

Table 1 Classical risk factors of cholecystolithiasis in multiple logistic regression

Classical risk factors	Odds ratio (OR)	95%CI	*P*
Age (per yr)	1.06	1.05–1.08	<0.001
Female sex	2.78	1.91–4.07	<0.001
BMI (per kg/m^2)	1.12	1.08–1.15	<0.001
Positive family history	1.89	1.30–2.75	<0.001

Table 2 History of pregnancy and the number of prior pregnancies in the multiple logistic regression model (only females)

Factor tested	Odds ratio (OR)	95%CI	*P*
Age (per yr)	1.06	1.04–1.08	<0.001
BMI (per kg/m^2)	1.11	1.07–1.15	<0.001
Positive family history	1.99	1.28–3.07	0.002
Positive history of pregnancy	0.76	0.44–1.31	0.321

Prevalence in relation to pregnancy

All female study subjects were questioned about their pregnancy status. Fifteen women declined to provide information on prior pregnancy. Of the remaining 1 096 subjects included in this analysis, 26.3% (n = 288) reported never having been pregnant. The group of women with positive history of pregnancy (n = 808, 73.7%) was broken down into the group with one to two pregnancies (560 women, 51.1%) and those with three or more pregnancies (248 women, 22.6%). Gallbladder stones were detected in 22 of 288 nulliparae (7.6%). In the group of 560 women with one or two pregnancies, 55 subjects (9.8%) were positive for past or present cholecystolithiasis, compared to 43 subjects (17.3%) in the group of patients with three or more pregnancies.

Multiple logistic regression analysis

Multiple logistic regression showed a strong association of the factor "age" with the development of gallbladder stones (OR 1.11 per year of age; 95%CI: 1.05-1.08; P<0.001; Table 1). The comparison of females to males yielded an odds ratio of 2.78 (95%CI: 1.91-4.07; P<0.001; Table 1). Body mass index (BMI in kg/m^2) also was an important risk factor (OR 1.12 per-unit; 95%CI: 1.08-1.15; P<0.001; Table 1). Compared to study subjects without known gallbladder stone disease in the biological parents, persons with a positive parental history of cholecystolithiasis showed an odds ratio of 1.89 (95%CI: 1.30-2.75; P<0.001; Table 1).

In separate logistic regression models for females including the risk factors age, BMI, and family history, neither pregnancy nor number of pregnancies showed an association with the development of gallbladder stone disease. The first model revealed an OR of 0.76 for pregnancy yes *vs* no (95%CI: 0.44-1.31; P = 0.321; Table 2) and the second model an OR of 0.65 for one or two pregnancies *vs* no pregnancy and an OR of 1.04 for three or more pregnancies *vs* no pregnancy (95%CI: 0.37-1.15 and 0.56-1.94; P = 0.104; Table 3).

Table 3 Number of pregnancies in the multiple logistic regression model (only females)

Factor tested	Odds ratio (OR)	95%CI	*P*
Age (per yr)	1.06	1.04–1.08	<0.001
BMI (per kg/m^2)	1.11	1.07–1.15	<0.001
Positive family history	2.09	1.34–3.25	<0.001
Number of pregnancies			0.104
One or two *vs* none	0.65	0.37–1.15	
Three or more *vs* none	1.04	0.56–1.94	

DISCUSSION

The present ultrasound-based epidemiological survey is, to our knowledge, the first study conducted in a collective drawn from an urban population in Germany. The prevalence of gallbladder stone disease in our unselected collective stands at 8.0%. Our findings are comparable, on one hand, with those documented in a rural population and in a collective of blood donors in Germany[19,20], and, on the other, with the prevalence figures reported from Italian, British, and Danish studies[21,27,33,37], but our results are not comparable with the low prevalences from Eastern countries such as China, India, Japan, Taiwan, and Thailand[14,16,17,23,38] (Figure 4).

The prevalence of gallbladder stone disease (predominantly cholesterol gallstones) reported from a majority of European and American studies shows a clear female dominance. In Asian countries with a higher prevalence of pigment gallstones, the female dominance is less distinct[6,23,38]. In the present study, female sex was also found to be a clear risk factor (OR = 2.78; 95%CI: 1.91-4.07; P<0.001) and the ratio of males with gallbladder stone disease to females stood at 1 to 2.3. Due to the great importance of the risk factors, age and especially female sex, the selection modalities of study collectives gain paramount importance[6,37]. Comparing gallbladder stone prevalence in women in relation to the method of selecting the study population, the highest prevalence is observed in studies conducted as a cross sectional sample of the total population[20–22] or large random samples[14,24] (Table 4). Most large European studies were conducted either as random samples[24,39,40] or as surveys of entire factories or governmental departments[32,41] (Table 4).

Our findings from Leutkirch (total prevalence 8.0%) are comparable to those reported for populations in Römerstein (7.8%) and blood donors in Ulm (6.0%) as well as to Italian studies conducted in Sirmione (6.9%) and Chianciano (5.9%), all of which were conducted as cross-sectional sample of the total population[19–22] (Figure 4).

The prevalence of gallbladder stones in our study collective is lower in younger persons than in those belonging to older age groups. Similar trends toward higher gallbladder stone prevalence in older persons have been described in nearly all sonographic studies[6,19,20,23,25], as well as in autopsy studies[37] and in studies based on clinical symptoms[42] (Figure 4).

Using multiple regression analysis under consideration

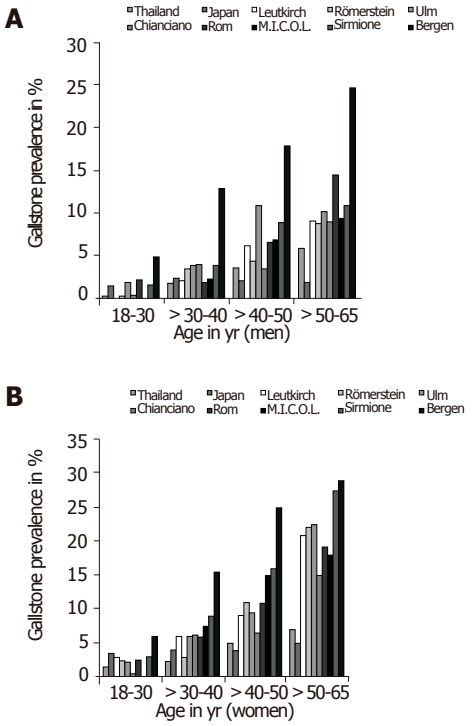

Figure 4 Age adjusted gallbladder stone prevalence in males and females in comparable large studies. **A:** in men; **B:** in women.

of the known risk factors age, sex, and family history, we found an association with study participants' current BMI (OR 1.12/unit; 95%CI: 1.08-1.15; $P<0.0001$). As in most other European studies, our findings showed an increased prevalence of gallbladder stone disease in overweight subjects[6,25–28]. The prevalence of gallbladder stones in subjects with a positive family history in the biological parents (12.7%) is more than twice as high as that in subjects with negative family history (6.3%). Our findings point to a strong effect for genetic factors in the pathogenesis of cholecystolithiasis (OR = 1.89; 95%CI: 1.30-2.75; $P<0.001$), although the mechanism of inheritance is not known. A familial accumulation of cholecystolithiasis cases has been observed in other sonographic screening studies in first-degree relatives of persons suffering from gallbladder stones[6,29-31,39].

The multiple logistic regression model failed to show an increased prevalence of gallbladder stones for female subjects with prior pregnancy (prevalence 12.1% *vs* 7.6%). One reason might be the much lower average age of the nulliparae (33.6±13.7 years) compared to women who had borne children (46.0±10.7 years), suggesting that the higher prevalence may actually be an age-related phenomenon. This effect is also apparent in the increased prevalence of gallbladder stones in women with three or more pregnancies (average age 48.9±9.7 years) compared with women who had been pregnant only one or two times (average age 44.7±10.9 years). The analysis of pregnancy as a risk factor for cholecystolithiasis has lead to different results in the literature[12,21,32–34] which range from no effect to a prevalence that is reduced by a factor of 40 in comparison of nulliparae to women who have been pregnant[15,43] (Table 5). The old clinical experience of an increased prevalence of gallbladder stones in women who have borne children could not be substantiated by the findings of the present study.

Table 4 Relative risk for gall bladder stones in relation to selection of study population

Place/region	Population selection	*n*	Sex distribution male:female
Chiayi[23]	Random sample	923	1:1.0
Rome[32]	Factory	2 325	1:1.1
Bergen[40]	Random sample	1 371	1:1.1
Ulm[19]	Blood donors	1 116	1:1.1
Copenhagen[44]	Random sample	4 807	1:1.4
Chiang Mai[38]	Random sample	6 146	1:1.5
Schwedt[41]	Factory	1 616	1:1.6
Okinawa[17]	Inhabitants of an island	2 584	1:1.7
Jiaotong[14]	Random sample	15 856	1:2.0
M.I.C.O.L[24]	Random sample	29 739	1:2.0
Römerstein[20]	Total survey	2 498	1:2.1
Sirmione[21]	Total survey	1 911	1:2.2
Cianciano[22]	Total survey	1 804	1:2.3
Leutkirch	Random sample	2 401	1:2.3

In conclusion, the classical risk factors age, female sex, body mass index (BMI), and positive family history have been confirmed by the findings of the present study. The female-specific factors of prior pregnancy and number of prior pregnancies, however, could not be shown to exert measurable influence on the prevalence of gallbladder stones. The selection of study populations affects study results i.e. the strength of the effect of female sex on the development of gallbladder stones. There does not appear to be a difference between the prevalence of gallbladder stones in urban and rural populations.

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Table 5 Review of published studies addressing the effect of the factor "pregnancy" on the prevalence of gallbladder stones

Factor by which the prevalence of gallstone disease in women with prior pregnancy is increased	1.0–1.5 times	1.6–2.5 times	2.6–10 times	11 – 50 times
Studies showing a quantitative relation between cholecystolithiasis and pregnancy	1966 Framingham[42] 1988 Sirmione[21] 1985 San Antonio[45] 1985 Maastricht[46]	1982 Oxford[47] 1982 Kopenhagen[44]	1979 Stockholm[48] 1982 Rom[32] 1986 Schwedt[41] 1986 Chianciano[22] 1991 Santiago[49]	1988 Srinagar[15]
Studies not showing a quantitative relation between cholecystolithiasis and pregnancy	1956 Birmingham[50] 1970 Pima reservation[51] 1980 Boston[52] 1982 Oberpfuss[53] 1983 Oxford[54] 1984 Adelaide[55] 1989 Soweto[12] 1990 Dublin[43] 1995 Ulm[19] 1996 Römerstein[20] 2002 Leutkirch			

W, Kratzer W, Kron M, Manfras B, Meitinger K, Mertens T, Oehme R, Pfaff G, Piechotowski I, Reuter S, Romig T, von Schmiesing AFA, Steinbach G, Tourbier M, Voegtle A, Walcher T, Wolff S.

REFERENCES

1 National Institutes of Health Consensus Development Conference Statement on Gallstones and Laparoscopic Cholecystectomy. *Am J Surg* 1993; 165: 390-398
2 **Russo MW,** Wei JT, Thiny MT, Gangarosa LM, Brown A, Ringel Y, Shaheen NJ, Sandler RS. Digestive and liver diseases statistics, 2004. *Gastroenterology* 2004; **126**: 1448-1453
3 **Diehl AK.** Epidemiology and natural history of gallstone disease. *Gastroenterol Clin North Am* 1991; **20**: 1-19
4 **Sandler RS,** Everhart JE, Donowitz M, Adams E, Cronin K, Goodman C, Gemmen E, Shah S, Avdic A, Rubin R. The burden of selected digestive diseases in the United States. *Gastroenterology* 2002; **122**: 1500-1511
5 **Kang JY,** Ellis C, Majeed A, Hoare J, Tinto A, Williamson RC, Tibbs CJ, Maxwell JD. Gallstones--an increasing problem: a study of hospital admissions in England between 1989/1990 and 1999/2000. *Aliment Pharmacol Ther* 2003; **17:** 561-569
6 **Kratzer W,** Mason RA, Kachele V. Prevalence of gallstones in sonographic surveys worldwide. *J Clin Ultrasound* 1999; **27**: 1-7
7 **Everhart JE**. US government printing office. *US government printing* 1994; 647-649
8 National Center for Health Statistics. National Hospital Discharge Survey. Advance Data from Vital and Health Statistics No 329, Hyatsville, MD 2002
9 Bundesministerium für Gesundheit. Morbidität - Krankenhausfälle und Arbeitsunfähigkeit. In: Der Bundesminister für Gesundheit, editor. Daten des Gesundheitswesens. Baden-Baden: Nomos, 1993: 34-43
10 Bundesministerium für Gesundheit. Gallensteine (ICD 574). In: Der Bundesminister für Gesundheit, editor. Ernährungsabhängige Krankheiten und ihre Kosten. Baden-Baden: Nomos, 1993: 187-193
11 **Safer L,** Bdioui F, Braham A, Ben Salem K, Soltani MS, Bchir A, Saffar H. [Epidemiology of cholelithiasis in central Tunisia. Prevalence and associated factors in a nonselected population] *Gastroenterol Clin Biol* 2000; **24:** 883-887
12 **Walker AR,** Segal I, Posner R, Shein H, Tsotetsi NG, Walker AJ. Prevalence of gallstones in elderly black women in Soweto, Johannesburg, as assessed by ultrasound. *Am J Gastroenterol* 1989; **84**: 1383-1385
13 **Shi JS,** Ma JY, Zhu LH, Pan BR, Wang ZR and Ma LS. Studies on gallstone in China. *World J Gastroenterol* 2001; **7**: 593-596
14 **Zhao Y,** Zhang R, Hu Y, Li R, Liang L, Gang Y. An epidemiological survey of gallstones with gray-scale ultrasound *Huaxi Yike Daxue Xuebao* 1990; **21:** 217-220
15 **Khuroo MS,** Mahajan R, Zargar SA, Javid G, Sapru S. Prevalence of biliary tract disease in India: a sonographic study in adult population in Kashmir. *Gut* 1989; **30:** 201-205
16 **Dhar SC,** Ansari S, Saha M, Ahmad MM, Rahman MT, Hasan M, Khan AK. Gallstone disease in a rural Bangladeshi community. *Indian J Gastroenterol* 2001; **20:** 223-6
17 **Nomura H,** Kashiwagi S, Hayashi J, Kajiyama W, Ikematsu H, Noguchi A, Tani S, Goto M. Prevalence of gallstone disease in a general population of Okinawa, *Japan. Am J Epidemiol* 1988; **128:** 598-605
18 **Zhu X,** Zhang S, Huang Z. [The trend of the gallstone disease in China over the past decade] *Zhonghua Waike Zazhi* 1995; **33:** 652-658
19 **Kratzer W,** Kachele V, Mason RA, Hill V, Hay B, Haug C, Adler G, Beckh K. Gallstone prevalence in Germany: the Ulm Gallbladder Stone Study. *Dig Dis Sci* 1998; **43**: 1285-1291
20 **Kratzer W,** Kron M, Hay B, Pfeiffer MM, Kachele V. Prevalence of cholecystolithiasis in South Germany--an ultrasound study of 2,498 persons of a rural population. *Z Gastroenterol* 1999; **37**: 1157-1162
21 **Barbara L,** Sama C, Morselli Labate AM, Taroni F, Rusticali AG, Festi D, Sapio C, Roda E, Banterle C, Puci A. A population study on the prevalence of gallstone disease: the Sirmione Study. *Hepatology* 1987; **7:** 913-917
22 **Loria P,** Dilengite MA, Bozzoli M, Carubbi F, Messora R, Sassatelli R et al. Prevalence rates of gallstone disease in Italy. The Chianciano population study. *Eur J Epidemiol* 1994; **10**: 143-150
23 **Lu SN,** Chang WY, Wang LY, Hsieh MY, Chuang WL, Chen SC, Su WP, Tai TY, Wu MM, Chen CJ. Risk factors for gallstones among Chinese in Taiwan. A community sonographic survey. *J Clin Gastroenterol* 1990; **12:** 542-526
24 **Attili AF,** Carulli N, Roda E, Barbara B, Capocaccia L, Menotti A, Okoliksanyi L, Ricci G, Capocaccia R, Festi D. Epidemiology of gallstone disease in Italy: prevalence data of the Multicenter Italian Study on Cholelithiasis (M.I.COL.) *Am J Epidemiol* 1995; **141:** 158-165
25 **Mendez-Sanchez N,** Chavez-Tapia NC, Motola-Kuba D, Sanchez-Lara K, Ponciano-Rodriguez G, Baptista H, Ramos MH, Uribe M. Metabolic syndrome as a risk factor for gallstone disease. *World J Gastroenterol* 2005; **11:** 1653-7
26 **Kono S,** Shinchi K, Todoroki I, Honjo S, Sakurai Y, Wakabayashi K, Imanishi K, Nishikawa H, Ogawa S, Katsurada M. Gallstone disease among Japanese men in relation to obesity,

glucose intolerance, exercise, alcohol use, and smoking. *Scand J Gastroenterol* 1995; **30**: 372-376
27 **Heaton KW,** Braddon FE, Mountford RA, Hughes AO, Emmett PM. Symptomatic and silent gall stones in the community. *Gut* 1991; **32**: 316-320
28 **Kodama H,** Kono S, Todoroki I, Honjo S, Sakurai Y, Wakabayashi K, Nishiwaki M, Hamada H, Nishikawa H, Koga H, Ogawa S, Nakagawa K. Gallstone disease risk in relation to body mass index and waist-to-hip ratio in Japanese men. *Int J Obes Relat Metab Disord* 1999; **23**: 211-216
29 **Barbara L,** Festi D, Morselli AM, Labate, Roda E, Rusticali AG et al. The sirmione study: familial frequency of gallstone disease. *Hepatology* 1984; **4**:1086
30 **Nurnberg D,** Berndt H, Pannwitz H. [Familial incidence of gallstones]. *Dtsch Med Wochenschr* 1989; **114**: 1059-1063
31 **Sarin SK,** Negi VS, Dewan R, Sasan S, Saraya A. High familial prevalence of gallstones in the first-degree relatives of gallstone patients. *Hepatology* 1995; **22**: 138-141
32 Prevalence of gallstone disease in an Italian adult female population. Rome Group for the Epidemiology and Prevention of Cholelithiasis (GREPCO). *Am J Epidemiol* 1984; **119**: 796-805
33 **Jorgensen T.** Gall stones in a Danish population: fertility period, pregnancies, and exogenous female sex hormones. *Gut* 1988; **29**: 433-439
34 **Hossain GA,** Islam SM, Mahmood S, Chakrabarty RK, Akhter N. Gall stone in pregnancy. *Mymensingh Med J* 2003; **12**: 112-116
35 WHO Expert Committee on Physical Status: the Use and Interpretation of Anthropometry: report of a WHO expert committee (WHO technical report series). *Genf: World Health Organization* 1995; 427-437
36 **Hosmer DWJr**, Lemeshow S. Applied logistic regression. *New York: Wiley*; 1989
37 **Brett M,** Barker DJ. The world distribution of gallstones. *Int J Epidemiol* 1976; **5**: 335-341
38 **Prathnadi P,** Miki M, Suprasert S. Incidence of cholelithiasis in the northern part of Thailand. J Med Assoc Thai 1992; 75: 462-470
39 **Jorgensen T.** Gallstones in a Danish population: familial occurrence and social factors. *J Biosoc Sci* 1988; **20**: 111-120
40 **Glambek I,** Kvaale G, Arnesjo B, Soreide O. Prevalence of gallstones in a Norwegian population. *Scand J Gastroenterol* 1987; **22**: 1089-1094
41 **Berndt H,** Nurnberg D, Pannwitz H. [Prevalence of cholelithiasis. Results of an epidemiologic study using sonography in East Germany] *Z Gastroenterol* 1989; **27**: 662-666
42 **Friedman GD,** Kannel WB, Dawber TR. The epidemiology of gallbladder disease: observations in the Framingham Study. *J Chronic Dis* 1966; **19**: 273-292
43 **Basso L,** McCollum PT, Darling MR, Tocchi A, Tanner WA. A study of cholelithiasis during pregnancy and its relationship with age, parity, menarche, breast-feeding, dysmenorrhea, oral contraception and a maternal history of cholelithiasis. *Surg Gynecol Obstet* 1992; **175**: 41-46
44 **Jorgensen T.** Prevalence of gallstones in a Danish population. *Am J Epidemiol* 1987; **126**: 912-921
45 **Diehl AK,** Rosenthal M, Hazuda HP, Comeaux PJ, Stern MP. Socioeconomic status and the prevalence of clinical gallbladder disease. *J Chronic Dis* 1985; **38**:1019-1026
46 **Thijs C,** Knipschild P, Leffers P. Pregnancy and gallstone disease: an empiric demonstration of the importance of specification of risk periods. *Am J Epidemiol* 1991; **134**: 186-195
47 **Layde PM,** Vessey MP, Yeates D. Risk factors for gall-bladder disease: a cohort study of young women attending family planning clinics. *J Epidemiol Community Health* 1982; **36**: 274-278
48 **Ahlberg J,** Angelin B, Einarsson K, Hellstrom K, Leijd B. Prevalence of gallbladder disease in hyperlipoproteinemia. *Dig Dis Sci* 1979; **24**: 459-464
49 **Valdivieso V,** Covarrubias C, Siegel F, Cruz F. Pregnancy and cholelithiasis: pathogenesis and natural course of gallstones diagnosed in early puerperium. *Hepatology* 1993; **17**: 1-4
50 **Horn G.** Observations on the aetiology of cholelithiasis. *Br Med J* 1956; **12**: 732-737
51 **Sampliner RE,** Bennett PH, Comess LJ, Rose FA, Burch TA. Gallbladder disease in pima indians. Demonstration of high prevalence and early onset by cholecystography. *N Engl J Med* 1970; **283**: 1358-1364
52 **Maclure KM,** Hayes KC, Colditz GA, Stampfer MJ, Speizer FE, Willett WC. Weight, diet, and the risk of symptomatic gallstones in middle-aged women. *N Engl J Med* 1989; **321**: 563-569
53 **Rhomberg HP,** Judmair G, Lochs A. How common are gallstones? *Br Med J (Clin Res Ed)* 1984; **289**: 1002
54 **Pixley F,** Wilson D, McPherson K, Mann J. Effect of vegetarianism on development of gall stones in women. *Br Med J (Clin Res Ed)* 1985; **291**: 11-12
55 **Scragg RK,** McMichael AJ, Seamark RF. Oral contraceptives, pregnancy, and endogenous oestrogen in gall stone disease--a case-control study. *Br Med J (Clin Res Ed)* 1984; **288**: 1795-1799

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• *CLINICAL RESEARCH* •

Characteristics of patients with columnar-lined Barrett's esophagus and risk factors for progression to esophageal adenocarcinoma

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Abstract

AIM: To determine the risk factors for the development of esophageal adenocarcinoma in these patients with columnar-lined esophagus (CLE).

METHODS: Data collected retrospectively on 597 consecutive patients diagnosed at endoscopy and histology to have CLE at Leeds General Infirmary between 1984 and 1995 were analyzed. Factors evaluated included age, sex, length of columnar segment, smoking, and drinking habits, history of non-steroidal ingestion, presence of endoscopic esophagitis, ulceration or benign strictures and presence of *Helicobacter pylori* in esophageal biopsies. Univariate and multivariate analyses were performed to identify risk factors for the development of adenocarcinoma.

RESULTS: Forty-four patients presented or developed esophageal adenocarcinoma during follow-up. Independent risk factors for the development of adenocarcinoma in patients with CLE were males (OR 5.12, 95%CI 2.04–12.84, $P = 0.0005$), and benign esophageal stricture (OR 4.37, 95%CI 2.02–9.45, $P = 0.0002$). Male subjects and patients who developed benign esophageal stricture constituted 86% ($n = 38$) of all patients who presented or developed esophageal adenocarcinoma. The presence of esophagitis was associated with a significant reduction in the development of esophageal carcinoma (OR 0.28, 95%CI 0.13–0.57, $P = 0.0006$). No other clinical characteristics differentiate between the non-malignant and malignant group.

CONCLUSION: In patients with CLE, endoscopic surveillance for the early detection of adenocarcinoma may be restricted to male subjects, as well as patients who develop benign esophageal strictures.

Key words: Barrett's esophagus; Adenocarcinoma; Risk factors; Esophageal adenocarcinoma; Esophageal stricture

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INTRODUCTION

Columnar-lined (Barrett's) esophagus (CLE) is defined as the replacement of the normal squamous lining of the lower esophagus by a unique metaplastic columnar epithelium usually as a consequence of chronic gastro-esophageal reflux (GER). The prevalence of CLE has been estimated to occur in 1 in 400 of the general population[1], and in 10–16% of patients with reflux esophagitis[2,3]. Cameron *et al.* suggested that there are 20 times as many cases of CLE in the general population as are clinically diagnosed[4].

Patients with CLE are at increased risk of esophageal adenocarcinoma[5]. The incidence of the latter varies between one in 46 to one in 441 patient-years follow-up[6,7] with an annual incidence of 1%, and is increasing more rapidly than any other type of malignancy[8,9]. Endoscopic surveillance programs are therefore instituted. Nonetheless, the increased cost and workload associated with the surveillance adds to the pressures on available resources. The identification of risk factors for the development of adenocarcinoma in these patients may allow for the selection of patients for intense endoscopic surveillance and a more efficient utilization of health care services and resources.

The current paper examines our experience with 597 consecutive patients diagnosed to have CLE for over a 11-year period. Our aim was to determine clinical factors, which could identify a subgroup of patients who are at a higher risk for the development of adenocarcinoma and who therefore would benefit most from being in a surveillance program. Examining the characteristics of

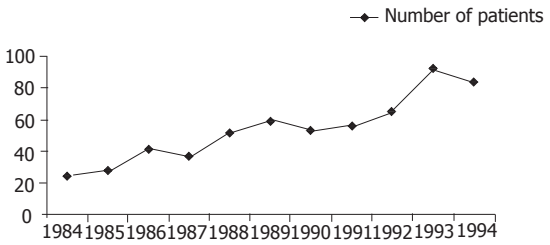

Figure 1 Number of patients diagnosed with columnar-lined esophagus during the study period.

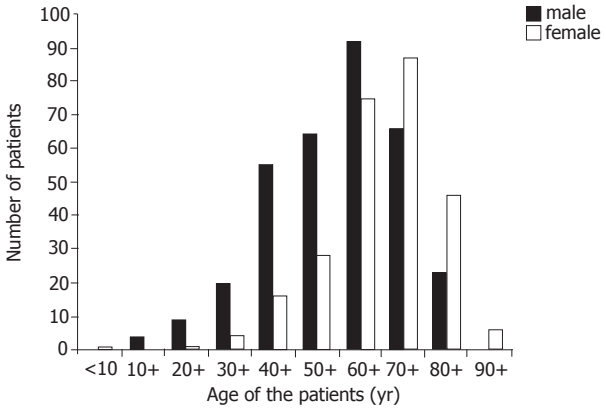

Figure 2 Age of patients at the time of diagnosis of columnar-lined esophagus.

patients with CLE and comparing them between the non-malignant group and both the prevalence and incident cases of adenocarcinoma might serve this purpose.

PATIENTS AND METHODS

Between January 1984 and December 1995, 626 consecutive patients were diagnosed at endoscopy and histology to have a CLE under the care of the Center for Digestive Diseases at the General Infirmary at Leeds. Patients were identified from a computer registry at the Institute of Pathology and their records were retrospectively reviewed. Data, including patient demographic characteristics, endoscopic findings, and histology reports were entered into a computer database.

Columnar-lined esophagus was defined as the presence of columnar-lined epithelium at least 3 cm above the endoscopically determined gastro-esophageal junction or the presence of specialized columnar epithelium (SCE) anywhere in the esophagus. Twenty-nine patients were excluded from this study; 17 patients did not fulfill the definition criteria mentioned above and the medical records of 12 patients could not be located.

Factors that may be associated with increased risk of development of adenocarcinoma were examined. These included age, sex, smoking, regular alcohol use, the ingestion of non-steroidal anti-inflammatory drugs (NSAIDs), length of columnar segment, the presence of hiatul hernia, esophagitis, benign esophageal stricture or ulcers at endoscopy, and the presence of SCE or *H pylori* in esophageal biopsies. Mean follow-up was 43 (range 1-155 mo) mo. Data related to the size of hiatul hernia and body mass index of the patients were incomplete and excluded from analysis. Data related to the result of the surveillance program for these patients were published elsewhere[6].

Patients were defined as smokers if they regularly smoked more than 10 cigarettes per day for at least one year at any time before the diagnosis of CLE. Patients were defined as regular alcohol users if they have a history of drinking 10 units of alcohol or more weekly for at least one year at any time before the diagnosis of CLE. Patients who have a history of regular ingestion of NSAIDs for at least 6 mo at any time before the diagnosis of CLE were regarded as NSAIDs users. *H pylori* colonization was determined upon the basis of hematoxylin and eosin and the use of a modified Giemsa stain of esophageal and gastric biopsy specimens.

All cases with malignant stricture were not regarded as cases of benign esophageal stricture and were not considered for the analysis. Only cases where the stricture was away from the cancer and histologically not involved with cancer were regarded as benign esophageal ulcer.

Statistical analysis

Univariate analysis was performed utilizing the χ^2 test and the Mann–Whitney *U* test as appropriate. Statistical significance was accepted at a $P<0.05$. Stepwise logistic regression analysis was used to identify independent risk factors. Statistical analyses were performed using SPSS for Windows version 10.

RESULTS

Five hundred and ninety-seven patients with histologically confirmed diagnosis of CLE were seen at our institute from 31st January 1984 to 31st January 1995. The number of new patients diagnosed each year during the study period showed an increasing trend (Figure 1). There were 333 (56%) males and 264 (44%) females. The mean age for the total group was 63.4 years (SD 14.86; range 2-94 years). Seventy-three percent of the male patients (244/333) had their diagnosis below the age of 70, while 81% (214/264) of female patients were diagnosed when they were above 60 years of age (Figure 2).

Of the 597 patients included in the analysis, 31 (5.2%) presented or developed adenocarcinoma within 6 mo of initial diagnosis of CLE and 13 (2.2%) developed adenocarcinoma after a mean follow-up of 55 mo (range 8-155 mo). Patients were divided into two groups: Non-malignant group: 553 patients had no esophageal adenocarcinoma either at initial presentation or during follow-up. There were 299 (54%) males and 254 (46%) females. Malignant group: 44 patients presented (31 patients) or developed (13 patients) esophageal adenocarcinoma. There were 34 (77%) males and 10 (23%) females.

Table 1 summarizes the details of patients and the results of the univariate analysis of the risk factors studied. Table 2 summarizes the results of multivariable analysis.

Table 1 Univariate analysis of potential risk factors for the development of esophageal adenocarcinoma in patients with columnar-lined esophagus

	Non-malignant group (n = 553)	Malignant group (n = 44)	P
Age			
<60 (yr: *n,* %)	189 (34.2)	13 (29.5)	0.532
>60 (yr: *n,* %)	364 (65.8)	31 (70.5)	
Sex: male (%)	299 (54.1)	34 (77.3)	0.003
Smoking+: *n* (%)	161 (45.2)	21 (51.2)	0.466
Regular alcohol use±: *n* (%)	52 (15.2)	8 (20)	0.430
NSAIDs: *n* (%)	304 (55)	25 (56.8)	0.813
Specialized epithelium: *n* (%)	467 (84.4)	42 (95.5)	0.047
Length of CLE Median (cm)	5	6	0.001
Hiatul hernia: *n* (%)	314 (56.8)	22 (50)	0.383
Esophagitis: *n* (%)	330 (59.7)	13 (29.5)	<0.0001
Esophageal ulcer	117 (21.2)	11 (25)	0.550
Esophageal stricture	77 (13.9)	16 (36.4)	<0.0001
Hp in esophageal biopsy	42 (7.6)	2 (4.5)	0.456

CLE – columnar-lined esophagus; NSAIDs – non-steroidal anti-inflammatory drugs; Hp – *Helicobacter pylori*. +Information regarding smoking was available in only 356 patients of the non-malignant group and 41 patients of the malignant group; ‡information regarding alcohol consumption was available in only 342 patients of the non-malignant group and 40 patients of the malignant group.

Table 2 Risk factors associated with the development of adenocarcinoma in patients with CLE; results of multivariable regression analysis

Risk factors	Odds ratio	95%CI for Odds ratio	P
Age ≥60 yr	1.65	0.75–3.64	0.216
Male sex	5.12	2.04–12.84	0.0005
Regular alcohol use	1.15	0.46–2.90	0.760
NSAIDs	1.41	0.68–2.93	0.352
Esophagitis	0.28	0.13–0.57	0.0006
Esophageal stricture	4.37	2.02–9.45	0.0002

CLE – columnar lined esophagus; NSAIDs – non-steroidal anti-inflammatory drugs

Significant independent risk factors for the development of esophageal adenocarcinoma in patients with CLE were male sex, and benign esophageal stricture. Male subjects and patients who developed benign esophageal stricture constituted 86% (n = 38) of all patients who presented or developed esophageal adenocarcinoma. Among the 24 patients who presented with esophageal adenocarcinoma, 9 patients had histologically proven benign peptic stricture above and away from the cancer and the strictures were located at the junction of the columnar mucosa with the squamous epithelium. Among the seven patients who developed esophageal adenocarcinoma within 6 mo after the diagnosis of CLE, four of them had histologically proven benign peptic stricture at the time of diagnosis of CLE. Among the 13 patients who developed esophageal adenocarcinoma after a mean follow-up of 55 (range 8-155 mo) mo, three patients had histologically proven benign peptic stricture at the time of diagnosis of CLE, and these three patients developed adenocarcinoma 17, 24, and 35 mo after the diagnosis of CLE, respectively.

The presence of esophagitis was associated with a significant reduction in the development of esophageal carcinoma. Although univariate analysis identified the length of columnar-lined esophageal segment and the presence of SCE on endoscopic biopsies as significant variables (P = 0.001, P = 0.047 respectively), these did not reach significance on multivariable analysis. Age >60 years, smoking, alcohol consumption, the presence of hiatul hernia or esophageal ulcer, and the presence of *H pylori* in the esophageal biopsies were insignificant risk factors for malignant progression.

Symptoms as a risk factor

For the purpose of comparison between the two groups, the main principal symptom for each patient was considered at the time of diagnosis, although many patients had more than one symptom. In the non-malignant group, the main symptoms at presentation were as follows: In 259 patients (46.8%), the main symptoms were those of GER (heartburn, regurgitation) or dyspepsia. Anemia or gastro-intestinal bleeding was the main symptom in 137 patients (24.8%). Dysphagia was the main symptom in 111 patients (20%) and chest pain was the main symptom in 26 patients (4.7%). Weight loss was the main symptom in only 20 patients (3.6%). Ninety-four patients (17%) in the non-malignant group had no esophageal symptoms at the time of diagnosis and CLE was diagnosed when endoscopy was performed to investigate iron deficiency anemia. In the malignant group, the main symptom was dysphagia in 21 patients (47.7%). Anemia or gastrointestinal bleeding was the main symptom in nine patients (20.5%). GER symptoms were the main symptoms in 10 patients (22.7%). Weight loss was the main symptom in four patients (9%). Dysphagia rather than reflux symptom was the main complaint of the patients in the malignant group at the time of diagnosis. This was mainly due to dysphagia being prominent in the prevalent adenocarcinoma cases (61%; 19/31) as would be expected; in contrast among the 13 patients who developed adenocarcinoma during follow-up, dysphagia was the main symptom in only two patients (15%; 2/13).

Length of the columnar segment

The length of the columnar segments for all patients is shown in Figure 3. Twenty-three patients had short segment (<3 cm) of SCE; none of this group were adenocarcinoma patients. The mean length of the columnar segment in the non-malignant group was 5.8 cm; range 2-20 cm and for the malignant group was 7.2 cm; range 3-15 cm. There was no correlation between the extent of the columnar segment and the presence or absence of symptoms of GER.

Esophageal and gastric H pylori

Forty-four patients only (7.4%) had *H pylori* detected in their esophageal biopsies. Only 234 patients of this series had gastric biopsies in addition to their esophageal specimens. Among this subgroup who had biopsy demonstrated CLE and from whom concomitant gastric biopsies were taken, 77 patients (32.9%) had *H pylori* in

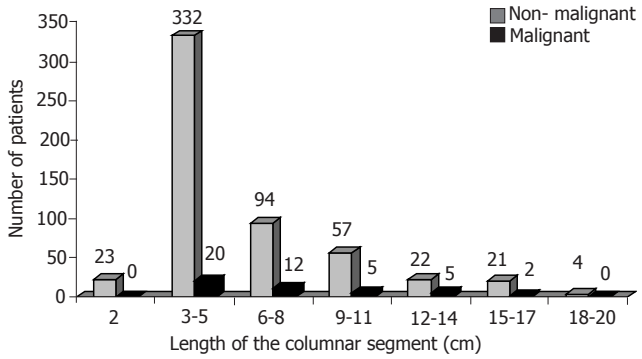

Figure 3 Length of the columnar segments in all patients.

their gastric biopsies, 20 of them had *H pylori* in both the esophageal and gastric specimens. The finding of *H pylori* in CLE biopsies was not associated with increase in the prevalence of esophagitis (52% *vs* 58%), esophageal strictures (11% *vs* 16%), esophageal ulcers (23% *vs* 21%) or esophageal adenocarcinoma (4.5% *vs* 7.6%).

DISCUSSION

The present series confirms the findings of other studies[10,11] that the prevalence of CLE increases with age and reaches a peak in late middle age. Eighty-two percent of our patients (487/597) were diagnosed when they were over 50 years of age. The high prevalence of CLE occurs mainly in later middle age and the elderly as shown by our population with a mean age of 59 in males and 68 in females, although it can be seen in younger patients also. However, only 2.5% of patients (15/597) were under 30 years of age at the time of diagnosis. One patient had a diagnosis of CLE below the age of 10 and this was a 3-year old girl who presented with repeated vomiting. Endoscopic and histological examination confirmed the diagnosis of CLE and anti-reflux surgery was performed on this patient at the age of four. This case could be explained on the basis of the few cases of congenital Barrett's esophagus reported previously[12].

Although there were slightly more males than females in the whole of our series (56% *vs* 44%), we have found that below the age of 60, there is a clear male predominance of 3:1. In contradiction to other published data, which showed either a male predominance[13] or an equal sex distribution[10,14], we observed a female predominance of 1.5:1 in patients above the age of 70.

A large proportion of patients with CLE in the general population will remain undiagnosed unless complications or carcinoma develop. CLE itself causes no symptoms[15] whereas the main symptoms, which bring patients to medical attention, are related to the reflux symptoms or complications of CLE such as ulceration, stricture, bleeding or adenocarcinoma. There is no correlation between severity of symptoms and histological findings, as about 17% of our patients with CLE had no esophageal symptoms. It is clear therefore that the selection of patients for a surveillance program cannot be based on symptomatology. This conclusion was also made in earlier patient series[10,16,17].

Our study confirms that patients with CLE have an increased incidence of esophageal adenocarcinoma. There were 7 patients who developed adenocarcinoma within 6 mo of initial diagnosis, before their first annual review endoscopy. This is likely to reflect the presence of undetected malignancy at the time of initial endoscopy. This probably represents a sampling error in the biopsies taken and therefore these seven patients were included in the prevalence rather than the incidence data[6].

This study identified male sex, and benign esophageal stricture to be independent risk factors (at least four-fold increased risk) for the development of esophageal adenocarcinoma in patients with CLE. Male patients with benign esophageal stricture constituted 86% of all patients who developed esophageal malignancy. The risk of malignancy was not related to the age of the patients, smoking, presence of SCE, length of columnar-lined segment, or the presence of esophageal ulceration or hiatul hernia on endoscopy or *H pylori* in esophageal or gastric biopsies. Previous reports identified white ethnicity[18], older age[19], male sex[13,14,20], SCE[21,22], long columnar segment[10,19,23-28], large hiatal hernia size[26,27], esophageal ulcer or stricture[10,29-31], severe acid reflux[26,32], obesity[33,34] and smoking and alcohol[20,35] to be associated with the progression from columnar epithelium to adenocarcinoma. However, some of these reports were based on observations or univariate analyses, and did not control for other variables as in the current study.

There was no significant association between age >60 years and the risk of malignant progression. Most patients are diagnosed with CLE in their sixth or seventh decade of life. Patients with Barrett's are often reported to be of the same age as those who develop cancer[26]. On the other hand, other studies have reported that the risk of esophageal adenocarcinoma increases with age. Gopal *et al.* reported that the risk of dysplasia increased by 3.3% per year of age[19]. A potential problem is that the mean age of patients with dysplasia is quite high, increasing the expected operative mortality and tending to preclude operative intervention. Clearly, surveillance should be offered only for those patients in whom esophagectomy is considered as a therapeutic option, if early carcinoma is detected. On the other hand, with the availability of ablation therapy, one can argue that even patients who are not fit for esophagectomy will benefit from the treatment of their high-grade dysplasia or early adenocarcinoma using photoablation irrespective of their age.

The increased susceptibility of male subjects with CLE to the development of esophageal malignancy has been reported previously[20,36,37]. In these series, men constituted 67–100% of all patients who developed esophageal adenocarcinoma (77% in this report). Our finding that benign stricture formation, which complicated 16% of all patients with CLE, was a risk factor for malignant transformation that supports previous reports[10,30,31,38,39]. Careful endoscopic surveillance of patients with benign

esophageal strictures is therefore required, despite the fact that malignant progression occurred in only one in eight patients.

We found no evidence to support the suggestion that a longer CLE segment is associated with a greater risk of carcinoma. Although the length of columnar segment was significantly greater in patients who developed esophageal adenocarcinoma compared with those who did not, this was not an independent risk factor for malignant transformation. This finding is in concordance with that of Robertson *et al*. who showed that the progression of the metaplastic epithelium up the esophagus, which occurred in 20% of their patients, was not associated with an increased risk of malignancy, as out of the 11 patients who had progression up to 6 cm, only 1 developed carcinoma[16]. Additionally, the marked association between adenocarcinoma of the gastroesophageal junction and 'short-segment' Barrett's mucosa[21,22,25,40] underscores the importance of the 'short-segment', and calls for follow-up program similar to that of longer segments of CLE. Rudolph *et al*. reported that segment length was not related to cancer risk in the full cohort of patients with CLE, and when patients with high-grade dysplasia at baseline were excluded; however, a non-significant trend was observed; a 5 cm difference in segment length was associated with a 1.7-fold increase in cancer risk. These authors concluded that the risk for esophageal adenocarcinoma in patients with short-segment Barrett esophagus was not substantially lower than that in patients with longer segments. They suggested that until more data are available, the frequency of endoscopic surveillance should be selected without regard to segment length[28].

There are some indications that the risk of cancer is proportionate to the anatomic extent of CLE[19,26,27]. A few studies have shown that the risk of adenocarcinoma increases with the length of the columnar segment and they suggested considering patients with CLE of more than 8 or 10 cm in length for surveillance. For example, Iftikhar *et al*.[24] reported that among 102 patients with CLE, 12 were found to have dysplasia; all of them had a columnar segment of 8 cm or more at the time of diagnosis and no patient with a columnar segment of less than 8 cm was found to have dysplasia or adenocarcinoma. In two retrospective studies conducted by Harle *et al*.[41] and Rosenberg *et al*.[42], 94% and 88% of patients with adenocarcinoma had long segments (>10 cm) of columnar epithelium. Both series suggested that an extended length of Barrett's esophagus is associated with a higher risk of malignant progression. Schnell *et al*.[23] reported a series of 238 patients with CLE. Adenocarcinoma was found in 7% of the 129 patients with segments less than 2 cm in length, in 12% of 50 patients with 3-5 cm segments, in 31% of 45 patients with 6-10 cm Barrett's segments and in 43% of the 14 patients who had segments of more than 10 cm in length. They concluded that there is a strong association between the length of CLE and risk of adenocarcinoma. Gopal *et al*. reported that the risk of dysplasia increased by 14%/cm of increased length[19], and similarly Avidan *et al*. reported that each 1-cm elongation of Barrett's mucosa carried with it a 17% increase in the risk of developing high-grade dysplasia or carcinoma[26]. On the other hand, other studies have suggested that shorter lengths of CLE may have been obscured by the tumor and therefore missed at resection. For example, Hamilton *et al*.[40] found that 64% of the resected specimens of adenocarcinoma of the esophagus and GEJ were associated with Barrett' s esophagus although the Barrett's mucosa was identified by endoscopic biopsies in only 38% of cases. When adenocarcinoma develops in a short segment of specialized epithelium, the tumor may destroy the area of specialized epithelium leaving no trace of such epithelium. Schnell *et al*.[21] did indeed report four patients with adenocarcinoma in short segment of specialized epithelium. Cameron *et al*.[22] reported that SCE was found in 9 of 9 (100%) cases of esophageal adenocarcinomas and in 10 of 24 (42%) of cases of junctional adenocarcinomas. SCE was found in 8 of 12 (67%) of junctional adenocarcinoma of 6 cm or less in length but in only 2 of 12 (17%) of larger tumors. Again they concluded that junctional adenocarcinomas are associated with both short and long segments of Barrett' s esophagus and larger tumors probably overgrow and conceal the underlying SCE from which they arise. In 38% of the patients who developed cancer, the metaplastic segment was less than 3 cm[43] Schnell *et al*.[21] emphasizes that patients with short segments should be considered at risk and should be followed in the same way as their counterparts with longer segments of CLE. The above studies clearly demonstrate that carcinoma can develop in short as well as long segment of CLE. It is now established that short segment CLE does carry a risk of malignant progression, *albeit* currently this is difficult to quantify[44].

Specialized epithelium is the most common and distinctive type of columnar epithelium found in CLE. Although dysplasia and carcinoma develop mainly in the presence of SCE, we cannot regard this type of epithelium as the sole indication for surveillance because histology has shown that it is present in most patients with CLE. Indeed nowadays its presence is rather a criterion to establish the diagnosis of CLE. There are, however, a few cases of adenocarcinoma, which can develop in columnar epithelium of other histological types (junctional or fundic type).

The reported data concerning the influence of alcohol consumption on the malignant progression of CLE is controversial. Most published reports including the current study suggest that alcohol ingestion has no or only little effect[26]. The reported decline in the incidence of oral cavity and pharynx cancers, which are traditionally, related to alcohol consumption contrasts with the increase in the incidence of esophageal adenocarcinoma[45]. Gammon *et al*. reported a decrease in the risk associated with wine drinking and no increase in the risk by the use of other alcoholic beverages[46]. Similarly, Garridou *et al*. reported that wine might have a protective effect[47]. On the other hand, other investigators have reported an increased risk of esophageal adenocarcinoma with high alcohol intake[20,35,48-50]. Zhang *et al*. reported a statistically not significant twofold increase in the risk of esophageal

adenocarcinoma in those who consume alcohol when compared with nondrinkers[48]. Kabat *et al*. reported that only hard liquor intake was associated with esophageal adenocarcinoma in males, and only daily beer intake was associated with adenocarcinoma in females[49]. Barrett's esophagus was reported to occur more frequently among subjects who consume large amounts of alcohol and alcohol consumption was also a risk factor for an increased length of Barrett's mucosa[51,52]. There is a well-known association between alcohol intake and risk of upper digestive tract cancers from epidemiological studies[53]. One possible mechanism is that this increased risk is due to a direct exposure of the esophageal mucosa to high alcohol concentrations but systemic effects could also be important.

There were no differences between smokers and non-smokers with regard to the length of the columnar segment and the presence or absence of Barrett's complications such as ulcer, stricture or adenocarcinoma. Several reports[26,54,55], as well as the current one, did not support the suggested increased risk of malignant transformation with smoking. Cooper and Barbezat[54] reported a series of 52 patients with CLE and 25 of them were smokers or ex-smokers. They also found no difference in the clinical characteristics between the smokers and non-smokers. Other investigators have shown a higher proportion of smokers in patients with CLE[56] and some suggested that the malignant progression of Barrett's metaplasia was higher in patients who smoked[20,35,50]. On the other hand, Levi *et al*.[55] evaluated the relationship between tobacco, alcohol and the risk of esophageal adenocarcinoma in CLE in an endoscopy-clinic-based case-control study of 30 cases of adenocarcinoma and 140 controls with non-malignant CLE. Among the cases, 18 (60%) were non-smokers and 14 (47%) non-drinkers, the corresponding proportions in the controls being 52% and 44%. Thus, there was no apparent relation between tobacco, alcohol and the risk of esophageal adenocarcinoma. They suggested that the findings of their study, although based on a limited number of cases, indicate that alcohol and tobacco are unlikely to play a major role in the etiology of adenocarcinoma in CLE. Overall, alcohol and tobacco may be risk factors for esophageal adenocarcinoma, but are not as important as they are in the etiology of esophageal squamous cell carcinoma[57].

Esophagitis and hiatal hernia were more common in the non-malignant group than the malignant group. But these differences have no clinical value, as it will not differentiate between the two groups. The apparent protective effect of esophagitis against malignant progression reflects the natural history of the disease. Esophagitis precedes the replacement of squamous with columnar epithelium, which in turn may become progressively dysplastic with final transformation into adenocarcinoma[12,58,59]. Additionally, columnar epithelium, unlike squamous, is less sensitive to injury secondary to GER and thus more resistant to inflammation[60].

There was an association between the prevalence of esophagitis and esophageal ulcerations with the history of NSAIDs ingestion within the non-cancer patients; however, there was no significant difference between the non-malignant group and malignant group regarding the prevalence of NSAIDs ingestion. It is likely that NSAIDs are prescribed or self-prescribed for esophagitis. Fifty-five percent of patients in the non-malignant group were taking these drugs at the time of diagnosis and 62% of them were found to have esophagitis or esophageal ulcerations. Cooper and Barbezat[54] reported similar findings and suggested that patients with CLE should avoid taking NSAIDs. However, NSAIDs are now proposed in intervention trials for patients with CLE. Increased expression of the cyclooxygenase 2 enzyme is proposed to be central to the development of esophageal cancer. Since this enzyme is inhibited by NSAIDs, these drugs hold promise as cancer chemopreventive agents in Barrett's esophagus patients[61]. Several preventive strategies against esophageal adenocarcinoma are under investigation using NSAIDs[62].

We found that esophageal ulcerations were present in 25% of patients in the carcinoma group (11/44) and in 21% of the patients who had no carcinoma (117/553); a difference that was not statistically significant. No case of esophageal perforation due to these ulcers was found in our series. These findings disagree with previous series, which suggested that the risk of developing carcinoma is increased in the presence of esophageal ulcers[10,29–31].

In concordance with previous work suggesting that stricture formation in CLE is a risk factor of malignant progression[10,30,31,39], we observed that esophageal strictures were present in 36% of patients in the carcinoma group (16/44) and in only 14% of the patients without carcinoma (77/553); a difference that was statistically significant. Theoretically it is possible that some of the strictures of the patients who presented or developed adenocarcinoma within 6 mo of CLE diagnoses could be malignant strictures (missed cancers) which were not detected histologically due to sampling error.

We found that *H pylori* colonization of the esophagus was not a risk factor for malignant progression. Our data indicate that *H pylori* can colonize the CLE, but its prevalence rate in the esophageal biopsies of these patients or in the subgroups that had complications or carcinoma is low and it is unlikely that *H pylori* has a significant role in the pathogenesis of CLE or its complications. Similar conclusion was reported before[63,64]. The prevalence rate of *H pylori* in the gastric biopsies of the same patients is similar or less than that in the normal population. Weston *et al*.[65] found that Barrett's high-grade dysplasia and adenocarcinoma were significantly more prevalent in patients who are not infected with *H pylori*. They suggested that *H pylori* appear to have a protective effect against the development of Barrett's adenocarcinoma.

While the role of endoscopic surveillance in low-risk patients with CLE is controversial, efficient and effective screening would target high-risk patients. This study suggests that such programs may be largely directed to male subjects and those who develop benign esophageal strictures. We have previously shown that

immunohistochemical detection of cyclin D1 in esophageal biopsies of patients with CLE is a sensitive tool for identifying subgroup of patients who may be at a higher risk[66]. But given that multiple genetic alterations, which are implicated in the natural history of esophageal adenocarcinoma, a combination of clinical risk factors and carefully validated biomarkers including cyclin D1, might improve still further the predictive value of the molecular approach[66,67].

In conclusion, we have found that 44 patients presented or developed esophageal adenocarcinoma of which 34 (77%) were males. Independent risk factors for progression from columnar metaplasia to esophageal adenocarcinoma were male sex, and the development of benign esophageal stricture. No other clinical characteristics differentiate between the non-malignant and malignant group. Most esophageal adenocarcinoma occurs in men with specialized epithelium. This subgroup may constitute a clinically recognized group at a high risk of cancer and particularly suitable for endoscopic surveillance. In patients with CLE, endoscopic surveillance for the early detection of adenocarcinoma may be restricted to male subjects, as well as patients who develop benign esophageal strictures. A large proportion of patients with CLE have no esophageal symptoms making recruitment into endoscopic surveillance programs problematic.

REFERENCES

1 **Stein HJ,** Siewert JR. Barrett's esophagus: pathogenesis, epidemiology, functional abnormalities, malignant degeneration, and surgical management. *Dysphagia* 1993; **8:** 276-288

2 **Naef AP,** Savary M, Ozzello L. Columnar-lined lower esophagus: an acquired lesion with malignant predisposition. Report on 140 cases of Barrett's esophagus with 12 adenocarcinomas. *J Thorac Cardiovasc Surg* 1975; **70:** 826-835

3 **Bartlesman JF,** Hameeteman W, Tytgat GN. Barrett's oesophagus. *Eur J Cancer Prev* 1992; **1:** 323-325

4 **Cameron AJ,** Zinsmeister AR, Ballard DJ, Carney JA. Prevalence of columnar-lined (Barrett's) esophagus. Comparison of population-based clinical and autopsy findings. *Gastroenterology* 1990; **99:** 918-922

5 **Spechler SJ**. Endoscopic surveillance for patients with Barrett' s esophagus: Does the cancer risk justify the practice? *Ann Intern Med* 1987; **106:** 902-904

6 **Bani-Hani K,** Sue-Ling H, Johnston D, Axon AT, Martin IG. Barrett's oesophagus: results from a 13-year surveillance programme. *Eur J Gastroenterol Hepatol* 2000; **12:** 649-654

7 **Cameron AJ**, Ott BJ, Payne WS. The incidence of adenocarcinoma in columnar-lined (Barrett's) esophagus. *N Engl J Med* 1985; **313:** 857-859

8 **Spechler SJ.** The frequency of cancer in patients with Barrett's esophagus. *Acta Endoscopica* 1992; **22:** 541-4

9 **Blot WJ**, Devesa SS, Kneller RW, Fraumeni JF Jr. Rising incidence of adenocarcinoma of the esophagus and gastric cardia. *JAMA* 1991; **265:** 1287-1289

10 **Macdonald CE,** Wicks AC, Playford RJ. Ten years' experience of screening patients with Barrett's oesophagus in a university teaching hospital. *Gut* 1997; **41:** 303-307

11 **Cameron AJ**, Lomboy CT. Barrett's esophagus: age, prevalence, and extent of columnar epithelium. *Gastroenterology* 1992; **103:** 1241-1245

12 **Borrie J**, Goldwater L. Columnar cell-lined esophagus: assessment of etiology and treatment. A 22 year experience. *J Thorac Cardiovasc Surg* 1976; **71:** 825-834

13 **Wright TA**, Gray MR, Morris AI, Gilmore IT, Ellis A, Smart HL, Myskow M, Nash J, Donnelly RJ, Kingsnorth AN. Cost effectiveness of detecting Barrett's cancer. *Gut* 1996; **39:** 574-579

14 **Menke-Pluymers MB,** Hop WC, Dees J, van Blankenstein M, Tilanus HW. Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The Rotterdam Esophageal Tumor Study Group. *Cancer* 1993; **72:**1155-1158

15 **Sjogren RW Jr**, Johnson LF. Barrett's esophagus: a review. *Am J Med* 1983; **74:** 313-321

16 **Robertson CS**, Mayberry JF, Nicholson DA, James PD, Atkinson M. Value of endoscopic surveillance in the detection of neoplastic change in Barrett's oesophagus. *Br J Surg* 1988; **75:** 760-763

17 **Achkar E**, Carey W. The cost of surveillance for adenocarcinoma complicating Barrett's esophagus. *Am J Gastroenterol* 1988; **83:** 291-294

18 **Rogers EL**, Goldkind SF, Iseri OA, Bustin M, Goldkind L, Hamilton SR, Smith RL. Adenocarcinoma of the lower esophagus. A disease primarily of white men with Barrett's esophagus. *J Clin Gastroenterol* 1986; **8:** 613-618

19 **Gopal DV**, Lieberman DA, Magaret N, Fennerty MB, Sampliner RE, Garewal HS, Falk GW, Faigel DO. Risk factors for dysplasia in patients with Barrett's esophagus (BE): results from a multicenter consortium. *Dig Dis Sci* 2003; *48:* 1537-1541

20 **Skinner DB,** Walther BC, Riddell RH, Schmidt H, Iascone C, DeMeester TR. Barrett's esophagus. Comparison of benign and malignant cases. *Ann Surg* 1983; **198:** 554-566

21 **Schnell TG,** Sontag SJ, Chejfec G. Adenocarcinomas arising in tongues or short segments of Barrett's esophagus. *Dig Dis Sci* 1992; **37:** 137-143

22 **Cameron AJ,** Lomboy CT, Pera M, Carpenter HA. Adenocarcinoma of the esophagogastric junction and Barrett's esophagus. *Gastroenterology* 1995; **109:** 1541-1546

23 **Schnell T**, Sontag S, Chejfec G, Miller T, Kurucar C, O' Connell S, Brand L. Does length (L) of Barrett's esophagus (BE) correlate with age, cigarette or alcohol consumption or risk of adenocarcinoma (adCa) (abstract)? *Gastroenterology* 1990; **98:** A120.

24 **Iftikhar SY,** James PD, Steele RJ, Hardcastle JD, Atkinson M. Length of Barrett's oesophagus: an important factor in the development of dysplasia and adenocarcinoma. *Gut* 1992; **33:** 1155-1158

25 **Clark GW,** Smyrk TC, Burdiles P, Hoeft SF, Peters JH, Kiyabu M, Hinder RA, Bremner CG, DeMeester TR. Is Barrett's metaplasia the source of adenocarcinomas of the cardia? *Arch Surg* 1994; **129:** 609-614

26 **Avidan B**, Sonnenberg A, Schnell TG, Chejfec G, Metz A, Sontag SJ. Hiatal hernia size, Barrett's length, and severity of acid reflux are all risk factors for esophageal adenocarcinoma. *Am J Gastroenterol* 2002; **97:** 1930-1936

27 **Weston AP**, Badr AS, Hassanein RS. Prospective multivariate analysis of clinical, endoscopic, and histological factors predictive of the development of Barrett's multifocal high-grade dysplasia or adenocarcinoma. *Am J Gastroenterol* 1999; **94:** 3413-3419

28 **Rudolph RE**, Vaughan TL, Storer BE, Haggitt RC, Rabinovitch PS, Levine DS, Reid BJ. Effect of segment length on risk for neoplastic progression in patients with Barrett esophagus. *Ann Intern Med* 2000; **132:** 612-620

29 **Van der Veen AH**, Dees J, Blankensteijn JD, Van Blankenstein M. Adenocarcinoma in Barrett's oesophagus: an overrated risk. *Gut* 1989; 30: 14-18

30 **Moghissi K,** Sharpe DA, Pender D. Adenocarcinoma and Barrett's oesophagus. A clinico-pathological study. *Eur J Cardiothorac Surg* 1993; **7:** 126-131

31 **Lerut T,** Coosemans W, Van Raemdonck D, Dillemans B, De Leyn P, Marnette JM, Geboes K. Surgical treatment of Barrett's carcinoma. Correlations between morphologic findings and prognosis. *J Thorac Cardiovasc Surg* 1994; **107:** 1059-65;

discussion 1065-1066
32 **Lagergren J**, Bergstrom R, Lindgren A, Nyren O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med* 1999; **340:** 825-831
33 **Brown LM,** Swanson CA, Gridley G, Swanson GM, Schoenberg JB, Greenberg RS, Silverman DT, Pottern LM, Hayes RB, Schwartz AG. Adenocarcinoma of the esophagus: role of obesity and diet. *J Natl Cancer Inst* 1995; **87:** 104-109
34 **Lagergren J**, Bergstrom R, Nyren O. Association between body mass and adenocarcinoma of the esophagus and gastric cardia. *Ann Intern Med* 1999; 130: 883-890
35 **Gray MR,** Donnelly RJ, Kingsnorth AN. The role of smoking and alcohol in metaplasia and cancer risk in Barrett's columnar lined oesophagus. *Gut* 1993; **34:** 727-731
36 **Allison PR,** Johnstone AS. The oesophagus lined with gastric mucous membrane. *Thorax* 1953; **8:** 87-101
37 **Haggitt RC**, Tryzelaar J, Ellis FH, Colcher H. Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. *Am J Clin Pathol* 1978; 70: 1-5
38 **Chow WH,** Finkle WD, McLaughlin JK, Frankl H, Ziel HK, Fraumeni JF Jr. The relation of gastroesophageal reflux disease and its treatment to adenocarcinomas of the esophagus and gastric cardia. *JAMA* 1995; **274:** 474-477
39 **van der Burgh A,** Dees J, Hop WC, van Blankenstein M. Oesophageal cancer is an uncommon cause of death in patients with Barrett's oesophagus. *Gut* 1996; **39:** 5-8
40 **Hamilton SR,** Smith RR, Cameron JL. Prevalence and characteristics of Barrett's esophagus in patients with adenocarcinoma of the esophagus or esophagogastric junction. *Hum Pathol* 1988; **19:** 942-948
41 **Harle IA**, Finley RJ, Belsheim M, Bondy DC, Booth M, Lloyd D, McDonald JW, Sullivan S, Valberg LS, Watson WC, *et al*. Management of adenocarcinoma in a columnar-lined esophagus. *Ann Thorac Surg* 1985; **40:** 330-336
42 **Rosenberg JC**, Budev H, Edwards RC, Singal S, Steiger Z, Sundareson AS. Analysis of adenocarcinoma in Barrett' s esophagus utilizing a staging system. *Cancer* 1985; **55:** 1353-1360
43 **Levine DS**, Haggitt RC, Blount PL, Rabinovitch PS, Rusch VW, Reid BJ. An endoscopic biopsy protocol can differentiate high-grade dysplasia from early adenocarcinoma in Barrett's esophagus. *Gastroenterology* 1993; **105:** 40-50
44 **Sharma P**. Short segment Barrett esophagus and specialized columnar mucosa at the gastroesophageal junction. *Mayo Clin Proc* 2001; **76:** 331-334
45 **Reynolds JC**, Rahimi P, Hirschl D. Barrett's esophagus: clinical characteristics. *Gastroenterol Clin North Am* 2002; **31:** 441-460
46 **Gammon MD**, Schoenberg JB, Ahsan H, Risch HA, Vaughan TL, Chow WH, Rotterdam H, West AB, Dubrow R, Stanford JL, Mayne ST, Farrow DC, Niwa S, Blot WJ, Fraumeni JF Jr. Tobacco, alcohol, and socioeconomic status and adenocarcinomas of the esophagus and gastric cardia. *J Natl Cancer Inst* 1997; **89:** 1277-1284
47 **Garidou A**, Tzonou A, Lipworth L, Signorello LB, Kalapothaki V, Trichopoulos D. Life-style factors and medical conditions in relation to esophageal cancer by histologic type in a low-risk population. *Int J Cancer* 1996; **68:** 295-299
48 **Zhang ZF**, Kurtz RC, Sun M, Karpeh M Jr, Yu GP, Gargon N, Fein JS, Georgopoulos SK, Harlap S. Adenocarcinomas of the esophagus and gastric cardia: medical conditions, tobacco, alcohol, and socioeconomic factors. *Cancer Epidemiol Biomarkers Prev* 1996; **5:** 761-768
49 **Kabat GC**, Ng SK, Wynder EL. Tobacco, alcohol intake, and diet in relation to adenocarcinoma of the esophagus and gastric cardia. *Cancer Causes Control* 1993; **4:** 123-132
50 **Spechler SJ,** Robbins AH, Rubins HB, Vincent ME, Heeren T, Doos WG, Colton T, Schimmel EM. Adenocarcinoma and Barrett's esophagus. An overrated risk? *Gastroenterology* 1984; **87:** 927-933
51 **Avidan B**, Sonnenberg A, Schnell TG, Sontag SJ. Hiatal hernia and acid reflux frequency predict presence and length of Barrett's esophagus. *Dig Dis Sci* 2002; **47:** 256-264
52 **Conio M,** Filiberti R, Blanchi S, Ferraris R, Marchi S, Ravelli P, Lapertosa G, Iaquinto G, Sablich R, Gusmaroli R, Aste H, Giacosa A. Risk factors for Barrett's esophagus: a case-control study. *Int J Cancer* 2002; **97:** 225-229
53 **Gronbaek M**, Becker U, Johansen D, Tonnesen H, Jensen G, Sorensen TI. Population based cohort study of the association between alcohol intake and cancer of the upper digestive tract. *BMJ* 1998; **317:** 844-847
54 **Cooper BT,** Barbezat GO. Barrett's oesophagus: a clinical study of 52 patients. *Q J Med* 1987; **62:** 97-108
55 **Levi F**, Ollyo JB, La Vecchia C, Boyle P, Monnier P, Savary M. The consumption of tobacco, alcohol and the risk of adenocarcinoma in Barrett's oesophagus. *Int J Cancer* 1990; **45:** 852-854
56 **Herlihy KJ**, Orlando RC, Bryson JC, Bozymski EM, Carney CN, Powell DW. Barrett's esophagus: clinical, endoscopic, histologic, manometric, and electrical potential difference characteristics. *Gastroenterology* 1984; **86:** 436-443
57 **Day NE**, Munoz N. Esophagus. In: Schottenfeld D, Fraumeni JF Jr, eds. Cancer epidemiology and prevention Philadelphia: WB Saunders Co, 1982: 596-623
58 **Goldman MC,** Beckman RC. Barrett syndrome. Case report with discussion about concepts of pathogenesis. *Gastroenterology* 1960; **39:** 104-110
59 **Halvorsen JF**, Semb BK. The "Barrett syndrome" (the columnar-lined lower oesophagus): an acquired condition secondary to reflux oesophagitis. A case report with discussion of pathogenesis. *Acta Chir Scand* 1975; **141:** 683-687
60 **Jankowski J**. Gene expression in Barrett's mucosa: acute and chronic adaptive responses in the oesophagus. *Gut* 1993; **34:** 1649-1650
61 **Morgan G**, Vainio H. Barrett's oesophagus, oesophageal cancer and colon cancer: an explanation of the association and cancer chemopreventive potential of non-steroidal anti-inflammatory drugs. *Eur J Cancer Prev* 1998; 7: 195-199
62 **Heath EI,** Limburg PJ, Hawk ET, Forastiere AA. Adenocarcinoma of the esophagus: risk factors and prevention. *Oncology (Williston Park)* 2000; **14:** 507-14; discussion 518-520, 522-523
63 **Paull G**, Yardley JH. Gastric and esophageal Campylobacter pylori in patients with Barrett's esophagus. *Gastroenterology* 1988; **95:** 216-218
64 **Talley NJ**, Cameron AJ, Shorter RG, Zinsmeister AR, Phillips SF. Campylobacter pylori and Barrett's esophagus. *Mayo Clin Proc* 1988; **63:** 1176-1180
65 **Weston AP**, Badr AS, Topalovski M, Cherian R, Dixon A, Hassanein RS. Prospective evaluation of the prevalence of gastric Helicobacter pylori infection in patients with GERD, Barrett's esophagus, Barrett's dysplasia, and Barrett's adenocarcinoma. *Am J Gastroenterol* 2000; **95:** 387-394
66 **Bani-Hani K**, Martin IG, Hardie LJ, Mapstone N, Briggs JA, Forman D, Wild CP. Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma. *J Natl Cancer Inst* 2000; **92:** 1316-1321
67 **Wild CP,** Forman D. Surveillance for Barrett's oesophagus. It is too early to dismiss surveillance programmes. *BMJ* 2001; **322:** 1125; author reply 1126

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•*CLINICAL RESEARCH*•

Association between cag-pathogenicity island in *Helicobacter pylori* isolates from peptic ulcer, gastric carcinoma, and non-ulcer dyspepsia subjects with histological changes

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Abstract

AIM: To investigate the presence of the *cag*-pathogenicity island and the associated histological damage caused by strains with complete *cag*-PAI and with partial deletions in correlation to the disease status.

METHODS: We analyzed the complete *cag*-PAI of 174 representative *Helicobacter pylori* (*H pylori*) clinical isolates obtained from patients with duodenal ulcer, gastric ulcer, gastric cancer, and non-ulcer dyspepsia using eight different oligonucleotide primers viz *cag*A1, *cag*A2, *cag*AP1, *cag*AP2, *cag*E, *cag*T, LEC-1, LEC-2 spanning five different loci of the whole *cag*-PAI by polymerase chain reaction (PCR).

RESULTS: The complete screening of the genes comprising the *cag*-PAI showed that larger proportions of subjects with gastric ulcer (97.8%) inhabited strains with complete *cag*-PAI, followed by gastric cancer (85.7%), non-ulcer dyspepsia (7.1%), and duodenal ulcer (6.9%), significant differences were found in the percentage distribution of the genes in all the clinical groups studied. It was found that strains with complete *cag*-PAI were able to cause severe histological damage than with the partially deleted ones.

CONCLUSION: The *cag*-PAI is a strong virulent marker in the disease pathogenesis as it is shown that a large number of those infected with strain with complete *cag*-PAI had one or the other of the irreversible gastric pathologies and interestingly 18.5% of them developed gastric carcinoma. The presence of an intact *cag*-PAI correlates with the development of more severe pathology, and such strains were found more frequently in patients with severe gastroduodenal disease. Partial deletions of the *cag*-PAI appear to be sufficient to render the organism less pathogenic.

Key words: *Helicobacter pylori*; *cag*-pathogenicity island; Genetic diversity; Gastro-duodenal diseases

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INTRODUCTION

Gastric cancer is the second most deadly malignant neoplasia worldwide. According to the presently available statistics, approximately 74% of those diagnosed succumb to this disease every year[1]; this is because of poor prognosis as it is often made when the disease has assaulted the muscularis propria. Evidences show that the pathogenesis of gastric cancer is a multistep process[2,3]. This 'cascade' is believed to be triggered by *Helicobacter pylori* (*H pylori*) infection, a Gram negative pathogen. Chronic infection with this gastric pathogen is known to be the major factor driving the precancerous process via mechanisms including direct transformation of cells, induction of immunosuppression with consequently reduced cancer immunosurveillance, or by causing chronic inflammation[4,5]. In 1994, the International Agency for Research on Cancer (IARC) declared *H pylori* a Class 1 (definite) carcinogen based on the epidemiological and interventional studies in human beings[6] and convinced that this bacterial infection indeed plays a key role in the initiation of the neoplastic process in the stomach.

Although many attempts in the past have been made to understand and associate the causal link between *H pylori* infection and the sequelae that leads to gastric carcinoma[7,8], there are conflicting data in the literature due

to differences in the study population and designs[9,10].

In the past few years, many *H pylori* virulence factors have been described that contribute to the survival of this pathogen in an extremely hostile acidic milieu of the stomach and its colonization in that organ[11,12]. Other putative virulence determinants such as vacuolating cytotoxin gene A (vacA), cytotoxin associated gene pathogenicity island (*cag*-PAI) and induced by contact with epithelium gene A (iceA) are not present in all *H pylori* strains or are known to exhibit different allelic variations[13]. The cytotoxin-associated gene island also referred to as the *cag*-PAI is an approximately 40-kb cluster of genes and is the most studied marker of the *H pylori*. In many studies[20,22,24], this large fragment has been a criterion of typing *H pylori* into pathogenic and non-pathogenic strains (Type I and Type II). Studies emphasizing on the functional importance of this island have reported that strains possessing *cag*-PAI induce more notable phenotypic changes *in vivo*, such as higher levels of IL-8 production than *cag*-PAI negative ones[30]. Recent studies made from within our institute[14,15] and other parts of the world[16,17] have mainly focused on the presence of this large fragment and its association with the disease status. Therefore, in a continuing attempt to further establish a strong epidemiologic relation between the *cag*-PAI and the disease conditions with reference to the histopathologic changes lead to the inception of the present study. As it is reported[18] that the presence of *cag* island and the consequent *cag* instability may produce differences in the pathogenicity and host adaptability within a bacterial strain, detailed analyses of the genes of the *cag* island in human beings isolates with reference to histologic damage and disease outcome would be essential. Therefore, compelled by these observations, the present study was designed to identify the distribution of different genes of the *cag*-PAI in clinical *H pylori* isolates by assessing the presence of representative genes located in different segments of the *cag*-island and its correlation with the histologic changes and disease status.

MATERIALS AND METHODS

Patients and sampling

The study population consisted of 174 patients (100 males and 74 females) with a mean age of 48.4 years (range 21-73 years). The patients were classified at the time of endoscopy into those suffering from active ulcers disease [duodenal ulcers (n = 58), gastric ulcers (n = 46), gastric carcinoma (n = 14)] and those with no evidence of mucosal ulcer and gastritis, but suffering from mild or severe dyspeptic symptoms, i.e. non-ulcer dyspepsia (n = 56). None of the patients included in the study had received NSAIDs or antibiotics within the previous 2 mo. Informed consents were taken from the patients who underwent upper gastrointestinal endoscopy at the department of gastroenterology, Deccan College of Medical Sciences, Hyderabad.

Four gastric biopsies were collected: one in urea solution for the rapid urease test (RUT), one in supplemented broth for isolating culture, one in phosphate-buffered saline (PBS) for testing by PCR assay, and one in 10% buffered formalin for histological examination by modified Giemsa stain for the presence of *H pylori*.

H pylori strains

A total of 174 clinical *H pylori* strains were screened for the presence of *cag*-PAI genes. These strains were recovered from individual subjects undergoing upper gastrointestinal endoscopy presenting with various symptoms. This included 43 live strains and 131 genomic DNA isolated from the gastric biopsy.

Extraction of genomic DNA

H pylori culture and DNA extraction from the culture and biopsy was carried out as described elsewhere[19].

PCR analysis of the *cag*-PAI genes

The genes of the *cag*-PAI were PCR amplified under the conditions described by Ikenoue *et al*.[20]. One microliter of the extracted genomic DNA was used in a 20 μL reaction volume containing 1´ PCR buffer, 1 U Taq DNA polymerase, 1.5 mmol/L $Mg2^{+}$, 200 μmol/L each dNTP and 10 pmol/L of each primer.

The cycling parameters were optimized and are as follows: Initial denaturation at 95 °C for 5 min, followed by 40 cycles each of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1.5 min and finally after the last cycle, extension was continued for another 7 min.

Eight sets of oligonucleotide primers spanning the 40 kb *cag*-PAI were used in the study. Appropriate positive and negative controls were included in each set to avoid misinterpretation of results. The details of the primers used with their product sizes are enlisted in Table 1. Amplicons were separated by electrophoresis on 2% agarose gel and stained by ethidium bromide.

Histopathological analysis

The biopsy specimen collected from the gastric antrum was used for histopathologic examination to grade

Table 1 List of primers

Target gene	Primer	Sequence (5'- 3')	Product size (bp)
	CagA-F1	AACAGGACAAGTAGCTAGCC	
CagA1	CagA-R1	TATTAATGCGTGTGTGGCTG	701
	CagA-F2	GATAACAGGCAAGCTTTTGA	
CagA2	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	349
	CagAP-F1	GTGGGTAAAAATGTGAATCG	
CagAP1	CagA-R1	TATTAATGCGTGTGTGGCTG	730
	CagAP-F2	CTACTTGTCCCAACCATTTT	
CagAP2	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	1 181
	CagE-F1	GCGATTGTTATTGTGCTTGTAG	
CagE	CagE-R1	GAAGTGGTTAAAAAATCAATGCCCC	329
	CagT-F1	CCATGTTTATACGCCTGTGT	
CagT	CagT-R1	CATCACCACACCCTTTTGAT	301
	LEC-F1	ACATTTTGGCTAAATAAACGCTG	
LEC-1	LEC-R1	TCTCCATGTTGCCATTATGCT	384
	LEC-F2	ATAGCGTTTTGTGCATAGAA	
LEC-2	LEC-R2	ATCTTTAGTCTCTTTAGCTT	877

the severity of disease after they were embedded in paraffin and stained with hematoxylin and eosin. A single experienced pathologist (ZA), who was blinded to the patient's history and molecular data of the isolate, evaluated all histological data. The grade of gastritis was determined on the basis of the updated Sydney system[21].

Statistical analysis

The data were analyzed by means of χ^2 test and the Mann –Whitney *U* test.

RESULTS

In a total of 174 isolates screened, only 65 (37.4%) was found to carry the complete *cag*-PAI, while 109 (62.6%) carried the *cag*-PAI with partial deletions. No isolate was found with completely deleted *cag*-PAI (Table 2).

With reference to the clinical status, we found majority of the duodenal ulcer (DU) isolates and non-ulcer dyspepsia (NUD) isolates to possess partially deleted *cag*-PAI, while on the contrary we found ~97.8% of the gastric ulcer (GU) isolates and 85.7% of the gastric carcinoma (GC) isolates possessed the intact *cag*-PAI. The details of the results obtained are given in Table 2 and Figure 1. Statistically these differences were highly significant.

Table 2 Relationship between the presence of cag-PAI and the clinical status

Clinical status (*n*)	Intact PAI (%)	*cag*-PAI type Partially deleted PAI (%)	Completely deleted PAI (%)
DU (58)	4 (6.9)	54 (93.1)	0
GU (46)	45 (97.8)	1 (2.2)	0
NUD (56)	4 (7.1)	52 (92.9)	0
GC (14)	12 (85.7)	2 (14.3)	0
Total (174)	65 (37.4)	109 (62.6)	0

χ^2=130.71 .

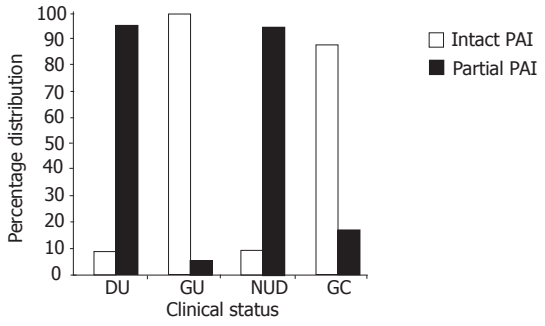

Figure 1 Distribution of *cag*-PAI in dyspeptic subjects.

When checked for the presence of each locus, we could find that the total *cag*A gene (i.e. with promoters) to be present in 69 (39.7%) of all the isolates screened, 4 (2.3%) isolates had total deletion of the gene and 101 (58%) were carrying partial deletions. Of the 69-*cag*A positive isolates, prevalence of *cag*A gene was predominant among gastric ulcer (GU) and gastric carcinoma (GC) strains (Table 3 and Figure 2) simultaneously *cag*A gene with partial deletions were more prevalent among *H pylori* isolated from duodenal ulcer (DU) and non-ulcer dyspepsia (NUD) subjects.

Table 3 *cag*A status of *H pylori* isolates

Clinical status (*n*)	*cagA* +ve (%)	Partial *cagA* +ve (%)	*cagA* –ve (%)
DU (58)	8 (13.8)	46 (79.3)	4 (6.9)
GU (46)	45 (97.8)	1 (2.2)	0
NUD (56)	4 (7.1)	52 (92.9)	0
GC (14)	12 (85.7)	2 (14.3)	0
Total (174)	69 (39.7)	101 (58)	4 (2.3)

DU *vs* GU, DU *vs* GC, GU *vs* NUD and NUD *vs* GC combinations are highly significant at 0.1% level. DU *vs* NUD and GU *vs* GC combinations are not significant at 0.1% level.

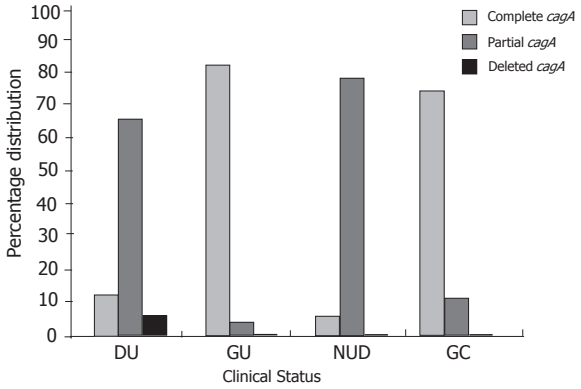

Figure 2 Distribution of *cag*A in clinical isolates.

Observing the prevalence patterns of *cag*A deletion mutant strains, we further analyzed the predominance of deletions in the promoter and body part of the *cag*A gene. The body part of *cag*A gene (i.e. A1+A2) was present in a maximum number of isolates i.e. 134 (77%), among which a considerable number of isolates lacked the promoter region (i.e. AP1+AP2) (Table 4).

Screening of the *cag*II region of the *cag*-PAI revealed *cag*E, *cag*T, LEC-1, and LEC-2 to be present in 143 (82.1%), 145 (87.3%), 142 (81.6%) and 111 (63.7%) of the total isolates, respectively. The distribution of these genes with reference to disease status is illustrated in detail in Table 5 and Figure 3. We found the difference between DU and GU, GU and NUD, GC and NUD to be highly significant statistically.

In the present study, histopathological examination of antral biopsies from a total of 174 subjects was carried out

Table 4 Distribution of *cag*A gene and *cag*A promoter in *H pylori* isolates

Clinical status (*n*)	A1+A2 (%)	AP1+AP2 (%)
DU (58)	43 (74.1)	8 (13.8)
GU (46)	45 (97.8)	46 (100)
NUD (56)	32 (57.1)	6 (10.7)
GC (14)	14 (100)	12 (85.7)
Total (174)	134 (77)	72 (41.4)

Table 5 Distribution of *cag*E, T, LEC1, and LEC2 in *H pylori* isolates

Clinical status (*n*)	*cag*E (%)	*cag*T (%)	LEC1 (%)	LEC2 (%)
DU (58)	47 (81)	56 (96.5)	44 (75.8)	33 (56.8)
GU (46)	46 (100)	46 (100)	46 (100)	46 (100)
NUD (56)	36 (64.2)	36 (64.2)	38 (67.8)	18 (32.1)
GC (14)	14 (100)	14 (100)	14 (100)	14 (100)
Total (174)	143 (82.1)	152 (87.3)	142 (81.6)	111 (63.7)

For *cag*E, GU *vs* NUD is highly significant at 0.1% level ($P<0.001$), DU *vs* GU, NUD *vs* GC combinations are significant at 1% level ($P<0.01$), DU *vs* NUD combination is significant at 5% level ($P<0.05$) and DU *vs* GC . For *cag*T, DU *vs* NUD, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$), NUD *vs* GC combination is significant at 1% level ($P<0.01$) and DU *vs* GU, DU *vs* GC, GU *vs* GC are not significant at 1% level. For LEC1, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$), NUD *vs* GC is significant at 1% level ($P<0.01$), DU *vs* GC is significant at 5% level ($P<0.05$). For LEC2, DU *vs* GU, GU *vs* NUD, NUD *vs* GC combinations are highly significant at 0.1% level ($P<0.001$), DU *vs* NUD, DU *vs* GC combinations are significant at 1% level ($P<0.01$) and GU *vs* GC is not significant at 1% level.

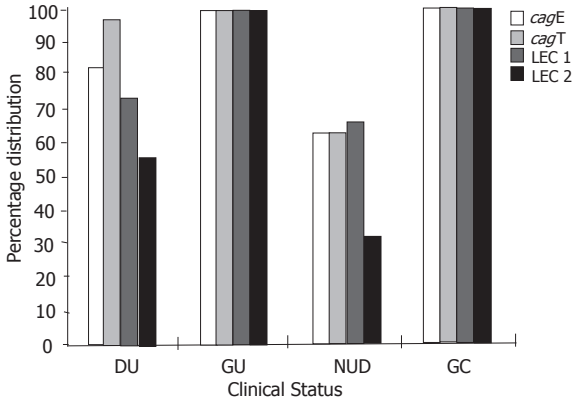

Figure 3 Distribution of *cag*E, T, LEC1, LEC2

to check the grade of gastritis and the differences between the pathologies of intact *cag*-PAI and partial *cag*-PAI infection. Of the 174 subjects included for the study, we screened 160 subjects as 14 of them had endoscopically proven gastric carcinoma and hence histopathology confirmed features of carcinoma. Out of the 160 subjects screened, all showed chronic gastritis and on further screening for any other advanced type of gastritis, we found 112 (70%) of the total 160 to possess topographic chronic superficial gastritis, 20 (12.5%) showed atrophic changes, whereas 18 (11.25%) and 10 (6.25%) of the subjects showed IM and dysplasia respectively (Table 6). When each of these histological lesions were scored individually in relation to their respective clinical status, we found that among the 58 duodenal ulcer cases, 52 (89.7%) showed chronic superficial gastritis, 2 (3.4%) showed atrophy and 4 (6.9%) showed intestinal metaplasia while none of the subjects had dysplasia (Table 6).

In the gastric ulcer category, we found that 9 (19.6%) showed chronic superficial gastritis, 13 (28.3%) showed atrophy, 14 (30.4%) showed intestinal metaplasia and 10 (21.7%) showed dysplasia. For atrophy, metaplasia and dysplasia, the difference between GU and DU was statistically highly significant ($P<0.001$, Table 6).

In the NUD category, we found that 51 (91.1%) showed chronic superficial gastritis and 5 (8.9%) showed atrophic gastritis while none had intestinal metaplasia and dysplasia. For metaplasia and dysplasia, the difference between GU and NUD, was statistically highly significant ($P<0.001$, Table 6).

Table 6 Frequency of atrophy, metaplasia, and dysplasia in chronic gastritis subjects

Clinical status (*n*)	Acute gastritis (%)	Chronic gastritis			
		Superficial gastritis	Gastritis with atrophy (%)	Gastritis with metaplasia (%)	Gastritis with dysplasia (%)
DU (58)	0	52 (89.7)	2 (3.4)	4 (6.9)	0
GU (46)	0	9 (19.6)	13 (28.3)	14 (30.4)	10 (21.7)
NUD (56)	0	51 (91.1)	5 (8.9)	0	0
Total (160)	0	112 (70)	20 (12.5)	18 (11.25)	10 (6.25)

For atrophy, DU *vs* GU combination is highly significant at 0.1% level ($P<0.001$), GU *vs* NUD is significant at 1% level ($P<0.01$) and DU *vs* NUD combination is not significant at 1% level. For metaplasia, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$) and DU *vs* NUD combination is significant at 5% level ($P<0.05$). For dysplasia, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$) and DU *vs* NUD combination is not significant at any level.

When we analyzed the histological status, with reference to the strain infecting with either intact PAI or partially deleted ones, we found a significant difference among them. As evidenced from Table 7, we found that, of the 65 subjects infected with intact PAI, 8 (12.3%) showed chronic superficial gastritis, 17 (26.1%) showed atrophic changes, 18 (27.7%) showed intestinal metaplasia (all of them were Type III), 10 (15.4%) showed high grade dysplastic changes and 12 (18.5%) showed intestinal type gastric carcinoma. On the contrary, when we checked those subjects, who inhabited partially deleted strains, we found 104 (95.4%) to possess chronic superficial gastritis, 3 (2.8%) showed atrophy while 2 (1.8%) showed gastric carcinoma and these differences were statistically very significant ($P<0.001$, Table 7).

When a correlation between the histological status of the subjects infected with either intact *cag*-PAI or partially deleted strains isolated from subjects with varied disease status was made, we found that among 58 DU subjects, 4 had intact *cag*-PAI and all of them were Type III intestinal metaplasia, among the remaining 54, which had partial deletions in the *cag*-PAI, 52 (96.3%) showed chronic superficial gastritis and 2 (3.7%) showed atrophic changes. Among the GU subjects, of the 46 infected, 45 were known to possess the *cag*-PAI and 8 (17.8%) of them showed chronic superficial gastritis, 13 (28.9%) showed atrophic gastritis, 14 (31.1%) showed Type III intestinal metaplasia and 10 (22.2%) showed high grade dysplastic changes (Table 8). Among the 56 NUD subjects, 4 with intact *cag*-PAI showed atrophic changes and of the rest with partial deletions 51 (98.1%) showed chronic superficial gastritis and 1 (1.9%) showed atrophic gastritis (Table 8) while among the 14 isolated from

Table 7 Histological status of subjects with intact *cag*-PAI (*n* = 65) and partial *cag*-PAI (*n* = 109)

Cag-type	Chronic gastritis							Gastric carcinoma	
(*n*)	Superficial gastritis	Gastritis with atrophy	Gastritis with metaplasia (%)			Gastritis with dysplasia(%)		Intestinal (%)	Diffuse (%)
	(%)	(%)	Ty-1	Ty-2	Ty-3	Low grade	High grade		
Intact (65)	8 (12.3)	17 (26.1)[b]	0	0	18 (27.7)	0	10 (15.4)	12[b] (18.5)	0
Partial (109)	104 (95.4)	3 (2.8)	0	0	0	0	0	2 (1.8)	0

[b]*P*<0. 001 *vs* others.

Table 8 Correlation of the histological status of the subjects with intact and partially deleted *cag*-PAI from varied disease status

Clinical status	Chronic gastritis								Gastric carcinoma	
(*n*)	cag status	Superficial gastritis	Gastritis with atrophy (%)	Gastritis with metaplasia (%)			Gastritis with dysplasia (%)		Intestinal (%)	Diffuse (%)
	(*n*)	(%)		Ty-1	Ty-2	Ty-3	Low grade	High grade		
DU (58)	Intact (4)	0	0	0	0	4 (100)	0	0	0	0
	Partial (54)	52 (96.3)	2 (3.7)	0	0	0	0	0	0	0
GU (46)	Intact (45)	8(17.8)[a]	13 (28.9)	0	0	14 (31.1)	0	10 (22.2)	0	0
	Partial (1)	1 (100)	0	0	0	0	0	0	0	0
NUD (56)	Intact (4)	0	4(100)[a]	0	0	0	0	0	0	0
	Partial (52)	51 (98.1)	1 (1.9)	0	0	0	0	0	0	0
GC (14)	Intact (12)	0	0	0	0	0	0	0	12 (100)[b]	0
	Partial (2)	0	0	0	0	0	0	0	2 (100)	0

[a]*P* <0.05, [b]*P* <0.001 *vs* others.

gastric carcinoma 12 had intact *cag*-PAI while the other 2 partial deletions and when scored for the type of gastric carcinoma all of them showed intestinal type of carcinoma (Table 8).

DISCUSSION

The *cag*-PAI is an approximately 40-kb cluster of genes in *H pylori* chromosome, and a quite conservative entity. Censini *et al*.[22] in 1996 first identified strains with partially deleted *cag*-PAIs. The molecular mechanism of these genetic rearrangements was explained by incorporation of an insertion element, IS605, in *cag*-PAI. Recently, the composition of the *cag*-PAI in clinical *H pylori* isolates has been studied in different populations by various methods, including PCR, dot blotting and long distance PCR[20,23,24].

In the present study, we used simple PCR for structural screening of *cag*-PAI in clinical isolates of *H pylori*. Out of the 174 clinical isolates, we found only 37.4% were carrying the complete *cag*-PAI (Table 2), whereas Mukhopadhyay *et al*.[25] reported more than 96% in Calcutta strains of peptic ulcer and non-ulcer dyspepsia. In our study, 97.8% of gastric ulcer and 85.7% (Table 2 and Figure 1) of the gastric carcinoma strains were carrying complete PAI, which are considered to be severe forms of the gastro-duodenal diseases. Even though duodenal ulcer is also considered to be a severe form of the gastro-duodenal disease, the proportion of DU strains that carried was just 6.9%. Jenks *et al*.[23] reported that the presence of certain genes (*cag*A, *cag*E, *cag*M, T, ORF 6, 10, 13) in the *cag*-PAI is highly associated with duodenal ulcers. We too found a similar type of observation, but not for all the seven genes which they selected. We observed the same kind of correlation with only two genes i.e. *cag*E and *cag*T, where DU cases carried *cag*E with 81% and *cag*T with 96.5% (Table 5 and Figure 3). Whereas NUD isolates were carrying the genes with an average of 65% (Table 5 and Figure 3). Further, Day *et al*.[26] revealed that isolates containing *cag*E were associated with duodenal ulceration.

Among non-ulcer dyspepsia strains, we found 7.1% to carry the complete *cag*-PAI, which is statistically almost equal to DU percentage, and a report from Sweden[27] showed 58% of *cag*-PAI positivity in NUD isolates. In the same report, the authors showed that 5% of isolates from severe pathology i.e. gastric carcinoma and duodenal ulcer, and 15% of the isolates from NUD lacking the *cag*-PAI. Not only the data from other continents, but from the same Indian sub-continent showed total deletions from ulcer group and non-ulcer group[25], whereas we could not come across a single isolate with entirely deleted *cag*-PAI from any of the disease condition indicating strain diversity. This kind of diversity, i.e. absence of completely deleted PAIs and presence of just 6.9% complete *cag*-PAIs in duodenal ulcer cases, might be particularly true for the south Indian of Telugu linguistic group who are mainly Dravidian and married consanguineously for millennia[28]. Their genetic separation from other Indian communities during much of the human history has already been reported[29].

Strains with intermediate genotypes, lacking parts of the *cag*-PAI, were found in 62.6% (Table 2) and more frequently found in patients with non-ulcer dyspepsia and duodenal ulcer (Figure 1). A probable mechanism for the establishment of these internal deletions within the *cag*-PAI would be that the short repeated sequences found by Nilsson *et al*.[27] may serve as homologies enabling slipped strand mispairing and consequently excision of the enclosed DNA fragment especially in DU and NUD subjects. Moreover, in our observation, the partially deleted *cag*-PAI represented a genotype more common than a complete *cag*-PAI and no strain was found with completely

deleted *cag*-PAI.

Conventionally *cag*A was used and is still used as a marker for the presence of an intact *cag*-PAI and for virulence. Recently, Backert *et al*.[30] showed a good correlation of *cag*A with the presence of *cag*-PAI. In this study, we found that 39.7% of the strains carried the complete *cag*A gene (Table 3) and the presence of *cag*A gene did not correlate with the genetic presence of complete *cag*-PAI. Further, for - 4 kb *cag*A gene, we used four sets of primers i.e. two primers for body of the gene and two primers for promoter, designed from various locations of the body region and promoter, whereas other studies taken up earlier had used a single set of primer for complete *cag*-PAI. This might be the reason for the correlation, which they obtained. The typical observation was when 77% of the isolates were carrying the body region (A1+A2) only 41.4% of them carried promoter region (Table 4), which means that in our isolates even though the strains carried the *cag*A gene, most of them lacked the promoter of the gene, without which *cag*A is not functional. Further, four isolates i.e. 2.3% completely lacked the *cag*A gene and all the four isolates belonged to duodenal ulcer cases.

Among many virulence markers present in the *H pylori* genome, *cag*-PAI is the major virulence factor and is associated with severe gastroduodenal pathology[31,32] that includes both duodenal and gastric ulcers along with carcinomas. Some studies have identified a correlation between an intact *cag*-PAI and development of disease[20,23,24,27], as we are trying to show with this present study in Indian scenario, whereas others could not find such a relationship[33,34].

"Infection with *H pylori* always causes chronic active gastritis"[35]. This phrase has become true in our observation. As it is observed in Table 6, there are no acute gastritis subjects. Moreover, the subjects infected with *cag*-PAI positive strains were found to show severe forms of histopathological changes, like atrophic gastritis, intestinal metaplasia, and neoplasia. This might be the reason for IARC-WHO to designate *H pylori* a class I (definite) carcinogen[6].

Out of the 174 isolates, 65 (37.4%) had complete *cag*-PAI and 109 (62.6%) had partial deletions in the *cag*-PAI (Table 2). As evident from Table 7, it can be observed that subjects infected with *H pylori* strains with intact *cag*-PAI had many remarkable histopathological changes, when compared to those who had partially deleted *cag*-PAI. It is quite clear from the statistics ($P<0.001$) that among 65 *H pylori* strains with intact *cag*-PAI, 18.5% subjects had advanced cancerous lesions, while only 1.8% of the 109 subjects, who harbored *H pylori* with partial *cag*-PAI had advanced to carcinoma thus allowing us to delineate that persons with intact *cag*-PAI are 10-fold more prone to develop carcinoma in comparison to the partially deleted *cag*-PAI strains.

Parallelly, a small group of population, i.e. 12.3% with intact *cag*-PAI, were shown to have only chronic superficial gastritis, but according to Ohkuma *et al*.[36] *H pylori* positive cases with chronic gastritis have increased risk of atrophy and intestinal metaplasia. On the other hand, partial *cag*-PAI subjects were also shown to have chronic gastritis (Table 6), but the percentage of disease progression was very high among *cag*-PAI positive subjects (Table 7) than those with partially deleted ones. Moreover, Type-3 metaplasia and high-grade dysplasia were seen only in *cag*-PAI positive subjects, where Type-3 metaplasia is closely linked to carcinoma[37] and dysplasia is nothing but a non-invasive type of neoplasia[38]. The results of this study are in contrast to those obtained by Keates *et al*.[39] who determined that gene products of the *cag* pathogenicity island are required for maximal activation of mitogen-activated protein kinases (MAPK) in gastric epithelial cells, which regulate cell proliferation, differentiation, inflammatory responses, stress, and programmed death, leading to induce gastroduodenal inflammation, ulceration and neoplasia. Further, Naumann *et al*.[40] stated that the integrity of whole *cag*-PAI is also a pre-requisite for efficient activation of early transcription factor AP-1, which is known for its immuno-stimulatory function.

In relation with disease status, among DU, GU, and NUD, gastric ulcers are considered to be more prone to the gastric carcinoma[41]. The observations of this study are in correlation to that obtained by Hansson *et al*.[41]. When we compare GU subjects with DU and NUD, the percentage of predisposing factors was much more among GU subjects. Recently, Wanatabe *et al*.[42] proved this in animal models. In their study at 26 wk, Mongolian gerbils developed chronic gastritis, ulceration and metaplasia. At 62 wk, 31% of them developed adenocarcinoma. Interestingly the inoculum used for the infection was obtained from a patient with gastric ulcer. Moreover, there have been reports that gastric cancer mortality rates bear an inverse relationship to duodenal ulcer disease rates[43], suggesting that they are directly relating with gastric ulcer in ulcer groups.

Further, in partial *cag*-PAI subjects, 2.8% showed atrophy, 1.8% showed carcinoma (Table 7) suggesting the role of other virulence genes and risk factors. Parallelly high incidence rate of gastric carcinoma among the gastric ulcer cases might be true, but it should not be assigned to a single determinant such as *cag*-PAI, but it is a result of many factors such as host genetic factors, environment, low socio-economic status, irregular dietary habits in addition to *H. pylori* with complete *cag*-PAI[44].

Hence, we can suggest that the *cag*-PAI is a strong virulent marker in the disease pathogenesis because more than 85% of the *cag*-PAI positive subjects were shown to have one or the other of the irreversible gastric pathologies and interestingly 18.5% of them developed gastric carcinoma and GU is the major risk/predisposing factor for the gastric carcinoma. Moreover, duodenal ulcer is not at all a risk factor for severe gastric pathologies and it is not a severe kind of disease, like gastric ulcer in our population.

REFERENCES

1 **Leung WK**, Lin SR, Ching JY, To KF, Ng EK, Chan FK, Lau

JY, Sung JJ. Factors predicting progression of gastric intestinal metaplasia: results of a randomised trial on Helicobacter pylori eradication. *Gut* 2004; **53:** 1244-1249

2 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52:** 6735-6740

3 **Kuipers EJ**, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, Festen HP, Meuwissen SG. Long-term sequelae of Helicobacter pylori gastritis. *Lancet* 1995; **345:** 1525-1528

4 **Correa P**. Is gastric cancer preventable? *Gut* 2004; **53:** 1217-1219

5 **Shimoyama T**, Fukuda S, Liu Q, Nakaji S, Fukuda Y, Sugawara K. Helicobacter pylori water soluble surface proteins prime human neutrophils for enhanced production of reactive oxygen species and stimulate chemokine production. *J Clin Pathol* 2003; **56:** 348-351

6 International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans, vol. **61:** Schistosomes, liver flukes and *Helicobacter pylori*. Lyons, France: IARC, 1994

7 **Wong BCY**, Ching CK, Lam SK. Helicobacter pylori and gastric cancer. *Hong Kong Med J* 1999; **5 :** 175-179

8 **Blaser MJ**. Linking Helicobacter pylori to gastric cancer. *Nat Med* 2000; **6:** 376-377

9 ***Uemura N***, Mukai T, Okamoto S, Yamaguchi S, Mashiba H, Taniyama K, Sasaki N, Haruma K, Sumii K, Kajiyama G. Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer Epidemiol Biomarkers Prev* 1997; **6:** 639-642

10 **Sung JJY**, Lin S-R, Ching JYL, Zhou L–Y, To KF, Wang R–T, Leung WK, NG EKW, Lau JYW, Lee YT, Yeung CK, Chao W, Chung SCS. Atrophy and intestinal metaplasia one year after cure of *H pylori* infection: a prospective, randomized study. *Gastroenterology* 2000; **119:** 7-14

11 **Koehler CI**, Mues MB, Dienes HP, Kriegsmann J, Schirmacher P, Odenthal M. Helicobacter pylori genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. *Mol Pathol* 2003; **56:** 36-42

12 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; ***347:*** 1175-1185

13 **Covacci A**, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. Helicobacter pylori virulence and genetic geography. *Science* 1999; **284:** 1328-1333

14 **Tiwari SK**, Khan AA, Ahmed KS, Ali M, Ahmed I, Habeeb A, Kauser F, Hussain MA, Ahmed N and Habibullah CM. PCR based analysis of Helicobacter pylori isolated from saliva: An approach for rapid molecular genotyping in correlation with disease status. *J Gastroenterol Hepatol* (In press).

15 **Kauser F**, Khan AA, Hussain MA, Carroll IM, Ahmad N, Tiwari S, Shouche Y, Das B, Alam M, Ali SM, Habibullah CM, Sierra R, Megraud F, Sechi LA, Ahmed N. The cag pathogenicity island of Helicobacter pylori is disrupted in the majority of patient isolates from different human populations. *J Clin Microbiol* 2004; **42:** 5302-5308

16 **Covacci A**, Falkow S, Berg DE, Rappuoli R. Did the inheritance of a pathogenicity island modify the virulence of Helicobacter pylori? *Trends Microbiol* 1997; **5:** 205-208

17 **Occhialini A**, Marais A, Urdaci M, Sierra R, Munoz N, Covacci A, Megraud F. Composition and gene expression of the cag pathogenicity island in Helicobacter pylori strains isolated from gastric carcinoma and gastritis patients in Costa Rica. *Infect Immun* 2001; **69:** 1902-1908

18 **Tomasini ML**, Zanussi S, Sozzi M, Tedeschi R, Basaglia G, De Paoli P. Heterogeneity of cag genotypes in Helicobacter pylori isolates from human biopsy specimens. *J Clin Microbiol* 2003; **41:** 976-890

19 **Li C**, Musich PR, Ha T, Ferguson DA Jr, Patel NR, Chi DS, Thomas E. High prevalence of Helicobacter pylori in saliva demonstrated by a novel PCR assay. *J Clin Pathol* 1995; **48:** 662-666

20 **Ikenoue T**, Maeda S, Ogura K, Akanuma M, Mitsuno Y, Imai Y, Yoshida H, Shiratori Y, Omata M. Determination of Helicobacter pylori virulence by simple gene analysis of the cag pathogenicity island. *Clin Diagn Lab Immunol* 2001; **8:** 181-186

21 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20:** 1161-1181

22 **Censini S**, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A. cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and diseaseassociated virulence factors. *Proc Natl Acad Sci USA* 1996; **93:** 14648-14653

23 **Jenks PJ**, Megraud F, Labigne A. Clinical outcome after infection with Helicobacter pylori does not appear to be reliably predicted by the presence of any of the genes of the cag pathogenicity island. *Gut* 1998; **43:** 752-758

24 **Maeda S**, Yoshida H, Ikenoue T, Ogura K, Kanai F, Kato N, Shiratori Y, Omata M. Structure of cag pathogenicity island in Japanese Helicobacter pylori isolates. *Gut* 1999; **44:** 336-341

25 **Mukhopadhyay AK**, Kersulyte D, Jeong JY, Datta S, Ito Y, Chowdhury A, Chowdhury S, Santra A, Bhattacharya SK, Azuma T, Nair GB, Berg DE. Distinctiveness of genotypes of Helicobacter pylori in Calcutta, India. *J Bacteriol* 2000; **182:** 3219-3227

26 **Day AS**, Jones NL, Lynett JT, Jennings HA, Fallone CA, Beech R, Sherman PM. cagE is a virulence factor associated with Helicobacter pylori-induced duodenal ulceration in children. *J Infect Dis* 2000; **181:** 1370-1375

27 **Nilsson C**, Sillen A, Eriksson L, Strand ML, Enroth H, Normark S, Falk P, Engstrand L. Correlation between cag pathogenicity island composition and Helicobacter pylori-associated gastroduodenal disease. *Infect Immun* 2003; **71:** 6573-581

28 **Ahmed N**, Khan AA, Alvi A, Tiwari S, Jyothirmayee CS, Kauser F, Ali M, Habibullah CM. Genomic analysis of Helicobacter pylori from Andhra Pradesh, South India: molecular evidence for three major genetic clusters. *Current Sci* 2003; **85 :** 1579-1586

29 **Bamshad M**, Fraley AE, Crawford MH, Cann RL, Busi BR, Naidu JM, Jorde LB. mtDNA variation in caste populations of Andhra Pradesh, India. *Hum Biol* 1996; **68:** 1-28

30 **Backert S**, Schwarz T, Miehlke S, Kirsch C, Sommer C, Kwok T, Gerhard M, Goebel UB, Lehn N, Koenig W, Meyer TF. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. *Infect Immun* 2004; **72:** 1043-56

31 **Crabtree JE**, Kersulyte D, Li SD, Lindley IJ, Berg DE. Modulation of Helicobacter pylori induced IL-8 synthesis in gastric epithelia cells mediated by cag-PAI encoded VirD4 homologoue. *J Clin Pathol* 1999; **52:** 653-657

32 **Guillemin K**, Salama NR, Tompkins LS, Falkow S. Cag pathogenicity island-specific responses of gastric epithelial cells to Helicobacter pylori infection. *Proc Natl Acad Sci USA* 2002; **99:** 15136-15141

33 **Audibert C**, Burucoa C, Janvier B, Fauchere JL. implication of the structure of the Helicobacter pylori cag pathogenicity island in induction of interleukin-8 secretion. *Infect Immun* 2001; **69:** 1625-1629

34 **Peters TM**, Owen RJ, Slater E, Varea R, Teare EL, Saverymuttu S. Genetic diversity in the Helicobacter pylori cag pathogenicity island and effect on expression of anti-CagA serum antibody in UK patients with dyspepsia. *J Clin Pathol* 2001; **54:** 219-223

35 **Meining A**, Riedl B, Stolte M. Features of gastritis predisposing to gastric adenoma and early gastric cancer. *J Clin Pathol* 2002; **55:** 770-773

36 **Ohkuma K**, Okada M, Murayama H, Seo M, Maeda K, Kanda M, Okabe N. Association of Helicobacter pylori infection with atrophic gastritis and intestinal metaplasia. *J Gastroenterol*

Hepatol 2000; ***15:*** 1105-1112

37 **Rokkas T**, Filipe MI, Sladen GE. Detection of an increased incidence of early gastric cancer in patients with intestinal metaplasia type III who are closely followed up. *Gut* 1991; **32:** 1110-1113

38 **Rugge M**, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H. Gastric dysplasia: the Padova international classification. *Am J Surg Pathol* 2000; **24:** 167-176

39 **Keates S**, Keates AC, Warny M, Peek RM Jr, Murray PG, Kelly CP. Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by cag+ and cag- Helicobacter pylori. *J Immunol* 1999; **163:** 5552-559

40 **Naumann M**, Wessler S, Bartsch C, Wieland B, Covacci A, Haas R, Meyer TF. Activation of activator protein 1 and stress response kinases in epithelial cells colonized by Helicobacter pylori encoding the cag pathogenicity island. *J Biol Chem* 1999; **274:** 31655-662

41 **Hansson LE**, Nyren O, Hsing AW, Bergstrom R, Josefsson S, Chow WH, Fraumeni JF Jr, Adami HO. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *N Engl J Med* 1996; **335:** 242–249

42 **Wanatabe T**, Tada M, Hirofumi N, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in Mongolian gerbils. *Gastroenterology* 1998; **115:** 642-648

43 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; ***347:*** 1175-1186

44 **Sipponen P**, Hyvarinen H, Seppala K, Blaser MJ. Review article: Pathogenesis of the transformation from gastritis to malignancy. *Aliment Pharmacol* Ther 1998; **12 Suppl 1:** 61-71

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• *CLINICAL RESEARCH* •

Single daily amikacin versus cefotaxime in the short-course treatment of spontaneous bacterial peritonitis in cirrhotics

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Abstract

AIM: To compare the efficacy and safety of single daily amikacin *vs*. cefotaxime in the 5-d treatment of spontaneous bacterial peritonitis (SBP).

METHODS: Thirty-seven cirrhotic patients with SBP, 19 in group A and 18 in group B, were studied. Group A received 1 g of cefotaxime every 6 h, and group B received 500 mg of amikacin qd. Both antibiotics were administered up to 5 d and the responses were compared.

RESULTS: Infection was cured in 15 of 19 patients (78.9%) treated with cefotaxime and in 11 of 18 (61.1%) treated with amikacin. Four patients of the Cefotaxime group (21.1%) and five patients of the Amikacin group (27.8%) died. Two in each group (10.5% *vs* 11.1%) had renal impairment during study period. One in each group (5.3% *vs* 5.6%) may be considered to suffer from nephrotoxicity due to increased urinary β_2-microglobulin concentration.

CONCLUSION: In this study, single daily doses of amikacin in the treatment of SBP in cirrhotics were not associated with an increased incidence of renal impairment or nephrotoxicity. However, a 5-d regimen of amikacin is less effective than a 5-d regimen of cefotaxime in the SBP treatment.

Key words: Spontaneous bacterial peritonitis; Amikacin; Cefotaxime

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INTRODUCTION

Some studies have suggested that liver disease is a risk factor for aminoglycoside-induced nephrotoxicity[1-4]. However, aminoglycosides are still frequently used to treat sepsis in patients with liver disease[5]. In recent studies[6-8], single daily parenteral aminoglycoside administrations have shown some benefits as compared with multiple daily doses. These benefits include reduced toxicity, possible enhanced efficacy, greater convenience, and reduced costs. However, results of single daily aminoglycoside treatments of bacterial infections in cirrhotics have not been evaluated.

Spontaneous bacterial peritonitis (SBP) is a common complication of cirrhotic ascites. In a recent study, 5-d cefotaxime treatment of SBP was as efficacious as a 10-d course[9].

Because of these reasons, we have designed this prospective randomized study to compare the efficacy and nephrotoxicity of single daily amikacin dosage versus that of cefotaxime in the 5-d treatment of SBP in cirrhotics.

MATERIALS AND METHODS

Materials

Between July 2000 and June 2002, patients admitted to the Kaohsiung Veterans General Hospital who fulfilled all of the following criteria were enrolled into this study: (1) had liver cirrhosis; (2) had an ascitic fluid absolute neutrophil count > 500 cells/mm^3 with SBP as the only suspected cause. Patients were excluded from the study for any of the following reasons: (1) had a history of allergy to penicillins, cephalosporins, or aminoglycoside; (2) considered to be a terminal or critical case with life expectancy of less than one month; (3) had secondary peritonitis or tumor rupture; (4) had a serum creatinine level >2 mg/dL; (5) had an antibiotic treatment during previous 2 wk.

Methods

Patients were randomly allocated into two different therapeutic groups. Group A received 1 g of cefotaxime every 6 h. Group B received 500 mg of amikacin qd or 8 mg/kg of body weight qd if patient's body weight was less than 60 kg. The subsequent dosages of amikacin were adjusted according to renal function so that the trough level of plasma amikacin remained ≤30 μg/mL Both antibiotics were administered by intravenous infusion for 30 min. The antibiotics were not changed in any case during the first 72 h unless a nonsusceptible organism was isolated in the initial cultures. Antibiotics

were administered up to 5 d to patients who responded to the treatment. For patients who did not respond to the treatment after 5 d, antibiotic treatment was changed according to antibiotic susceptibility tests when a resistant organism was isolated, or empirically when the causative bacteria was not cultured.

Blood, urine, and ascites samples were obtained for culture, routine cell counts, and chemistry screening before initiation of antibiotic treatment. Other body fluids were cultured when indicated.

Abdominal paracentesis was repeated every 72 h until the culture became sterile and the ascitic fluid neutrophil count decreased to <250 cells/mm^3. Clinical signs and symptoms of infection, e.g., fever, chills, abdominal pain, abdominal tenderness, ileus, and mental status change, were recorded daily. Patients infected by organisms resistant to cefotaxime or amikacin were treated with appropriate alternative antibiotics according to the culture result and susceptibility tests. Two days after completion of antibiotic therapy, abdominal paracentesis was performed for culture test and cell count. Blood culture was repeated if bacteremia had been documented previously. If signs or symptoms of infection developed after discontinuation of the antibiotic, paracentesis for cell count and culture of blood were also repeated.

Infection was considered cured when all clinical and laboratory signs of infection disappeared during therapeutic period and cultures performed 2 d after antibiotic withdrawal were negative. Antibiotic treatment was considered a failure when the symptoms and signs of infection did not improve, or worsened, or when a nonsusceptible bacteria was isolated in the initial cultures. Patients discharged alive were followed closely throughout their illness for 4 wk after completion of treatment. Recurrence within 4 wk after discontinuation of therapy was defined as recurrent SBP or bacteremia. Relapse within 4 wk after discontinuation of therapy was defined as recurrent infection of ascitic fluid or blood with the same organism (identical species) that caused the initial infection. Reinfection within 4 wk after discontinuation of therapy was defined as recurrent bacteremia or recurrence of SBP with an organism different from the original pathogen. Superinfection was defined as development of SBP or bacteremia caused by a different pathogenic bacterium from the original organism during therapy. Infection-related mortality was defined as death caused by bacterial infection of ascitic fluid or blood, with clinical or bacteriologic evidence of uncontrolled infection. Hospitalization mortality was defined as death due to any cause during the hospitalization. In evaluating antibiotic efficacy, patients who died within the first 3 d after inclusion in the study were not considered.

Serum and urine creatinine levels were measured before treatment, every 2 d during treatment, and 24 h after completion of therapy. The 24-h urine was collected every 2 d for assessment of the creatinine clearance.

For patients who were treated with amikacin, blood and ascites samples for the determination of the trough and peak levels of amikacin were obtained 30 min before and one hour after administration of the drugs for every alternate day during treatment. The samples were stored at - 30 ^{o}C until assay. The amikacin levels were measured by radioimmunoassay.

According to previous investigations[4], urinary β_2-microglobulin is a useful test to discriminate antibiotic-induced nephrotoxicity from functional renal failure (or hepatorenal syndrome) in cirrhotic patients. Therefore, in the current study, the urinary concentration of β_2-microglobulin was measured in all patients studied before therapy, 3 d after initiation of treatment, and 2 d after antibiotic withdrawal. Fresh urine samples were collected and stored at pH 6 to 7 (with the addition of 1 N sodium hydroxide) and at - 30 ^{o}C until assayed. The analysis was performed using a commercial radioimmunoassay. Results of β_2-microglobulin were not available during the study.

In this study, renal impairment was defined as a rise in serum creatinine of 0.5 mg/dL or a ≥50% fall in creatinine clearance during the period. In the absence of other possible causes of renal tubular damage, renal impairment was considered to be secondary to nephrotoxicity if urinary β_2-microglobulin concentration increased from normal values (before treatment) to more than 2 000 mg/L (during treatment). Otherwise, renal impairment was considered functional. Patient who died within the first 3 d after inclusion in the study were not considered in evaluating the incidence of nephrotoxicity.

The *t*-test with Yates' correction, χ^2 with Fisher's exact test, or the nonparametric Mann-Whitney *U* test were used for statistical analysis. Data are presented as mean±SD. In each instance a two-tailed test was used. A *P* value of < 0.05 was considered significant.

RESULTS

A total of fifty- seven patients met inclusion criteria. Twelve patients were excluded because of either critical case with shock on presentation (4), prior treatment with antibiotics (2), initial serum creatinine concentration > 2 mg/dL (4), evidence of secondary peritonitis (1), or tumor rupture (1). Forty-five patients were eligible for the study and were randomized. Twenty-two patients were randomized to cefotaxime treatment and twenty-three patients to amikacin treatment. Two patients in amikacin group were later disqualified, because secondary peritonitis and tuberculous peritonitis were diagnosed after evaluation. Three patients in each group were not considered in the analysis of the result, because they died or fled against medical advice within 48 h after entry into the study. The remaining 37 patients, 19 in cefotaxime group and 18 in amikacin group, were the subjects of this analysis.

There was no significant difference between patients of the two groups (Table 1), in relation to sex, age, etiology of cirrhosis, severity of cirrhosis as expressed by Child-Pugh score, and renal function before treatment (expressed by serum creatinine level). In each group only one patient was Child-Pugh class B. The others were class C. Only 22 patients (59.5%) had normal serum creatinine level

Table 1 Comparison of clinical and laboratory characteristics of the patients

Characteristics	Treatment regimen		
	Cefotaxime	Amikacin	*P*
Number of patients	19	18	
Male/female	17/2	11/7	NS
Age(yr)[1]	54 ± 17	58 ± 11	NS
Etiologies of cirrhosis (%)[2]			NS
Alcoholism	3 (16)	2 (11)	
Chronic hepatitis B	14 (74)	11 (61)	
Chronic hepatitis C	1 (5)	4 (22)	
Child-Pugh score[1]	11.4 ± 1.2	11.1 ± 1.1	NS
Serum creatinine(mg/dL)[1]	1.5 ± 0.5	1.4 ± 0.4	NS

[1]Data are presented as mean ± SD. [2]Data are presented as number and percentage of total. NS: not significant

(<1.5 mg/dL) before treatment.

Nine (24%) of the 37 patients grew a pathogen from their ascitic fluid, and 8 (21.6%) were bacteremic. The ascites and blood isolates were similar between the two groups (Table 2). Two pathogens in the blood (group B *Streptococcus* and *Vibrio amalonaticus*) were resistant to cefotaxime and amikacin. Although the clinical signs of infection disappeared during therapeutic period with cefotaxime, crystal penicillin and tetracycline were given according to the susceptibility tests since the sixth day. The other isolates were sensitive to cefotaxime and amikacin.

Table 2 Flora of ascites and blood

	Treatment regimen		
	Cefotaxime (%)	Amikacin (%)	*P*
Ascites			
Escherichia coli	4 (21)	3 (17)	NS
Klebsiella pneumoniae	0	1 (6)	NS
Citrobacter diversus	0	1 (6)	NS
Blood			
Escherichia coli	2 (11)	1 (6)	NS
Klebsiella pneumoniae	1 (5)	2 (11)	NS
Streptococcus group B	1 (5)	0	NS
Vibrio amalonaticus	1 (5)	0	NS

Data are presented as number and percentage of total. NS: not significant.

The clinical response to treatment and survival were similar between the groups (Table 3). Infection was cured in 15 of 19 patients (78.9%) treated with cefotaxime and in 11 of 18 (61.1%) treated with amikacin. However, there was no statistic significance between these two groups. Three patients in cefotaxime group had recurrent infection within 4 wk after completion of treatment. One was considered relapse due to recurrent bacteremia with the same organism that caused the initial bacteremia. The other two also suffered from bacteremia, but the previous infection episode was not bacteremic. Recurrent SBP concurrent with new episodes of bacteremia rather than relapse were considered in these two patients. Among the 8 bacteremic patients in the initial treatment, 2 in the cefotaxime group had resistant isolates. Although they became well during the initial therapeutic period and cured without recurrence within 4 wk after changing antibiotics, treatment failure was still considered according to the study's design. The other 6 bacteremic patients were bacteriologically cured by repeated culture after 5-d of antibiotic treatment. Only one patient (16.6%) in the cefotaxime group had relapse 10 d after completion of treatment.

Table 3 Results of treatment

	Treatment regimen		
	Cefotaxime (%)	Amikacin (%)	*P*
Number of patients	19	18	
Cure[2]	15 (78.9)	11 (61.1)	NS
Normalized PMN count[2]	18 (94.7)	15 (83.3)	NS
Serum creatinine(mg/dL)[1]	1.3 ± 0.8	1.5 ± 1.1	NS
Afebril in 72 h[2]	18 (94.7)	15 (83.3)	NS
Pain-free in 72 h[2]	19 (100)	17 (94.4)	NS
Recurrence[2]	3(15.8)	0	NS
Superinfection[2]	0	0	NS
Infection-related mortality[2]	0	3 (16.7)	0.105
Hospitalization mortality[2]	4 (21.1)	5 (27.8)	NS
Days of hospitalization[1]	12 ± 8	13 ± 9	NS

[1]Data are presented as mean ± SD. [2]Data are presented as number and percentage of total. NS: not significant

There was no significant difference between these two groups in the mortality rate. During the whole hospitalization period, 4 patients of the cefotaxime group (21.1%) and 5 patients of the amikacin group (27.8%) died. Although for most patients the cause of death was multifactorial, in three cases of the amikacin group infection was considered to be the main cause of death due to no other major event or infection identified.

There was no significant difference in the incidence of renal impairment or nephrotoxicity between patients treated with cefotaxime or amikacin (Table 4). Two in each group (10.5% *vs* 11.1%) had renal impairment during study period. The urinary β_2-microglobulin concentration increased in both groups during treatment and decreased after antibiotics withdrawal. One in each group (5.3% *vs* 5.6%) may be considered nephrotoxicity due to increased urinary β_2-microglobulin concentration from normal values (before treatment) to more than 2 000 mg/L (during treatment). The patient who developed nephrotoxicity in the amikacin group died on the 6th d of the study period.

Table 4 Evaluation of nephrotoxicity

	Treatment regimen		
	Cefotaxime(%)	Amikacin(%)	*P*
Number of patients	19	18	
Renal impairment (%)[2]	2 (10.5)	2 (11.1)	NS
Urinary β^2-microglobulin (mg/L)			
Before treatment[1]	402 ± 80	1220 ± 392	NS
3 d after initiation[1]	779 ± 2465	612 ± 814	NS
2 d after withdrawal[1]	126 ± 119	173 ± 44	NS
increase > 2 000 mg/L (%)[2]	1 (5.3)	1 (5.6)	NS

[1]Data are presented as mean ± SD. [2]Data are presented as number and percentage of total. NS: not significant.

The mortality was considered infection-related. The patient in the cefotaxime group died on the 5th d due to hepatic and renal failure even though the infection appeared under controlled.

Wide range of peak and trough levels of amikacin in patients treated with amikacin was noted in this study (Table 5).

Table 5 Amikacin concentration (mean ± SD)

Blood peak level (μg/mL)	19.6 – 127.3 (40 ± 32)
Blood trough level (μg/mL)	1.3 – 73.7 (12 ± 22)
Ascites peak level (μg/mL)	4.3 – 80.1 (20 ± 25)
Ascites trough level(μg/mL)	1.2 – 78.9 (15 ± 26)

DISCUSSION

The aminoglycosides are potent antibiotics, with peak concentration-dependent bactericidal activity against Gram-negative pathogens and staphylococci. They display trough concentration-dependent nephrotoxicity and ototoxicity. Aminoglycosides exhibit enduring antibacterial activity (especially against Gram-negative bacilli) many hours after tissue concentrations become negligible. Appreciation of this postantibiotic effect leads to replacement of conventional multiple daily doses by large single daily doses. The latter regimens confer at least equivalent efficacy and less risk of nephrotoxicity[7]. Among the aminoglycosides available in our hospital, we used amikacin in this study, because it is the least susceptible to degradation by bacterial enzymes and causes less nephrotoxicity than gentamicin and tobramycin[7]. Because some studies have suggested that liver disease is a risk factor for nephrotoxicity in patients treated with aminoglycoside[1-4], we used only about half of the recommended single daily dosage of amikacin (15 mg/kg q24 h in usual study[7]) in this study.

The optimal duration of antibiotic treatment for SBP had been investigated recently. Ten to fourteen days intravenous therapy had been recommended[10-12]. However, it had been argued that, because SBP had a low bacterial load (often only 1 organism/mm^3 of ascitic fluid), a shorter duration of treatment might suffice. A recent randomized controlled study comparing 5 d *vs* 10 d treatment with cefotaxime found no difference in efficacy and mortality rate [9].

In this study the cure rate was higher in the group of patients treated with cefotaxime (78.9%) than in the group of patients treated with amikacin (61.1%), although there was no significant difference. Larger sample sizes in further studies may confirm this finding. The cure rate for SBP in patients treated with cefotaxime in this study is similar to the previous studies. In Runyon's study[9], the cure rate for SBP treated by 5-d cefotaxime is 93.1%. On the other hand, the cure rate in patients treated with amikacin in this study is also similar to the previous studies that treated cirrhotic patients with severe infection using aminoglycosides combining with other antibiotics. In Felisart's study which compared cefotaxime *vs.* ampicillin-tobramycin in cirrhotics with severe infections (most were peritonitis), the cure rates were 85% and 56% respectively[13]. The response rate in the McCormick's study which used netilmicin plus mezlocillin in the empirical therapy of presumed sepsis in cirrhotic patients was 56%[5]. Single daily dosage of aminoglycoside in the treatment of infections in cirrhotic patients seemed as effective as combining with other antibiotics in traditional dosages but less effective than cefotaxime.

It is well known that in traditional dosages, the serum, the tissue and the body fluid levels of aminoglycosides are unpredictable, varying from one patient to another[14, 15]. We conducted this study by using a single daily dose of aminoglycoside for easy monitoring of the drug level. Just like previous reports, our study also showed that there were wide ranges of drug levels in blood and ascites between the patients regardless of whether their renal function were normal or not. Some levels might not achieve the bactericidal levels. For example, the MIC of amikacin for *E coli* was 2 mg/mL in this study. Only 13 of the 18 patients (72%) had 4-fold or higher for their peak level of ascites. On the other hand, it is well established that cefotaxime has a wide range between therapeutic and toxic dosages. Also, the ascitic fluid concentration of cefotaxime is several-fold higher than the MIC of most susceptible organisms at any time throughout the treatment[16]. This may explain the difference of efficacy of treatment between cefotaxime and amikacin.

The incidence of nephrotoxicity in this present study was 5.6% in patients treated with amikacin. This was similar with the Felisart's study in patients treated with ampicillin-tobramycin (7%)[13], but almost six times lower than the Cabrera's study in patients treated with cephalothin-gentamicin or cephalothin-tobramycin (32%)[4]. Previous investigations have suggested that combined therapy, i.e. cephalothin, might enhance the nephrotoxicity of aminoglycosides[17, 18]. Although some study suggested that the risk for aminoglycoside nephrotoxicity was 5 times higher in a patient with liver disease than without[2], we found that a single daily dosage of amikacin did not cause marked nephrotoxicity in cirrhotic patient in this study. The incidence was between 3% and 11% in patients treated with aminoglycosides, similar with previous reports[19, 20].

In this study, eight patients (22%) had positive blood culture concurrent with SBP. Two of them were resistant isolates to cefotaxime and had other antibiotic treatment. Six patients were all bacteriologic cured after 5-d of treatment. This was confirmed by negative culture result repeated after treatment. However, one of the six patients (17%) who was treated with cefotaxime had bacteremic relapse 10 d after completion of treatment. In Runyon's study, 9 bacteremic patients treated with 5-d cefotaxime were documented to become sterile during the first 72 h of therapy. No relapse was mentioned. 9 Because bacteremia in cirrhosis is a severe prognostic sign, it has been considered common practice to treat it for 10 to 14 d[21]. Do patients with SBP in addition to bacteremia require longer treatments than patients without bacteremia? Is a

5-d course adequate for treating bacteremia in cirrhotic patients? These issues remain to be clarified.

In spite of the lower antibiotic efficacy of amikacin, the hospitalization mortality rate resulting from this antibiotic regimen was similar to that observed in patients treated with cefotaxime. This may be explained by the fact that both groups of cirrhotics had a similar degree of liver failure. Most mortalities were related to infective complications in patients treated with amikacin and to noninfective complications in patients treated with cefotaxime.

In summary, we found that single daily doses of amikacin in the treatment of SBP in cirrhotics were not associated with an increased incidence of renal impairment or nephrotoxicity. However, the efficacy of a 5-d regimen of amikacin is less than a 5-d regiimen of cefotaxime in SBP treatment.

REFERENCES

1 **Garcia-Tsao G**. Current management of the complications of cirrhosis and portal hypertension: variceal hemorrhage, ascites, and spontaneous bacterial peritonitis. *Gastroenterology* 2001; **120:** 726-748

2 **Mowat C**, Stanley AJ. Review article: spontaneous bacterial peritonitis--diagnosis, treatment and prevention. *Aliment Pharmacol Ther* 2001; **15:** 1851-1859

3 **Follo A**, Llovet JM, Navasa M, Planas R, Forns X, Francitorra A, Rimola A, Gassull MA, Arroyo V, Rodes J. Renal impairment after spontaneous bacterial peritonitis in cirrhosis: incidence, clinical course, predictive factors and prognosis. *Hepatology* 1994; **20:** 1495-1501

4 **Cabrera J**, Arroyo V, Ballesta AM, Rimola A, Gual J, Elena M, Rodes J. Aminoglycoside nephrotoxicity in cirrhosis. Value of urinary beta 2-microglobulin to discriminate functional renal failure from acute tubular damage. *Gastroenterology* 1982; **82:** 97-105

5 **McCormick PA**, Greenslade L, Kibbler CC, Chin JK, Burroughs AK, McIntyre N. A prospective randomized trial of ceftazidime versus netilmicin plus mezlocillin in the empirical therapy of presumed sepsis in cirrhotic patients. *Hepatology* 1997; **25:** 833-836

6 **Prins JM**, Buller HR, Kuijper EJ, Tange RA, Speelman P. Once versus thrice daily gentamicin in patients with serious infections. *Lancet* 1993; **341:** 335-339

7 **Kumana CR**, Yuen KY. Parenteral aminoglycoside therapy. Selection, administration and monitoring. *Drugs* 1994; **47:** 902-913

8 **Mauracher EH**, Lau WY, Kartowisastro H, Ong KH, Genato VX, Limson B, Yusi GM, Liu CY, Suwangool P. Comparison of once-daily and thrice-daily netilmicin regimens in serious systemic infections: a multicenter study in six Asian countries. *Clin Ther* 1989; **11:** 604-613

9 **Runyon BA**, McHutchison JG, Antillon MR, Akriviadis EA, Montano AA. Short-course versus long-course antibiotic treatment of spontaneous bacterial peritonitis. A randomized controlled study of 100 patients. *Gastroenterology* 1991; **100:** 1737-1742

10 **Holland DJ**, Sorrell TC. Antimicrobial therapy and prevention of spontaneous bacterial peritonitis. *J Gastroenterol Hepatol* 1993; **8:** 370-374

11 **Bhuva M**, Ganger D, Jensen D. Spontaneous bacterial peritonitis: an update on evaluation, management, and prevention. *Am J Med* 1994; **97:** 169-175

12 **Garcia-Tsao G**. Spontaneous bacterial peritonitis. *Gastroenterol Clin North Am* 1992; **21:** 257-275

13 **Felisart J**, Rimola A, Arroyo V, Perez-Ayuso RM, Quintero E, Gines P, Rodes J. Cefotaxime is more effective than is ampicillin-tobramycin in cirrhotics with severe infections. *Hepatology* 1985; **5:** 457-462

14 **Kaye D**, Levison ME, Labovitz ED. The unpredictability of serum concentrations of gentamicin: pharmacokinetics of gentamicin in patients with normal and abnormal renal function. *J Infect Dis* 1974; **130:** 150-154

15 **Moore RD**, Lietman PS, Smith CR. Clinical response to aminoglycoside therapy: importance of the ratio of peak concentration to minimal inhibitory concentration. *J Infect Dis* 1987; **155:** 93-99

16 **Moreau L**, Durand H, Biclet P. Cefotaxime concentrations in ascites. J Antimicrob Chemother 1980; **6 Suppl A:** 121-122

17 Wade JC, Schimpff SC, Wiernik PH. Antibiotic combination-associated nephrotoxicity in granulocytopenic patients with cancer. *Arch Intern Med* 1981; **141:** 1789-1793

18 **Wade JC**, Smith CR, Petty BG, Lipsky JJ, Conrad G, Ellner J, Lietman PS. Cephalothin plus an aminoglycoside is more nephrotoxic than methicillin plus an aminoglycoside. *Lancet* 1978; **2:** 604-606

19 **Appel GB**, Neu HC. The nephrotoxicity of antimicrobial agents (second of three parts). *N Engl J Med* 1977; **296:** 722-728

20 **Kahlmeter G**, Dahlager JI. Aminoglycoside toxicity - a review of clinical studies published between 1975 and 1982. *J Antimicrob Chemother* 1984; **13 Suppl A:** suppl A 9-22

21 **Graudal N**, Hubeck B, Bonde J, Thomsen AC. The prognostic significance of bacteremia in hepatic cirrhosis. *Liver* 1987; **7:** 138-141

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• *RAPID COMMUNICATION* •

Scattered and rapid intrahepatic recurrences after radio frequency ablation for hepatocellular carcinoma

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Abstract

AIM: To evaluate a series of patients with hepatocellular carcinoma (HCC) treated with several different protocols and devices.

METHODS: We treated 138 patients [chronic hepatitis/liver cirrhosis (Child–Pugh A/B/C), 3/135 (107/25/3)] with two different devices and protocols: cool-tip needle [initial ablation at 60 W (standard method) (n = 37) or at 40 W (modified method) (n = 28)] or; ablation with a LeVeen needle using a standard single-step, full expansion (single-step) method (n = 39) or a multi-step, incremental expansion (multi-step) method.

RESULTS: Eleven patients experienced rapid and scattered recurrences 1 to 7 mo after the ablation. Nine patients were treated by the cool-tip original protocol (60 W) (9/37 = 24%) and the other two by the LeVeen single-step method (2/39 = 5%). The location of the recurrence was surrounding and limited to the site of ablation segment in three cases, and spread over one lobule or both lobules in the other eight cases. There was no recurrence in the patients treated with the modified cool-tip modified method (40 W) or the LeVeen multi-step method.

CONCLUSION: There is a risk of rapid and scattered recurrence after RFA, especially when the standard cool-tip procedure is used. Because such recurrence would worsen the prognosis, we recommend that modified protocols for the cool-tip and LeVeen needle methods should be used in clinical practice.

Key words: Radio frequency ablation; Hepatocellular carcinoma; Cool-tip needle; LeVeen needle; Recurrence

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INTRODUCTION

Image-guided radio frequency ablation (RFA) is an emerging technique for the treatment of hepatocellular carcinoma (HCC)[1-5], as well as for metastatic liver tumors[6]. The procedure has been adopted worldwide as a safe and effective method, and is replacing percutaneous ethanol injection therapy (PEIT) as the treatment of choice. Livraghi *et al*.[7] and Lencioni *et al*.[8] have compared RFA and PEIT for the treatment of small-sized HCC. Although both studies concluded that RFA resulted in a higher rate of complete necrosis, Livraghi *et al*.[7] also indicated that the complication rate was higher with RFA than with PEIT.

A number of reports have described complications associated with RFA[9-14]. The major complications reported were peritoneal bleeding, hepatic abscess, hemothorax, perforation of the gastrointestinal wall, and rapid hepatic decompensation. These complications occurred during or just after RFA, however, and delayed complications have been reported much less frequently. Takada *et al*.[15] described two cases in which rapid and aggressive recurrence accompanied by portal thrombus occurred 4 to 6 mo after RFA. Nicoli *et al*.[16] described a peculiar form of recurrence after RFA for HCC that was characterized by numerous and equally-sized recurrence nodules and which occurred after only one month post-treatment. More recently, Ruzzenente *et al*.[17] also described a series of patients with rapidly spreading recurrence after RFA.

In the past several years, we have observed cases similar to those presented by Nicoli *et al*.[16]. We have previously reported a significant increase in pressure in the ablated area during RFA[18], and concluded that scattered recurrence is attributable to an explosion caused by excessive increases in intra-tumor pressure. In our experience, intra-operative complications are easily avoidable by confirming the safety of a puncture route

before the treatment, whereas a rapid recurrence after RFA is a serious clinical problem that can influence the prognosis of patients.

In this report, we have evaluated a series of HCC cases treated by RFA with different devices and protocols, and have analyzed cases with rapid and scattered recurrence after RFA.

PATIENTS AND METHODS

RFA

Between April 2000 and December 2004, 138 patients with HCC were treated with the RFA procedure in Kyushu University Hospital or National Hospital Organization Kyushu Medical Center. Characteristics of the candidates are shown in Table 1. They consisted of 79 males and 59 females, aged 40 to 83 years with a mean of 68.2 years. All of them had chronic liver damage; 121 patients were hepatitis C virus (HCV) positive and 17 were hepatitis B virus (HBV) positive. Among them, 135 patients were diagnosed with liver cirrhosis by liver biopsy, clinical laboratory data, ultrasonography and/or computed tomography. According to the hepatic functional reserve evaluation for the cirrhotic patients just before RFA, 107, 25, and 3 were classified as Child–Pugh's class A, B, and C, respectively. The diagnosis of HCC was confirmed by aspiration tumor biopsy for all patients prior to treatment.

RFA was performed with either a LeVeen™ multipolar array needle in combination with an RF 2000 generator™ (Radio Therapeutics Corporation, Mountain View, CA, USA) or a Cool-tip™ RF System (3.0 cm exposure length) (Radionics, Burlington, MA, USA). One of the two devices was selected randomly for RFA. All procedures were performed by hepatologists who had at least 10 years of experience performing image-guided *in situ* tumor ablation therapy. The original standard protocol was used for cool-tip needle RFA until December 2002, and the modified protocol was used thereafter. For LeVeen needle RFA, the original standard protocol was used until April 2002, and the modified method was used thereafter. The details of each protocol are described below.

Original procedure with cool-tip needle: Cool-tip electrode with 3 cm of exposed tip was used to deliver RF (radio frequency) energy to the tumors. RF energy was delivered as described previously[19]. In short, after needle puncture of the tumor, generator output was increased to 100–120 W and maintained at this level until the end of the procedure. If an increase in impedance equal to or greater than 10 Ω above baseline was observed, the current was reduced, until stable impedance was observed and then increased again.

Modified procedure with cool-tip needle: The needle used was the same type as was used for the original procedure. The method differed from the original procedure in that the ablation was started at a low voltage of 40 W, and the electric power was increased by 10 W every minute. The maximum of electric power was 120 W, and the RF energy delivery was continued, until the impedance increased beyond the limit of the generator.

Original procedure with LeVeen needle: The electrode used for this procedure was a 3 or 3.5 cm LeVeen needle depending on the tumor size. Before delivery of RF power, the tumor was punctured with a needle and the ten tines were then fully expanded. The ablation was started at 40 W (3-cm needle) or 50 W (3.5-cm needle) RF power and was further increased by 10 W/min up to 75 W (3-cm needle) or 90 W (3.5-cm needle). If the impedance had not increased after 10 min, the RF power was again increased by 10 W increments. The procedure was terminated when a marked increase in impedance ("roll off") occurred.

Modified procedure with LeVeen needle: The needle used was the same type as was used for the original procedure. In the modified version, the tines of the electrode were expanded step by step in 10 steps, and at every step, the length of tine expansion was one-tenth of the full expansion length. Ablation at each step was continued, until the impedance increased to "roll off". Furthermore, at the first step, the ablation was started at a low voltage of 30 W. If it took more than 30 s for "roll off" at a step, the power was increased by 10 W before starting the next step. The maximum electric power for this protocol was 75 W (3-cm needle) or 90 W (3.5-cm needle), which was maintained until the final step.

After RFA treatment, all of the patients were followed up every one or two months with US or CT. When recurrence was detected by imaging examination, additional treatment was instituted with RFA, PEIT, transarterial chemoembolization (TACE) or a combination of these therapies. The prognosis was based on the data obtained up to December 2004.

Statistical analysis

Baseline characteristics of the patients prior to RFA treatment are shown as mean±SD and statistical comparisons were performed using χ^2 test for categorical data and non-paired *t*-test for numerical data.

Table 1 Characteristics of the patients

	Scattered recurrence (–)	Scattered recurrence (+)	Total
n	127	11	138
Age	67.5±8.6	69.2±8.1	67.6±8.5
Sex (male/female)	71/56	8/3	79/59
Virus (HBV/HCV)	14/113	3/8	17/121
Tumor size (mm)	24.3±13.7	20.1±5.7	24.0±13.5
Albumin (g/dL)	3.65±0.38	3.52±0.57	3.64±0.39
Bilirubin (mg/dL)	0.86±0.51	1.02±0.44	0.87±0.50
ALT (U/L)	48.4±28.3	56.0±17.8	49.0±28.5
Platelet (x10^4/mL)	10.9±5.5	9.0±3.6	10.7±5.5
Child–Pugh (CH[1]/A/B/C)[a]	3/102/20/2	0/5/5/1	3/107/25/3
Device and Protocol[b]			
Original cool-tip	28	9	37
Modified cool-tip	28	0	28
Single-step LeVeen	37	2	39
Multi-step LeVeen	34	0	34
Prognosis (alive/death)[b]	87/40	3/8	90/48
Observation period (mo)	27.1±11.0	24.0±11.5	26.9±11.1

[a]$P<0.05$, [b]$P<0.01$ between the patients with and without scattered recurrences (χ^2 test). [1]CH: chronic hepatitis.

Table 2 Details of the patients with scattered recurrence

n	Sex	Age	Background	Liver function (Child-Pugh)	Location/ Size (mm)	Device	Protocol	Form of recurrence	Interval between RFA and recurrence (mo)	Prognosis after RFA (mo)
1	F	75	LC (HCV)	C	S8/15	LeVeen	Original	Bilobular	3	Death (15)
2	M	61	LC (HBV)	A	S7/20	Cool-tip	Original	Bilobular	3	Alive (42)
3	F	79	LC (HCV)	B	S2/25	Cool-tip	Original	Bilobular	2	Death (36)
4	M	63	LC (HBV)	A	S4/15	Cool-tip	Original	Surrounding	7	Death (24)
5	M	69	LC (HCV)	B	S7/26	Cool-tip	Original	Bilobular	4	Death (27)
6	M	73	LC (HCV)	B	S5/13	Cool-tip	Original	Bilobular	5	Death (20)
7	M	74	LC (HCV)	A	S4/18	Cool-tip	Original	Bilobular	4	Death (7)
8	M	70	LC (HCV)	A	S8/30	Cool-tip	Original	Surrounding	5	Alive (36)
9	M	70	LC (HCV)	B	S8/25	Cool-tip	Original	Bilobular	6	Death (16)
10	M	51	LC (HBV)	B	S6/20	LeVeen	Original	Surrounding	2	Death (10)
11	F	76	LC (HCV)	A	S3/14	Cool-tip	Original	Lobular	7	Alive (31)

RESULTS

Eleven patients suffered from rapid and scattered intrahepatic recurrences, which occurred between one month and seven months after ablation. These 11 cases consisted of eight males and three females, ranging in age from 51 to 79 years. The ablated tumors were located variably in either lobe of the liver. Among the baseline characteristics of patients prior to RFA treatment, the cirrhosis stage was significantly more advanced in patients without scattered recurrences (Table 1). Of the patients with scattered recurrences, nine were treated with the cool-tip device according to the original protocol and the other two with the LeVeen needle and original full-expansion method (Tables 1 and 2). After switching from the original to the modified protocols, no scattered recurrences were observed.

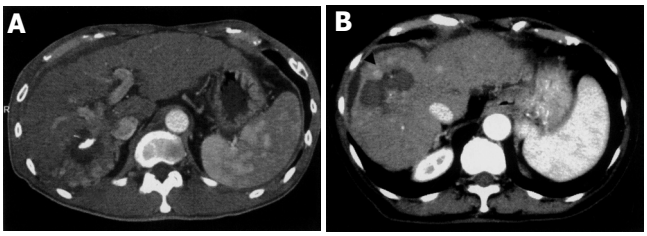

Figure 1 CT images of scattered recurrences after RFA. **A:** Recurrences around the ablated tumor after RFA (case 10 in Table 2); **B:** Multiple recurrences scattered over the whole liver after RFA (case 6 in Table 2). The white arrows indicate the ablated area without enhancement by contrast medium, and the black arrowheads indicate scattered recurrence with enhancement.

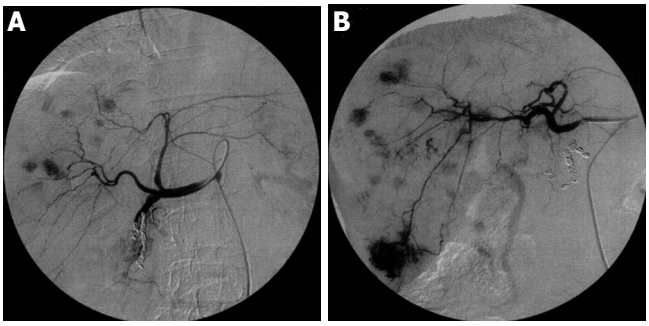

Figure 2 Angiographic images of scattered recurrences after RFA, which were treated by TACE in most of the cases. **A:** Multiple recurrences were located surrounding the ablated tumor (case 8 in Table 2). **B:** Multiple recurrences were scattered in the whole liver (case 9 in Table 2). In both patterns, recurrent tumors were almost equal in size.

Scattered recurrent tumors occurred in two different patterns. One pattern consisted of scattered tumors that were located in a single lobe (three patients). This pattern is shown in Figures 1A (CT) and 2A (angiography), appearing as multiple tumors around the ablated tumor that were localized in the segment. The other pattern consisted of tumors spread over a single lobule in the several segments (one patient) or both lobules (Figures 1B and 2B) (seven patients). Regardless of the scattering pattern, the tumors were roughly equal in diameter. When recurrences were found, neither tumor thrombus nor extrahepatic metastasis was observed by CT or US imaging.

Follow-up of patients to December 2004 showed that the prognosis was significantly worse for patients with scattered recurrences. Among the 127 patients without scattered recurrences, 40 patients died within the observation period; 8 due to liver failure caused by progression of liver cirrhosis, 2 from variceal rupture, and the remainder due to the progression of HCC. In contrast, 8 of 11 patients with scattered recurrences died and all had advanced HCC.

DISCUSSION

There are several common characteristics among the cases described in this study. First, recurrence occurred rapidly following RFA. Most of these cases were detected within 6 mo. Second, multiple recurrent tumors were almost equal in diameter. Finally, the recurrent tumors were either scattered around the ablated tumor or all over the lobe(s), and the location had no relation to the puncture route used for RFA.

Nicoli *et al.*[16] recently reported a similar form of recurrence that is, rapid and numerous bilobular recurrent tumors, and proposed that this type of recurrence would result from new communication formed between two vascular regions (arterial and venous-portal) as a result of RFA needle puncture. It was also suggested that the new communication facilitated the migration of tumor cells

from a high pressure arterial regions to a low pressure portal liver regions. However, we disagree with this speculation because the tumor treated with RFA in that study was only 3.5 cm in diameter, and the feeding artery would not likely have sufficient pressure to spread the malignant cells throughout the lobes. Ruzzenente *et al*.[17] also identified a series of patients with similar recurrences and suggested that increased intra-tumor pressure might be the cause.

In recent *in vitro* and *in vivo* experiments, we demonstrated that the pressure in an ablated area can increase drastically during RFA[18]. We assumed that the peculiar scattered recurrence was caused by an explosion due to increased intra-tumor pressure. The explosion could strew the malignant cells, as a large cluster, which would enable the metastatic tumors to grow in a short time.

A substantial increase in pressure would be necessary for the tumor to explode during RFA, and in rider for this to occur either of the two different conditions would be necessary; namely a fibrotic capsule around the tumor, or parenchymal fibrosis surrounding the tumor accompanied by cirrhosis. Without these conditions, the pressure produced by ablation would easily escape through the microvasculature or sinusoids adjacent to the ablated tumor. In our study, all of the patients with scattered recurrences also had liver cirrhosis, and the rate of advanced-stage cirrhosis was higher in patients with recurrence. This suggests that accumulated collagens in the liver of patients with cirrhosis could form a wall that traps in pressure.

Once a scattered recurrence occurs, focal treatment such as ablation therapy can no longer be used, and the only remaining treatment options are TACE or systemic chemotherapy. The incidence of recurrent tumors is also associated with a poor prognosis. We found that within the observation period, the mortality rate of patients with scattered recurrences was higher than that of patients without scattered recurrences.

In order to avoid scattered recurrences, we believe that modified protocols for RFA should be used. After changing to the modified protocols, we found no cases of scattered recurrence associated with either the cool-tip or LeVeen needle procedures. Another way to avoid scattered recurrence is to use alternative procedures to RFA such as ablation by PEIT, which is performed with a thinner needle than that used for RFA and has a lower risk of complications[7,8,20]. There are no reports that we are aware of to indicate that PEIT can cause scattered recurrences. PEIT is generally considered to be inferior to RFA for the following two reasons. First, in contrast with RFA, PEIT requires multiple sessions. Second, local recurrence following PEIT may be more likely when the surgeon is inexperienced, while a highly-skilled surgeon can achieve complete tumor necrosis.

We conclude that critical complications of rapid and scattered recurrences after RFA can be avoided by the use of modified protocols. If the need arises, it is also necessary to select PEIT, not to cling to use RFA procedure. In some cases, PEIT should be considered as a suitable alternative to RFA.

REFERENCES

1 **Giorgio A**, Francica G, Tarantino L, de Stefano G. Radio-frequency ablation of hepatocellular carcinoma lesions. *Radiology* 2001; **218:** 918-919

2 **Gazelle GS**, Goldberg SN, Solbiati L, Livraghi T. Tumor ablation with radio-frequency energy. *Radiology* 2000; **217:** 633-646

3 **Denys AL**, De Baere T, Mahe C, Sabourin JC, Sa Cunha A, Germain S, Roche A. Radio-frequency tissue ablation of the liver: effects of vascular occlusion on lesion diameter and biliary and portal damages in a pig model. *Eur Radiol* 2001; **11:** 2102-2108

4 **Maluf D**, Fisher RA, Maroney T, Cotterell A, Fulcher A, Tisnado J, Contos M, Luketic V, Stravitz R, Shiffman M, Sterling R, Posner M. Non-resective ablation and liver transplantation in patients with cirrhosis and hepatocellular carcinoma (HCC): safety and efficacy. *Am J Transplant* 2003; **3:** 312-317

5 **Livraghi T**. Treatment of hepatocellular carcinoma by interventional methods. *Eur Radiol* 2001; **11:** 2207-2219

6 **Solbiati L**, Livraghi T, Goldberg SN, Ierace T, Meloni F, Dellanoce M, Cova L, Halpern EF, Gazelle GS. Percutaneous radio-frequency ablation of hepatic metastases from colorectal cancer: long-term results in 117 patients. *Radiology* 2001; **221:** 159-166

7 **Livraghi T**, Goldberg SN, Lazzaroni S, Meloni F, Solbiati L, Gazelle GS. Small hepatocellular carcinoma: treatment with radio-frequency ablation versus ethanol injection. *Radiology* 1999; **210:** 655-661

8 **Lencioni RA**, Allgaier HP, Cioni D, Olschewski M, Deibert P, Crocetti L, Frings H, Laubenberger J, Zuber I, Blum HE, Bartolozzi C. Small hepatocellular carcinoma in cirrhosis: randomized comparison of radio-frequency thermal ablation versus percutaneous ethanol injection. *Radiology* 2003; **228:** 235-240

9 **Francica G**, Marone G, Solbiati L, D'Angelo V, Siani A. Hemobilia, intrahepatic hematoma and acute thrombosis with cavernomatous transformation of the portal vein after percutaneous thermoablation of a liver metastasis. *Eur Radiol* 2000; **10:** 926-929

10 **Choi H**, Loyer EM, DuBrow RA, Kaur H, David CL, Huang S, Curley S, Charnsangavej C. Radio-frequency ablation of liver tumors: assessment of therapeutic response and complications. *Radiographics* 2001; **21 Spec No:** S41-S54

11 **Dromain C**, de Baere T, Elias D, Kuoch V, Ducreux M, Boige V, Petrow P, Roche A, Sigal R. Hepatic tumors treated with percutaneous radio-frequency ablation: CT and MR imaging follow-up. *Radiology* 2002; **223:** 255-262

12 **Livraghi T**, Solbiati L, Meloni MF, Gazelle GS, Halpern EF, Goldberg SN. Treatment of focal liver tumors with percutaneous radio-frequency ablation: complications encountered in a multicenter study. *Radiology* 2003; **226:** 441-451

13 **Tamai F**, Furuse J, Maru Y, Yoshino M. Intrahepatic pseudoaneurysm: a complication following radio-frequency ablation therapy for hepatocellular carcinoma. *Eur J Radiol* 2002; **44:** 40-43

14 **Koda M**, Maeda Y, Matsunaga Y, Mimura K, Murawaki Y, Horie Y. Hepatocellular carcinoma with sarcomatous change arising after radiofrequency ablation for well-differentiated hepatocellular carcinoma. *Hepatol Res* 2003; **27:** 163-167

15 **Takada Y**, Kurata M, Ohkohchi N. Rapid and aggressive recurrence accompanied by portal tumor thrombus after radiofrequency ablation for hepatocellular carcinoma. *Int J Clin Oncol* 2003; **8:** 332-335

16 **Nicoli N**, Casaril A, Hilal MA, Mangiante G, Marchiori L, Ciola M, Invernizzi L, Campagnaro T, Mansueto G. A case of rapid intrahepatic dissemination of hepatocellular carcinoma after radiofrequency thermal ablation. *Am J Surg* 2004; **188:** 165-167

17 **Ruzzenente A**, Manzoni GD, Molfetta M, Pachera S, Genco B, Donataccio M, Guglielmi A. Rapid progression of hepatocellular carcinoma after Radiofrequency Ablation. *World J Gastroenterol* 2004; **10:** 1137-1140

18 **Kotoh K**, Nakamuta M, Morizono S, Kohjima M, Arimura E, Fukushima M, Enjoji M, Sakai H, Nawata H. A multi-step, incremental expansion method for radio frequency ablation: optimization of the procedure to prevent increases in intra-tumor pressure and to reduce the ablation time. *Liver Int*, in press

19 **Poon RT**, Ng KK, Lam CM, Ai V, Yuen J, Fan ST. Radiofrequency ablation for subcapsular hepatocellular carcinoma. *Ann Surg Oncol* 2004; **11:** 281-289

20 **Caturelli E**. Percutaneous ablative therapies for small hepatocellular carcinoma: radio-frequency or percutaneous ethanol injection? *Radiology* 2000; **216:** 304-306

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• *RAPID COMMUNICATION* •

Effect of pegylated interferon alpha 2b plus ribavirin treatment on plasma transforming growth factor-β1, metalloproteinase-1, and tissue metalloproteinase inhibitor-1 in patients with chronic hepatitis C

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Abstract

AIM: To evaluate the effect of antiviral treatment on plasma levels of transforming growth factor-β1 (TGF-β1), metalloproteinase 1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in patients with chronic hepatitis C.

METHODS: TGF-β1, MMP-1, and TIMP-1 plasma concentrations were measured by an enzyme immunoassay in 28 patients, during 48 wk of treatment with pegylated interferon-alpha 2b (PEG-IFN-α2b) plus ribavirin (RBV) and after 24 wk of follow-up. Patients were divided into two groups: responders (R) and non-responders (NR) related to achieved sustained virologic response. Normal values were evaluated in plasma samples of 13 healthy volunteers.

RESULTS: Baseline plasma concentrations of TGF-β1 and TIMP-1 (30.9±3.7 and 1 506±61 ng/mL respectively) measured in all subjects significantly exceeded the normal values (TGF-β1: 18.3±1.6 ng/mL and TIMP-1: 1 102±67 ng/mL). In contrast, pretreatment MMP-1 mean level (6.5±0.9 ng/mL) was significantly lower than normal values (11.9±0.9 ng/mL). Response to the treatment was observed in 12 patients (43%). TGF-β1 mean concentration measured during the treatment phase decreased to the control level in both groups. However at wk 72, values of NR patients increased and became significantly higher than in R group. TIMP-1 concentrations in R group decreased during the treatment to the level similar to normal. In NR group, TIMP-1 remained significantly elevated during treatment and follow-up phase and significant difference between both groups was demonstrated at wk 48 and 72. MMP-1 levels were significantly decreased in both groups at baseline. Treatment caused rise of its concentration only in the R group, whereas values in NR group remained on the level similar to baseline. Statistically significant difference between groups was noted at wk 48 and 72.

CONCLUSION: These findings support the usefulness of TGF-β1, TIMP-1, and MMP-1 in the management of chronic hepatitis C. Elevated TIMP-1 and low MMP-1 plasma concentrations during antiviral therapy may indicate medication failure.

Key words: HCV; Hepatitis; Liver; Interferons; Fibrosis

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INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is considered as a pivotal inducer of liver fibrosis acting through activation of hepatic stellate cells (HSCs) and their transformation to myofibroblasts, which are the main source of extracellular matrix (ECM) proteins[1,2]. Moreover, TGF-β1 stimulates the production of tissue inhibitor of TIMP-1 that inhibits MMP activity. This effect is responsible for the inhibition of ECM protein breakdown and its accumulation[3]. TGF-β1 inhibits DNA synthesis serving as a terminator of regenerative cell proliferation and induces apoptosis of hepatocytes[4]. Additionally, TGF-β1 may inhibit stellate cell apoptosis and promote their survival, at least in part as a result of anti-apoptotic effect of TIMP-1[5,6]. On the other hand, TGF-β1 exerts regulatory, mostly immunosuppressive effects on the immune system and as demonstrated recently can also suppress hepatitis C virus (HCV) replication[7,8]. Since HCV infection is related to an immune response, cell proliferation and fibrosis as well as modulation of TGF-β1 can affect the course of chronic hepatitis C. As demonstrated recently, HCV core and

nonstructural proteins regulate biological functions in HSC and increase the secretion of TGF-β1 and the expression of ECM proteins in both HSCs and parenchymal hepatic cells[9,10]. The possible role of TGF-β1, TIMP-1, and MMP-1 as predictive biomarkers of chronic hepatitis activity and progression is supported by recent clinical studies[11-19]. These studies demonstrated association with hepatic function impairment or fibrosis, and only few evaluated possible effects of antiviral treatment on growth factors, but they did not include possible metalloproteinase involvement[20,21].

We undertook this study to evaluate the effect of pegylated interferon-α2b plus ribavirin (PEG-IFN-α2b/RBV) treatment on plasma TGF-β1, TIMP-1, and MMP-1 levels in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

Ethical approval for the study was obtained from the Bioethical Committee of the Medical University of Bialystok. Informed consent was obtained from 28 patients (8 females and 20 males, mean age 49±12 years) with chronic hepatitis C, who were included into the protocol of PEG-IFN-α2b (Pegintron™, Schering-Plough) and RBV (Rebetol™, Schering-Plough) treatment. All patients had proven chronic hepatitis C through the presence of anti-HCV antibodies with elevated ALT activities demonstrated at least twice during a 6-mo observation period. Additionally, the disease activity was confirmed by the presence of viral replication and liver biopsy (Hepafix System, Braun, Melsungen, Germany). Patients with HBV infection and a history of alcohol abuse or psychiatric disorders were excluded from the study. Patients received combination therapy with weekly doses of 100 μg PEG-IFN-α2b administered subcutaneously and RBV administered orally at daily doses of 1 000 or 1 200 mg/d based on body weight <75 or ≥75 kg, respectively. The total duration of treatment was 48 wk. Liver biopsy was performed before and after antiviral therapy. Patients were divided into two groups related to sustained virologic response (SVR), defined as undetectable HCV RNA, 24 wk after the end of therapy. Patients who achieved SVR were included into the responder group (R) and those without SVR into non-responder group (NR). Paraffin-embedded biopsy specimens were stained and evaluated using the scoring system according to Scheuer[22]. TGF-β1, TIMP-1, and MMP-1 plasma concentrations were measured at baseline, 24 and 48 wk after treatment and additionally 24 wk after the termination of the treatment (wk 72). Serum liver function tests and scored histological changes were investigated for the possible correlation with TGF-β1, TIMP-1, and MMP-1. Normal values of TGF-β1, TIMP-1, and MMP-1 were collected from 13 healthy volunteers (5 females and 7 males , mean age: 48± 6 years).

Methods

Venous blood for plasma TGF-β1, TIMP-1, and MMP-1 was collected on ice using tubes with EDTA. Samples for TGF-β1 were immediately activated with acetic acid and urea and assayed with ELISA using recombinant human TGF-β soluble receptor Type II (TbR-II) as a solid phase precoated onto a microplate (Quantikine®, R&D Systems Inc., Minneapolis, USA) as described previously[23]. TIMP-1 and MMP-1 were assayed by the two-site ELISA sandwich technique (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) using specific antibodies as a solid phase. MMP-1 assay recognized total human MMP-1, namely free and complexed with TIMP-1. TIMP-1 assay recognized total human TIMP-1, including free and complexed with any of the metalloproteinases bound to the solid phase. TIMP-1 or MMP-1 bound to the solid phase was detected by peroxidase-labeled antibodies. There was no cross-reactivity between TIMP-1 and MMP-1 in these assays. Alanine and aspartate aminotransferase (ALT and AST) activity and bilirubin concentration were measured in serum using a Cobas Mira instrument (Roche).

Statistical analysis

Values were expressed as mean±SE. The significance of the difference was calculated by two-tailed Student's *t* test. For correlation analysis, the Pearson's product moment correlation was performed. $P<0.05$ was considered statistically significant.

RESULTS

Plasma concentrations of TGF-β1 and TIMP-1 measured before PEG-IFN-α2b/RBV treatment (mean: 30.9±3.7 and 1 506±61 ng/mL respectively) significantly exceeded the normal values (18.3±1.6 and 1 102±67 ng/mL respectively). Treatment resulted in a significant decrease of TGF-β1 by wk 24, and its further decline at the end of the treatment as well as 24 wk after its completion to the level similar to normal (Table 1). TIMP-1 plasma mean concentration also decreased, but did not differ significantly from baseline. Moreover, it remained on the level significantly exceeding controls during treatment and follow-up period (Table 1). Mean MMP-1 baseline level (6.5±0.9 ng/mL) was significantly lower than normal (11.9±0.9 ng/mL) but increased during the treatment. After treatment, its level still remained lower than normal but the difference was not significant (Table 1). There was a significant positive correlation between TIMP-1 and aminotransferases as well as between TGF-β1 and AST at baseline (Table 2). A significant correlation was also demonstrated between baseline TGF-β1 or TIMP-1 concentrations and scored fibrosis in pre-treatment liver biopsy specimens (Table 3). No association was

Table 1 Plasma concentrations of TGF-β1, TIMP-1, and MMP-1 during treatment (mean±SE)

	Controls	Weeks after starting treatment			
		0	24	48	72
TGF-β1 (ng/mL)	18.3±1.6	30.9±3.7[a]	21.2±2.8[c]	17.9±2.0[c]	21.0±2.5[c]
TIMP-1 (ng/mL)	1 102±67	1 506±61[a]	1 372±70[a]	1 389±51[a]	1410±66[a]
MMP-1 (ng/mL)	11.9±0.9	6.5±0.9[a]	7.2±1.5[a]	7.5±2.6	8.0±1.6

[a]$P<0.05$ *vs* normal, [c]$P<0.05$ *vs* baseline.

Table 2 Correlation expressed by r-value between biochemical indices of liver injury and TGF-β1, TIMP-1, or MMP-1 in chronic hepatitis C patients before treatment

	Bilirubin	ALT	AST
TGF-β1 (ng/mL)	0.240	0.163	0.388[a]
TIMP-1 (ng/mL)	0.023	0.393[a]	0.370[a]
MMP-1 (ng/mL)	–0.192	–0.130	–0.299

[a]$P<0.05$ biochemical indices *vs* TGF-β1, TIMP-1, and MMP-1.

Table 3 Correlation expressed by r-value between scored histological picture and TGF-β1, TIMP-1, or MMP-1 in chronic hepatitis C patients before treatmen

	Inflammation		Fibrosis
	Portal	Lobular	
TGF-β1 (ng/mL)	0.088	–0.132	0.495[a]
TIMP-1 (ng/mL)	0.091	0.326	0.404[a]
MMP-1 (ng/mL)	0.229	0.360	–0.018

[a]$P<0.05$ histological score *vs* TGF-β1, TIMP-1, and MMP-1

demonstrated between MMP-1 and biochemical or histological signs of liver injury (Tables 2 and 3).

SVR was observed in 12 among 28 patients (43%). Evaluation of baseline liver function tests showed no statistically significant differences between R and NR groups (Table 4). Treatment did not affect bilirubin levels in both groups. Responders demonstrated a significant decrease of ALT and AST activities during the treatment and follow-up. Decline of aminotransferases activity in NR group was only temporal and rose to values significantly higher than in R group after discontinuation of the treatment (Table 4). As demonstrated in Figure 1, scored values of histologic changes were similar before the treatment. There were no significant differences between biopsies preformed before and after the treatment in NR group. In contrast, responders demonstrated improvement after the treatment; however, statistically significant difference between score values at wk 0 and 48 was noted only in respect to portal inflammation.

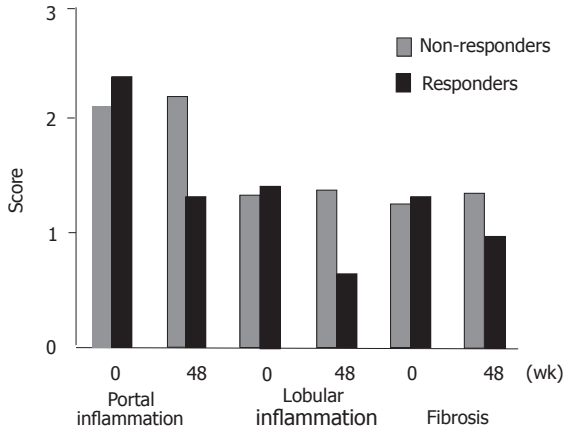

Figure 1 Mean score values of histologic changes in liver biopsy specimens before (wk 0) and after (wk 48) treatment. Statistically significant difference is indicated with arrows.

Table 4 Values of biochemical indices of liver injury during treatment (0, 24, and 48 wk) and 24 wk after its completion (wk 72) in both groups

		Weeks after the beginning of treatment			
		0	24	48	72
Bilirubin	Non-responders	1±0.1	1±0.1	0.7±0.1	0.9±0.1
(mg%)	Responders	1.1±0.1	1.1±0.1	0.9±0.1	1.0±0.1
ALT(U/L)	Non-responders	101±15	40±6[c]	55±20	98±18[a]
	Responders	96±13	35±9[c]	24±5[c]	22±2[c]
AST(U/L)	Non-responders	58±8	30±4[c]	34±6[a,c]	64±10[a]
	Responders	48±6	26±3[c]	20±1[c]	19±2[c]

[a]$P<0.05$ responders *vs* non-responders, [c]$P<0.05$ *vs* baseline.

There were no statistically significant differences in TGF-β1, TIMP-1, and MMP-1 concentrations between R and NR groups at the baseline and 24 wk after the treatment. As demonstrated in Figure 2A, TGF-β1 mean concentration decreased to the control level during treatment in both groups. However, 24 wk after the treatment (wk 72), values in NR patients increased (23.2± 2.3 ng/mL) and became significantly higher than those in R group (18.6±3.7 ng/mL). As shown in Figure 2B, mean concentration of TIMP-1 decreased during the treatment only in R group and there were no statistically significant differences in comparison with controls at wk 24, 48, and 72. In contrast, TIMP-1 concentration in NR group remained significantly elevated (above 1 500 ng/mL) during treatment and follow-up (Figure 2B). Significant difference between both groups was demonstrated at wk 48 and 72. As shown in Figure 2C, MMP-1 levels were significantly decreased in both groups at baseline. Treatment caused rise of its concentration only in the R group, whereas the values in NR group remained on the level similar to baseline. Statistically significant difference between groups was noted at wk 48 and 72 (Figure 2C).

DISCUSSION

The effect of TGF-β1 on liver fibrosis is at least in part related to stimulation of TIMP-1 that affects MMP activity and is responsible for inhibition of ECM protein breakdown[3]. The pivotal role of TGF-β1 in fibrogenesis is initially proved in transgenic mice with overexpression of TGF-β1, causing increase of its plasma levels up to 700 ng/mL and a marked upregulation of TIMP-1 gene expression[24-26]. Recent studies demonstrated that HCV proteins can stimulate secretion of TGF-β1 and production of ECM proteins by HSCs[9,10]. On the other hand, Murata *et al*.[8] showed that TGF-β suppresses viral HCV-RNA replication and can affect the mechanism of liver disease caused by HCV. Chronic liver injury leading to fibrosis displays diminished ECM degradation mainly through TIMP induction following MMP inhibition[3]. As demonstrated recently, TIMP-1 recombinant plasmid has inhibitory effects on the production of types I and III collagens secreted by activated rHSCs *in vitro*[27].

The most important factor affecting TGF-β1 measurement in human beings is from platelets which are an important source of this cytokine[28]. The Quantikine ELISA System is recommended because

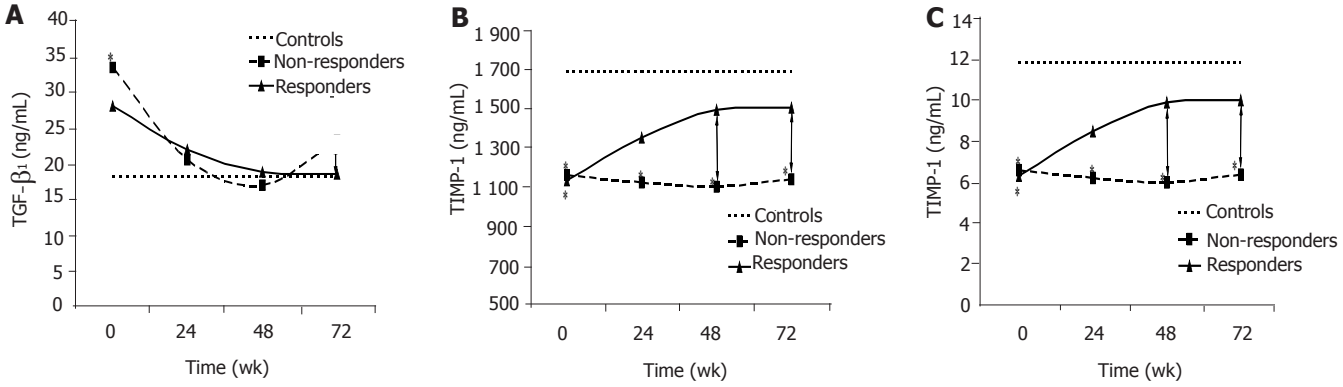

Figure 2 Mean TGF-β1 (**A**), TIMP-1 (**B**), and MMP-1 (**C**) plasma concentrations before and during IFN-α plus RBV therapy as well as 24 wk after its completion (wk 72) in respect to the treatment efficacy. Statistical significance in comparison to normal values is indicated with asterisks and between groups with arrows.

of quick and simple activation with acid and urea that disrupt the majority of TGF-β1 complexes. Mean plasma concentration of TGF-β1 measured in our healthy controls with this method is consistent with the range from more than 20 studies reviewed by Grainger *et al*.[28].

According to our previous research, TGF-β1 and TIMP-1 correlate with the degree of liver insufficiency, hepatocyte injury and degree of fibrosis in human beings with liver cirrhosis and chronic viral hepatitis[12,23,29]. Association between TGF-β1 mRNA in liver specimens and fibrogenic activity in chronic hepatitis is demonstrated for the first time by Castilla *et al*.[30]. Ten years after the association between circulating or tissue TGF-β and liver fibrosis in HCV infection has been confirmed by Kanzler *et al*.[13]. As demonstrated by Yoo *et al*.[31] and Lee *et al*.[32], HBV antigens also stimulate TGF-β1 synthesis. According to Neuman *et al*.[20,33], serum TNF-α reflects the progression of inflammation, whereas TGF-β reflects the degree of fibrosis in HCV patients. A similar relationship has been demonstrated with respect to primary biliary cirrhosis and alcoholic liver disease[34]. Our previous study showed that a positive predictive value of TGF-β1 plasma levels exceeding the upper normal range reaches 96% for liver cirrhosis[23]. According to Boeker *et al*.[11] measurement of plasma TIMP-1 detects cirrhosis with 100% sensitivity but a lower specificity. Lichtinghagen *et al*.[14] demonstrated that MMP-1 mRNA expression increases steadily with fibrosis progression during the course of chronic hepatitis C. Walsh *et al*.[17] who studied liver histology in patients with chronic hepatitis C have underlined the high sensitivity of TIMP-1 and TIMP-2 in detecting advanced liver disease. According to Nie *et al*.[35], there is a significant correlation between circulating and liver levels of TIMP-1 in cirrhotics, indicating that its measurement in plasma may be useful in fibrosis management. These observations indicate the usefulness of both TGF-β1 and TIMP-1 as possible early non-invasive biomarkers for liver fibrosis.

In this study, we confirmed the association between the degree of hepatocyte injury or liver fibrosis and plasma TGF-β1 or TIMP-1 levels in patients with chronic hepatitis C. As the levels of TGF-β1 showed a similar behavior in both groups during therapy, it is unclear whether its decrease is a direct effect of medication on the expression or an effect caused by HCV inhibition. However, measurement carried out 24 wk after treatment demonstrated an association with treatment efficiency. Similar effects on plasma TGF-β1 have been observed by Castilla *et al*.[30] and Neuman *et al*.[20] and in our previous study of chronic hepatitis B[29]. TIMP-1 and MMP-1 concentrations demonstrated significant differences between groups at the end of the treatment and after 24 wk of follow-up. Since plasma TIMP-1 and MMP-1 remained on the baseline level in non-responder group only, lack of their normalization should be considered as a possible indicator of ineffective antiviral therapy. Results of the present study are in accordance with our previous findings, demonstrating the strong association between TGF-β1 or TIMP-1 plasma levels and scored hepatic fibrosis evaluated in biopsy specimens of patients with chronic hepatitis B and C[12]. Since the findings of increased TGF-β1 and TIMP-1 are accompanied with an elevation in plasma carboxyterminal cross-linked telopeptide of type 1 procollagen (ICTP), indicating type I collagen degradation, collagenolytic mechanisms precede TGF-β1/TIMP-1 dependent stimulation of liver fibrosis[12]. Low MMP-1 plasma levels before the treatment in the present study are consistent with this observation as well as in accordance with Murawaki *et al*.[15] who demonstrated a decrease in MMP-1 concentration during histological progression of chronic hepatitis. Moreover, significantly decreased baseline plasma MMP-1 followed by an increase during treatment supports the role of TGF-β1/TIMP-1 dependent mechanism of liver fibrosis in patients with active chronic hepatitis C. Similar effects on MMP-1 and TIMP-1 in patients with chronic hepatitis C have been observed by Ninomiya *et al*.[16] who showed improvement of liver histology after treatment with IFN-α alone. Downregulation of the mechanism causing an increase of MMP-1 activity should be considered as the probable reason for this effect. As we demonstrated recently, treatment of chronic hepatitis B with lamivudine affects TGF-β1, TIMP-1, and MMP-1 plasma levels in a similar way and this mechanism should be recognized as an effect of response to the antiviral treatment,

irrespective of the etiology[29].

Results of this study support the role of TIMP-1 and MMP-1 balance in the TGF-β1 dependent mechanism of liver fibrosis related to HCV infection. Association between hepatic injury and antiviral treatment efficacy suggests their possible usefulness in chronic hepatitis C management. Elevated TIMP-1 and low MMP-1 plasma concentrations during antiviral therapy may indicate medication failure.

REFERENCES

1 **Knittel T**, Janneck T, Muller L, Fellmer P, Ramadori G. Transforming growth factor beta 1-regulated gene expression of Ito cells. *Hepatology* 1996; **24:** 352-360
2 **Williams EJ**, Gaca MD, Brigstock DR, Arthur MJ, Benyon RC. Increased expression of connective tissue growth factor in fibrotic human liver and in activated hepatic stellate cells. *J Hepatol* 2000; **32:** 754-761
3 **Knittel T**, Mehde M, Kobold D, Saile B, Dinter C, Ramadori G. Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1. *J Hepatol* 1999; **30:** 48-60
4 **Fausto N**, Liver regeneration. *J Hepatol* 2000; **32:** 19-31
5 **Saile B**, Matthes N, Knittel T, Ramadori G. Transforming growth factor beta and tumor necrosis factor alpha inhibit both apoptosis and proliferation of activated rat hepatic stellate cells. *Hepatology* 1999; **30:** 196-202
6 **Murphy FR**, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. **J Biol Chem** 2002; **277:** 11069-11076
7 **Prud'homme GJ**, Piccirillo CA. The inhibitory effects of transforming growth factor-beta-1 (TGF-beta1) in autoimmune diseases. *J Autoimmun* 2000; **14:** 23-42
8 **Murata T**, Ohshima T, Yamaji M, Hosaka M, Miyanari Y, Hijikata M, Shimotohno K. Suppression of hepatitis C virus replicon by TGF-beta. *Virology* 2005; ***331:*** 407-417
9 **Bataller R**, Paik YH, Lindquist JN, Lemasters JJ, Brenner DA. Hepatitis C virus core and nonstructural proteins induce fibrogenic effects in hepatic stellate cells. *Gastroenterology* 2004; **126:** 529-540
10 **Taniguchi H**, Kato N, Otsuka M, Goto T, Yoshida H, Shiratori Y, Omata M. Hepatitis C virus core protein upregulates transforming growth factor-beta 1 transcription. *J Med Virol* 2004; **72:** 52-59
11 **Boeker KH**, Haberkorn CI, Michels D, Flemming P, Manns MP, Lichtinghagen R. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. *Clin Chim Acta* 2002; **316:** 71-81
12 **Flisiak R**, Maxwell P, Prokopowicz D, Timms PM, Panasiuk A. Plasma tissue inhibitor of metalloproteinases-1 and transforming growth factor beta 1--possible non-invasive biomarkers of hepatic fibrosis in patients with chronic B and C hepatitis. *Hepatogastroenterology* 2002; **49:** 1369-1372
13 **Kanzler S**, Baumann M, Schirmacher P, Dries V, Bayer E, Gerken G, Dienes HP, Lohse AW. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-beta. *J Viral Hepat* 2001; **8:** 430-437
14 **Lichtinghagen R**, Bahr MJ, Wehmeier M, Michels D, Haberkorn CI, Arndt B, Flemming P, Manns MP, Boeker KH. Expression and coordinated regulation of matrix metalloproteinases in chronic hepatitis C and hepatitis C virus-induced liver cirrhosis. *Clin Sci* 2003; **105:** 373-382
15 **Murawaki Y**, Ikuta Y, Idobe Y, Kawasaki H. Serum matrix metalloproteinase-1 in patients with chronic viral hepatitis. *J Gastroenterol Hepatol* 1999; **14:** 138-145
16 **Ninomiya T**, Yoon S, Nagano H, Kumon Y, Seo Y, Kasuga M, Yano Y, Nakaji M, Hayashi Y. Significance of serum matrix metalloproteinases and their inhibitors on the antifibrogenetic effect of interferon-alfa in chronic hepatitis C patients. *Intervirology* 2001; **44:** 227-231
17 **Walsh KM**, Timms P, Campbell S, MacSween RN, Morris AJ. Plasma levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases -1 and -2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic hepatitis C: comparison using ROC analysis. *Dig Dis Sci* 1999; **44:** 624-630
18 **Patel K**, Gordon SC, Jacobson I, Hezode C, Oh E, Smith KM, Pawlotsky JM, McHutchison JG. Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. *J Hepatol* 2004; **41:** 935-942
19 **Leroy V**, Monier F, Bottari S, Trocme C, Sturm N, Hilleret MN, Morel F, Zarski JP. Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am J Gastroenterol* 2004; **99:** 271-279
20 **Neuman MG**, Benhamou JP, Bourliere M, Ibrahim A, Malkiewicz I, Asselah T, Martinot-Peignoux M, Shear NH, Katz GG, Akremi R, Benali S, Boyer N, Lecomte L, Le Breton V, Le Guludec G, Marcellin P. Serum tumour necrosis factor-alpha and transforming growth factor-beta levels in chronic hepatitis C patients are immunomodulated by therapy. *Cytokine* 2002; **17:** 108-117
21 **Anatol P**, Robert F, Danuta P. Effect of interferon alpha2b plus ribavirin treatment on selected growth factors in respect to inflammation and fibrosis in chronic hepatitis C. *World J Gastroenterol* 2005; **11:** 1854-1858
22 **Scheuer PJ**. Classification of chronic viral hepatitis: a need for reassessment. *J Hepatol* 1991; **13:** 372-374
23 **Flisiak R**, Pytel-Krolczuk B, Prokopowicz D. Circulating transforming growth factor beta(1) as an indicator of hepatic function impairment in liver cirrhosis. *Cytokine* 2000; **12:** 677-681
24 **Kanzler S**, Lohse AW, Keil A, Henninger J, Dienes HP, Schirmacher P, Rose-John S, zum Buschenfelde KH, Blessing M. TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis. *Am J Physiol* 1999; **276:** G1059-G1068
25 **Sanderson N**, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, Roberts AB, Sporn MB, Thorgeirsson SS. Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci USA* 1995; **92:** 2572-2576
26 **Clouthier DE**, Comerford SA, Hammer RE. Hepatic fibrosis, glomerulosclerosis, and a lipodystrophy-like syndrome in PEPCK-TGF-beta1 transgenic mice. *J Clin Invest* 1997; **100:** 2697-2713
27 **Liu WB**, Yang CQ, Jiang W, Wang YQ, Guo JS, He BM, Wang JY. Inhibition on the production of collagen type I, III of activated hepatic stellate cells by antisense TIMP-1 recombinant plasmid. *World J Gastroenterol* 2003; **9:** 316-319
28 **Grainger DJ**, Mosedale DE, Metcalfe JC. TGF-beta in blood: a complex problem. *Cytokine Growth Factor Rev* 2000; **11:** 133-145
29 **Flisiak R**, Al-Kadasi H, Jaroszewicz J, Prokopowicz D, Flisiak I. Effect of lamivudine treatment on plasma levels of transforming growth factor beta1, tissue inhibitor of metalloproteinases-1 and metalloproteinase-1 in patients with chronic hepatitis B. *World J Gastroenterol* 2004; **10:** 2661-2665
30 **Castilla A**, Prieto J, Fausto N. Transforming growth factors beta 1 and alpha in chronic liver disease. Effects of interferon alfa therapy. *N Engl J Med* 1991; **324:** 933-940
31 **Yoo YD**, Ueda H, Park K, Flanders KC, Lee YI, Jay G, Kim SJ. Regulation of transforming growth factor-beta 1 expression

by the hepatitis B virus (HBV) X transactivator. Role in HBV pathogenesis. *J Clin Invest* 1996; **97:** 388-395

32 **Lee DK**, Park SH, Yi Y, Choi SG, Lee C, Parks WT, Cho H, de Caestecker MP, Shaul Y, Roberts AB, Kim SJ. The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. *Genes Dev* 2001; **15:** 455-466

33 **Neuman MG**, Benhamou JP, Malkiewicz IM, Ibrahim A, Valla DC, Martinot-Peignoux M, Asselah T, Bourliere M, Katz GG, Shear NH, Marcellin P. Kinetics of serum cytokines reflect changes in the severity of chronic hepatitis C presenting minimal fibrosis. *J Viral Hepat* 2002; **9:** 134-140

34 **Neuman M**, Angulo P, Malkiewicz I, Jorgensen R, Shear N, Dickson ER, Haber J, Katz G, Lindor K. Tumor necrosis factor-alpha and transforming growth factor-beta reflect severity of liver damage in primary biliary cirrhosis. *J Gastroenterol Hepatol* 2002; **17:** 196-202

35 **Nie QH**, Cheng YQ, Xie YM, Zhou YX, Bai XG, Cao YZ. Methodologic research on TIMP-1, TIMP-2 detection as a new diagnostic index for hepatic fibrosis and its significance. *World J Gastroenterol* 2002; **8:** 282-287

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• *RAPID COMMUNICATION* •

Prevalence and risk factors of stress-induced gastrointestinal bleeding in critically ill children

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Abstract

AIM: To assess the frequency and the risk factors of stress-induced gastrointestinal (GI) bleeding in children admitted to a pediatric intensive care unit (PICU).

METHODS: The medical records of children aged between 1 month and 15 years admitted to the PICU between January 2002 and December 2002 were reviewed. Demographic data, indications for PICU admission, principle diagnosis, and basic laboratory investigations were recorded. Previously described factors for stress ulcer bleeding (mechanical ventilation, sepsis, acute respiratory distress syndrome, renal insufficiency, coagulopathy, thrombocytopenia, and intracranial pathology) were used as independent variables in a multivariate analysis.

RESULTS: One hundred and seventy of two hundred and five medical records were eligible for review. The most common indication for PICU admission was respiratory failure (48.8%). Twenty-five children received stress ulcer bleeding prophylaxis with ranitidine. The incidence of stress ulcer bleeding was 43.5%, in which 5.3% were clinically significant bleeding. Only mechanical ventilation and thrombocytopenia were significantly associated with stress ulcer bleeding using the univariate analysis. The odds ratio and 95% confidence intervals were 5.13 (1.86-14.12) and 2.26 (1.07-4.74), respectively. However, the logistic regression analysis showed that mechanical ventilation was the only significant risk factor with the odds ratio of 14.1.

CONCLUSION: The incidence of gastrointestinal bleeding was high in critically ill children. Mechanical ventilation was an important risk factor for gastrointestinal bleeding.

Key words: Gastrointestinal; Hemorrhage; Stress; Risk factor; Child

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INTRODUCTION

Stress ulcer bleeding is one of the common complications in critically ill patients admitted to the intensive care unit. Its incidence in adults ranges from 0.17% to 14%, depending on the diagnostic criteria, patient selection, and methods of investigation[1-4]. There have been a few reports of this condition in pediatric populations with the incidence varying from 10% in a pediatric intensive care unit to 53% in a neonatal intensive care unit[5,6]. This study was designed to assess the frequency and the risk factors of stress-induced upper gastrointestinal bleeding in critically ill children admitted to a pediatric intensive care unit.

MATERIALS AND METHODS

Patients

All medical records of children younger than 15 years admitted to the PICU between January 2002 and December 2002 at Chiang Mai University Hospital were retrospectively reviewed. Our hospital is a tertiary care center with a facility of six-bed PICU, taking care of approximately 83% of medical and 17% of surgical pediatric patients. The general indications for admission included respiratory/cardiovascular failure, shock, coma, post-operative care, and patients requiring intensive monitoring. The patients with duration of admission in PICU shorter than 48 h, positive previous history of GI bleeding, recent GI tract surgery, brain death, and epistaxis/oropharyngeal bleeding were excluded. Demographic data, indications for PICU admission, and principle diagnosis as well as basic laboratory investigations including hemoglobin level, platelet count, coagulation studies, blood urea nitrogen (BUN), creatinine (Cr), and liver function tests were recorded. Upper GI bleeding during PICU admission was categorized as overt and clinically significant bleeding. Overt GI bleeding (OB) was diagnosed, if there was evidence of hematemesis, coffee ground gastric content, or melena;

whereas clinically significant bleeding (CSB) was defined as overt GI bleeding associated with major changes in vital signs, namely a decrease in blood pressure greater than 20 mmHg, an increase in heart rate of >20 beats above the baseline value, and a decrease in hemoglobin level of more than 2 g/dL. Based on previous studies in adults, potential risk factors were used. These were the use of mechanical ventilation, sepsis, acute respiratory distress syndrome (ARDS), renal insufficiency, coagulopathy, thrombocytopenia, and intracranial pathology. The diagnostic criteria for these conditions were: sepsis-at least two of the following, body temperature >38 °C or <36 ° C, heart rate >160/min (infant) or >150/min (child) or >90/min (adolescent), WBC >15 000 or <4000/mm^3, and band form >10% or there was a positive blood culture; ARDS - positive alveolar infiltration in both lungs on chest X-ray and PaO_2/FiO_2 <200 without evidence of left-sided heart failure; *renal insufficiency* - Cr >2 mg/dL, or requiring dialysis; *coagulopathy* - prothrombin time (PT) >3s and partial thromboplastin time (PTT) >10 s above the control value; *thrombocytopenia* -'platelet count<100 000/mm^3; *intracranial pathology* - abnormal imaging study, meningitis, or encephalitis. This study protocol was approved by the Research Ethic Committee of Chiang Mai University.

Statistical analysis

All data were assessed by SPSS program. Cross-tabulations were analyzed using the χ^2 test, presented as the odds ratio and 95% confidence interval, in which a $P<0.05$ was considered statistically significant. Multivariate analysis of various independent variables was performed using logistic regression modeling.

RESULTS

Over the 12-month period, 205 of 258 medical records were available for review (53 records were missing). Thirty-five cases were excluded for the following reasons: duration of admission shorter than 48 h (n = 28), incomplete medical records (n = 3), epistaxis (n = 2), brain death (n = 1), and recent gastrointestinal surgery (n = 1). Therefore, a total of 170 charts were eligibly reviewed. There were 89 males (52.4%) with an average age of 3.8 years. The total duration of admission was 7.2 d (2-35 d). The most common indication for PICU admission was respiratory failure (48.8%). The demographic data of children with and without bleeding are shown in Table 1. Twenty-five children received stress ulcer bleeding prophylaxis, in which ranitidine was used in 22 cases with a dosage of 3 mg/kg/d; whereas the other three patients received antacids. In the subgroup of children who received stress ulcer prophylaxis, 14 cases developed upper GI hemorrhage (3 CSB and 11 OB); whereas stress ulcer bleeding occurred in 60 of 145 cases who did not receive the prophylactic treatment (6 CSB and 54 OB). GI bleeding complicated 43.5% of cases admitted to the PICU and 5.3% had clinically significant bleeding. Twenty-two percent of patients with CSB were diagnosed as dengue hemorrhagic fever, compared to none in the patients without bleeding.

Table 1 Demographic data of children with and without stress-induced GI bleeding

Characteristic	Bleeding (n = 74)	No bleeding (n = 96)	P
Age (yr)[1]	3.82 (0.44)	3.84 (0.40)	0.977
Sex, male	41	48	0.484
Duration of admission (d)[1]	8.23 (0.78)	6.41 (0.58)	0.062
Underlying diseases			
Respiratory system	8	15	0.445
Cardiovascular system	9	18	
Neurological system	19	25	
Hemato/oncologic system	11	11	
Infections/HIV	14	9	
Gastrointestinal system	5	6	
Others	8	12	

[1]Presented as mean±SE.

Among the independent variables, only mechanical ventilation and thrombocytopenia were significantly associated with stress ulcer bleeding using the univariate analysis. The odds ratio and 95%CI were 5.13 (1.86-14.12) and 2.26 (1.07-4.74), respectively (Table 2). Using multivariate analysis, only mechanical ventilation was found to be significantly associated with the development of gastrointestinal bleeding in critically ill patients ($P<0.05$). In our study, stress ulcer prophylaxis did not reduce the risk of bleeding. The overall mortality rate was 18.8%. Gastrointestinal bleeding and transfusion requirements were associated with high mortality ($P<0.05$).

DISCUSSION

Stress-induced gastrointestinal lesions, including gastritis, erosions, gastric, and duodenal ulcers, can result in significant upper gastrointestinal hemorrhage, increased morbidity and mortality[2]. The prevalence varies between studies[1-6]. In our series, 5% of the cases developed clinically significant bleeding which is considerably higher than that in previous studies performed in pediatric population[5,7]. This might result from a relatively high prevalence of hemorrhagic fever which commonly causes thrombocytopenia and subsequent gastrointestinal bleeding in our region. As noted in our report, 22% of the cases with CSB were diagnosed as dengue hemorrhagic fever. Additionally, we did not routinely use stress ulcer prophylaxis in all patients, and patients with ranitidine prophylaxis did not receive the recommended dose of 6 mg/kg/d[8]. This may explain the poor beneficial prophylactic effect noted in our study. Apart from clinically significant bleeding, we also found a high prevalence of overt upper gastrointestinal bleeding (38.2%), which is comparable to the retrospective report section from Kuusela *et al.* in neonates[6]. However, this figure could be exceptionally high due to a possibility of inclusion of traumatic nasogastric tube injuries which are very difficult to be documented in such a retrospective design and we did not routinely perform endoscopy in all children to delineate the cause of upper GI bleeding during the study period. Although we believe that the prevalence

Table 2 Risk factors for stress-induced GI bleeding in children (*n* = 170)

Risk factors		*n*	Gastrointestinal bleeding (%)	Odds ratio (95%CI) simple regression	Odds ratio (95%CI) multiple regression
Mechanical ventilation	Yes	139	49.6	5.126 (1.861–14.118)	14.096 (2.205–90.112)
	No	31	16.1	*P* = 0.001	*P* = 0.005
Thrombocytopenia	Yes	37	59.4	2.256 (1.073–4.745)	3.462 (0.843–14.216)
	No	132	39.4	*P* = 0.030	*P* = 0.085
Renal insufficiency	Yes	9	77.8	4.858 (0.978–24.124)	2.763 (0.326–23.426)
	No	160	43.1	*P* = 0.035	*P* = 0.351
Prolonged PT	Yes	34	64.7	2.292 (0.946–5.551)	1.222 (0.289–5.161)
	No	32	44.4	*P* = 0.064	*P* = 0.785
Prolonged PTT	Yes	37	62.2	2.071 (0.876–4.899)	1.198 (2.640–5.446)
	No	52	44.2	*P* = 0.095	*P* = 0.815
ARDS	Yes	96	46.9	2.050 (0.912–4.610)	0.772 (0.171–3.487)
	No	54	38.5	*P* = 0.080	*P* = 0.737
Sepsis	Yes	69	49.3	1.481 (0.799–2.748)	1.234 (0.325–4.691)
	No	101	39.6	*P* = 0.212	*P* = 0.757
Intracranial pathology	Yes	32	46.8	1.181 (0.546–2.556)	1.192 (0.283–5.010)
	No	138	42.8	*P* = 0.672	*P* = 0.811

reported in this study might be over-estimated, it discerns a significant magnitude of the problem that requires careful medical attention. Similar to previous studies in adults and children, the mechanical ventilation was found to be the most significant risk factor for stress-induced gastrointestinal bleeding in our study[1,2,5,6]. Although coagulopathy is also noted as a significant independent risk factor in some studies[1,2,5,7], this was not observed in our series using multivariate analysis. A further prospective study with a larger sample size is needed.

Imbalance between protective and destructive factors has been postulated as a basic pathophysiology of GI bleeding. Increased acid production and decreased gastric blood flow, secondary to hypotension and metabolic acidosis, are composed of major physiologic responses leading to mucosal injuries. Hemorrhagic gastritis affects mainly the gastric body as it is the most vulnerable area for ischemic injury[4]. As a result, prophylactic strategies with H_2RA and cytoprotective agents have been widely prescribed to the critically ill patients admitted to the intensive care unit. Lacroix *et al.*[9] have reported a significant increase in the gastric pH following cimetidine prophylaxis stress ulcer bleeding in children, but no prophylactic benefit was demonstrated in their study. Kuusela *et al.*[10] showed that short-term prophylactic ranitidine treatment could prevent gastric mucosal lesions in newborn infants under stress. Cook *et al.*[11] performed a meta-analysis and showed that H_2RA significantly reduces clinically important bleeding over sucralfate and antacids. However, overgrowth of Gram-negative bacteria following the increase of gastric pH by antisecretory agents can be associated with ventilator-associated pneumonia (VAP)[12,13]. Lopriore *et al.*[14] reported that 8.4% of mechanically ventilated children develop VAP. Among these, more children prophylactically treated with ranitidine tend to be associated with VAP than those in the control group (11.1% *vs* 6.2%), despite no statistical significance. The use of sucralfate, since its introduction, seems logically useful in preventing this complication. Unfortunately, this hypothesis was not supported by the large meta-analysis study[11].

Ben-Menachem *et al.*[15] did a cost-effective analysis on stress ulcer prophylaxis and suggested that the cost of prophylaxis is substantial and may be prohibitive in ICU patients at low risk of developing stress-related hemorrhage. Therefore, several authors have recently suggested that such prophylaxis should be selective and may be indicated only for patients at high risk, particularly in those with mechanical ventilation and coagulopathy[2,7]. However, the cost estimation in PICU on this issue has not been well studied.

In conclusion, the incidence of gastrointestinal bleeding is high in critically ill children. Mechanical ventilation is a significant risk factor for gastrointestinal bleeding.

REFERENCES

1 **Schuster DP**, Rowley H, Feinstein S, McGue MK, Zuckerman GR. Prospective evaluation of the risk of upper gastrointestinal bleeding after admission to a medical intensive care unit. *Am J Med* 1984; **76**: 623-630

2 **Cook DJ,** Fuller HD, Guyatt GH, Marshall JC, Leasa D, Hall R, Winton TL, Rutledge F, Todd TJ, Roy P. Risk factors for gastrointestinal bleeding in critically ill patients. Canadian Critical Care Trials Group. *N Engl J Med* 1994; **330:** 377-381

3 **Pimentel M**, Roberts DE, Bernstein CN, Hoppensack M, Duerksen DR. **Clinically significant gastrointestinal bleeding in** critically ill patients in an era of prophylaxis. *Am J Gastroenterol* 2000; **95**: 2801-2806

4 **Fusamoto H**, Hagiwara H, Meren H, Kasahara A, Hayashi N, Kawano S, SugimotoT, Kamada T. A clinical study of acute gastrointestinal hemorrhage associated with various shock states. *Am J Gastroenterol* 1991; **86**: 429-433

5 **Chaibou M**, Tucci M, Dugas MA, Farrell CA, Proulx F, Lacroix J. Clinically significant upper gastrointestinal bleeding

acquired in a pediatric intensive care unit: a prospective study. *Pediatrics* 1998; **102**: 933-938

6 **Kuusela AL**, Maki M, Ruuska T, Laippala P. Stress-induced gastric findings in critically ill newborn infants: frequency and risk factors. *Intensive Care Med* 2000; **26**: 1501-1506

7 **Lacroix J**, Nadeau D, Laberge S, Gauthier M, Lapierre G, Farrell CA. Frequency of upper gastrointestinal bleeding in a pediatric intensive care unit. *Crit Care Med* 1992; **20**: 35-42

8 **Harrison AM**, Lugo RA, Vernon DD. Gastric pH control in critically ill children receiving intravenous ranitidine. *Crit Care Med* 1998; **26**: 1433-1436

9 **Lacroix J**, Infante-Rivard C, Gauthier M, Rousseau E, van Doesburg N. Upper gastrointestinal tract bleeding acquired in a pediatric intensive care unit: prophylaxis trial with cimetidine. *J Pediatr* 1986; **108**: 1015-1018

10 **Kuusela AL**, Ruuska T, Karikoski R, Laippala P, Ikonen RS, Janas M, Maki M. A randomized, controlled study of prophylactic ranitidine in preventing stress-induced gastric mucosal lesions in neonatal intensive care unit patients. *Crit Care Med* 1997; **25**: 346-351

11 **Cook DJ**, Reeve BK, Guyatt GH, Heyland DK, Griffith LE, Buckingham L, Tryba M. Stress ulcer prophylaxis in critically ill patients: Resolving discordant meta-analyses. *JAMA* 1996; **275**: 308-314

12 **Tryba M**. Risk of acute stress bleeding and nosocomial pneumonia in ventilated intensive care unit patients: sucralfate versus antacids. *Am J Med* 1987; **83** :117-124

13 **Prod'hom G**, Leuenberger P, Koerfer J, Blum A, Chiolero R, Schaller MD, Perret C, Spinnler O, Blondel J, Siegrist H, Saghafi L, Blanc D, Francioli P. Nosocomial pneumonia in mechanically ventilated patients receiving antacid, ranitidine, or sucralfate as prophylaxis for stress ulcer. A randomized controlled trial. Ann Intern Med 1994; **120:** 653-662

14 **Lopriore E**, Markhorst DG, Gemke RJ. Ventilator-associated pneumonia and upper airway colonisation with Gram negative bacilli: the role of stress ulcer prophylaxis in children. *Intensive Care Med* 2002; **28**: 763-767

15 **Ben-Menachem T**, McCarthy BD, Fogel R, Schiffman RM, Patel RV, Zarowitz BJ, Nerenz DR, Bresalier RS. Prophylaxis for stress-related gastrointestinal hemorrhage: a cost effectiveness analysis. *Crit Care Med* 1996; **24**: 338-345

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• *RAPID COMMUNICATION* •

Favorable response to subcutaneous administration of infliximab in rats with experimental colitis

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Abstract

AIM: To investigate the influence of infliximab (Remicade) on experimental colitis produced by 2,4,6,trinitrobenzene sulfonic acid (TNBS) in rats.

METHODS: Thirty-six Wistar rats were allocated into four groups (three groups of six animals each and a fourth of 12 animals). Six more healthy animals served as normal controls (Group 5). Group 1: colitis was induced by intracolonic installation of 25 mg of TNBS dissolved in 0.25 mL of 50% ethanol and infliximab was subcutaneously administered at a dose of 5 mg/kg BW; Group 2: colitis was induced and infliximab was subcutaneously administered at a dose of 10 mg/kg BW; Group 3: colitis was induced and infliximab was subcutaneously administered at a dose of 15 mg/kg BW; Group 4: colitis was induced without treatment with infliximab. Infliximab was administered on d 2–6. On the 7th d, all animals were killed. The colon was fixed in 10% buffered formalin and examined by light microscopy for the presence and activity of colitis and the extent of tissue damage. Tumor necrosis factor-alpha (TNF-α) and malondialdehyde (MDA) were also measured.

RESULTS: Significant differences concerning the presence of reparable lesions and the extent of bowel mucosa without active inflammation in all groups of animals treated with infliximab compared with controls were found. Significant reduction of the tissue levels of TNF-α in all groups of treated animals as compared with the untreated ones was found (0.47±0.44, 1.09±0.86, 0.43±0.31 *vs* 18.73±10.53 respectively). Significant reduction in the tissue levels of MDA was noticed in group 1 as compared to group 4, as well as between groups 2 and 4.

CONCLUSION: Subcutaneous administration of infliximab reduces the inflammatory activity as well as tissue TNF-α and MDA levels in chemical colitis in rats. Infliximab at a dose of 5 mg/kg BW achieves better histological results and produces higher reduction of the levels of TNF-α than at a dose of 10 mg/kg BW. Infliximab at a dose of 5 mg/kg BW produces higher reduction of tissue MDA levels than at a dose of 15 mg/kg BW.

Key words: Experimental colitis; Infliximab; Inflammatory bowel disease; Tumor necrosis factor-alpha; Malondialdehyde; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis is a chronic relapsing inflammatory condition involving the large bowel of unknown etiology. Clinical manifestations are considered to be the result of an imbalance between proinflammatory and inflammatory cytokines, resulting in inflammation and clinical symptoms. Activated T-lymphocytes release cytokines, thereby recruiting a large number of inflammatory cells in the mucosa. Activation of these cells causes further production of cytokines, cell recruitment and inflammation. In addition to cytokines, leukotrienes, thromboxane, and reactive oxygen species are released from activated mucosal cells[1]. This uncontrolled immune system activation results in the sustained overproduction of reactive metabolites of oxygen and nitrogen[2]. It has been suggested that self-sustaining cycles of oxidant formation may amplify flare-ups of inflammation and mucosal injury in ulcerative colitis[3]. Treatment of ulcerative colitis includes a wide range of anti-inflammatory and immunosuppressant drugs

with satisfactory results.

TNF-α is a pleiotropic cytokine with important proinflammatory and immunomodulatory properties. This cytokine plays a significant role in a number of inflammatory disorders including inflammatory bowel disease[4]. It has been shown that administration of the chimeric anti-TNF-α antibody in patients with active Crohn's disease results in a dramatic improvement of many clinical and laboratory parameters[5,6]. One of the most striking findings of the initially performed clinical trials is the observation that infliximab administered at a dose of 5 mg/kg BW results in better patients' improvement than at the dose of 10 or 15 mg/kg BW. Infliximab has also been administered in severe ulcerative colitis patients with promising results[7-9], though the clinical benefit is not prominent in patients refractory to previous administration of steroids[10,11]. Experimental evidence suggests that TNF-α may also play a role in the pathogenesis of experimental colitis[12].

The aim of this study was to investigate the influence of infliximab on experimental colitis in rats, produced by TNBS and to estimate its influence on the oxidative stress accompanying this model of colitis.

METHODS

The experimental procedures described below were approved by the Animal Care Committee according to the European Union Act and Greek Law 160, A-64, May, 1991.

General preparation

Adult male Wistar rats weighing 200-240 g were allowed to adapt to our laboratory conditions 1 wk prior to the experiment. They were housed individually in cages at a constant temperature (22 °C) and in a 12-h d/night cycle with free access to food and water. A total number of 36 rats were used. They were randomly allocated into five groups. *Group 1*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 5 mg/kg BW ("Infliximab 5"); *Group 2*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 10 mg/kg BW ("Infliximab 10"); *Group 3*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 15 mg/kg BW ("Infliximab 15"); *Group 4*: experimental colitis was induced without treatment with infliximab (12 animals) ("Untreated"). Six more healthy animals served as controls (Group 5). On the 7th d, all animals were killed and the colon was removed. The same part of rat's colon was used for histology as well as for MDA and TNF-α estimation.

Induction of experimental colitis

Distal colitis was induced by intracolonic installation of 25 mg of TNBS dissolved in 0.25 mL of 50% ethanol. The solution was injected into the colon 8 cm proximal to the anus with a PE-50 cannula. In order to ensure that TNBS-ethanol solution was not immediately expelled by the rat, the cannula was left in place for 15 s prior to its removal.

Drug administration

Infliximab was administered subcutaneously only in groups 1, 2, and 3 on d 2-6 at the doses of 5, 10 and 15 mg/kg BW, respectively. The subcutaneous administration has not previously been tried in both men and animals. We chose to administer the drug for five consecutive d and not to follow the usual scheme of administration of infliximab in patients with Crohn's disease because we were unable to predict the exact serum levels of the drug following the subcutaneous administration. A second reason was the fact that in this model of experimental colitis, the recommended time to kill the animals was 7 d. Following two or more weeks, the macro- and microscopic lesions were usually not detectable in the survived animals. Healthy control animals were given a subcutaneous dose of normal saline from d 2 to 6.

Histology

Specimens were fixed in 10% buffered formalin and embedded in paraffin blocks (3-4 blocks for each case). Then hematoxylin-eosin stained sections were blindly examined by two pathologists. In each case, the extent of lesions (expressed as a percentage of tissue damage of the whole bowel length) was estimated. The histological lesions such as active ulcers and erosions and reparable lesions (newly re-epithelized lesions or granulation tissue beneath cylindrical epithelium) were estimated. The extent of mucosa without signs of active inflammation was also estimated as previously described[13-16].

Tissue malondialdehyde (MDA) estimation

The MDA measurement was based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole (MPI), with MDA at 45°C. One molecule of MDA reacted with two molecules of MPI to yield a stable chromophore with maximum absorbance at 586 nm. The reagents used included Reagent MPI, 10.3 mmol/L *N*-methyl-2-phenylindole in acetonitrile, MDA standard, 10 mmol/L 1,1,3,3-tetramethoxypropane in 20 mmol/L Tris-HCl, 500 mmol/L butylated-hydroxytoluene, in acetonitrile, 20 mmol/L Tris buffer pH 7.4, 0.9% NaCl, 37% (12 mol/L) HCl, methanol, HPLC grade, acetonitrile and HPLC grade. Before the procedure, three volumes of the MPI reagent were diluted with one volume of 100% methanol. Tissue samples were rinsed with ice-cold isotonic saline before homogenization which was carried-out using Tris buffer 20 mmol/L pH 7.4 and an ULTRA-TURRAX (IKA-Labortecnik) blender. One milliliter buffer was used for 0.1 g of tissue. Ten milliliters of 500 mmol/L BHT was added to 1 mL of tissue homogenate to prevent sample oxidation. The homogenate was centrifuged at 3 000 r/min at 4 °C for 10 min. Then 0.2 mL of sample (plasma or supernatant of tissue homogenate) and 0.65 mL of diluted MPI reagent were added to a polypropylene microcentrifuge tube. The mixture was vortexed and then 0.15 mL of 12 mol/L HCl was added. Tubes were incubated at 45 °C for 60 min and centrifuged at 6 000 r/min.

Table 1 Comparison of histological lesions in treated and untreated groups of animals (mean±SD)

Group	Active ulcers and erosions	*P* value	Reparative lesions	*P* value
1 (Infliximab 5, *n* = 6)	0.07±0.12	0.30	0.38±0.12	0.0001
2 (Infliximab 10, *n* = 6)	3.77±5.07	0.42	0.80±0.14	0.0001
3 (Infliximab 15, *n* = 6)	25.02±38.90	1.00	1.00±0.47	0.0001
4 (Untreated, *n* = 12)	25.00±38.91		15.00±5.48	

Table 2 Percentage of bowel area without active inflammation in treated and untreated groups of animals (mean±SD)

Group	Percentage of mucosa without active inflammation	*P* value
1 (Infliximab 5)	99.55±0.19	0.049
2 (Infliximab 10)	95.43±5.15	0.088
3 (Infliximab 15)	73.98±39.29	0.705
4 (Untreated)	60.00±37.18	

Table 3 Tissue TNF-α levels in treated and untreated groups of animals (mean±SD)

Group	Tissue TNF-α (pg/mL)	*P* value
1 (Infliximab 5)	0.47±0.44	<0.0001
2 (Infliximab 10)	1.09±0.86	<0.0001
3 (Infliximab 15)	0.43±0.31	<0.0001
4 (Untreated)	18.73±10.53	
5 (Healthy animals)	0.00+/–0.0	

Table 4 Mean value of tissue malondialdehyde in treated and untreated groups of animals (mean±SD)

Group	Serum malondialdehyde (μmol/l)	*P* value
1 (Infliximab 5)	1.85±0.20	0.017
2 (Infliximab 10)	1.84±0.37	0.011
3 (Infliximab 15)	2.29±0.56	0.272
4 (Untreated)	2.73±0.46	
5 (Healthy animals)	1.11+/–0.19	

for 15 min. Then 0.8 mL of the supernatant was measured at 586 nm. MDA standards for the standard curve were made by dilutions of the stock 10 mm TMOP solution. The final concentrations were 2.08, 4.16, 8.33, 12.5 and 16.66 μmol/L and the assay procedure was followed as for the samples. The absorbance was 0.059, 0.124, 0.264, 0.4, and 0.545 respectively.

Tissue TNF-α estimation

TNF-α was determined after tissue homogenization by ELISA. In order to avoid errors in the interpretation of results, a specific rat antibody was used (antirat, DIACLONE Research) instead of human antibody against TNF-α.

Statistical analysis

Data were presented as mean±SD. Statistical comparisons between groups were made by one-way ANOVA followed by Dunnett's (two-sided) test. A difference between treated (1-3 groups) and untreated (group 4) animals was considered statistically significant at the level of $P<0.05$. Computations were done using the statistical package SPSS (version 11.0).

RESULTS

Histology

Table 1 shows the percentage of bowel area with the presence of active ulcers and erosions and reparative lesions observed in the treated and untreated groups of animals. Significant differences concerning the presence of reparable lesions between all groups of animals treated with infliximab compared to the untreated ones were found. Though differences were obvious between groups 1 and 2, they did not reach statistical significance (Table 1).

Table 2 shows the extent of mucosa without active inflammation in all groups of animals expressed as a percentage (mean value). Significant differences between the three groups of treated animals compared to the untreated ones were observed.

TNF-α tissue levels (pg/mL) (mean value)

The levels of tissue TNF-α in all groups are shown in Table 3. A significant reduction of the tissue levels of TNF-α was found in all groups of treated animals compared to the untreated ones.

Tissue malondialdehyde levels (μM)

The levels of tissue MDA in the treated and untreated animals are shown in Table 4. A significant reduction was noticed in group 1 compared to group 4, as well as between groups 2 and 4. No significant differences between groups 3 and 4 were noticed.

DISCUSSION

The findings of this experimental study in rats suggested that subcutaneous administration of infliximab was biologically effective; infliximab at doses of 5, 10, and 15 mg/kg BW could reduce the histological changes as observed in this particular experimental model; infliximab could significantly reduce tissue levels of TNF-α and MDA; suggesting that this molecule has probably antioxidant properties though the latter could be the consequence of its anti-inflammatory action.

In more details, subcutaneous administration of infliximab resulted in a significant amelioration of inflammatory histological lesions, a finding which was more prominent in the group of animals receiving 5 mg/kg per d. Administration of the drug resulted in a statistically significantly smaller area of large bowel with reparable lesions compared to the untreated group of animals, although the percentage of bowel area with active ulcers and erosions did not differ significantly between treated and untreated animals. It was also shown that the percentage of bowel area with normal mucosa was

significantly larger only in the group treated with 5 mg/kg BW of infliximab as compared to untreated animals. The percentage of bowel area with normal mucosa did not differ significantly between groups receiving 10 and 15 mg/kg BW and the untreated group of animals. Wooddruff *et al*.[17] also showed that a single IV dose of infliximab before the induction of experimental colitis results in a significant reduction of the severity of the lesions.

In our study, all doses of infliximab significantly reduced the tissue levels of TNF-α, suggesting that TNF-α plays a significant pathogenetic role in this model of colitis as well. The IV dose of 5 mg/kg BW is the currently recommended one for the treatment of patients with active Crohn's disease. The beneficial effect of infliximab observed in this experimental model is in accordance with clinical observations showing beneficial clinical effects on some patients with severe ulcerative colitis[7-9]. TNF-α is a 17-ku proinflammatory cytokine produced by monocytes, macrophages, and T cells. The biological actions of this cytokine include induction of acute-phase response, cachexia, and potentially lethal shock[18]. Furthermore, TNF-α stimulates secretion of IL-1 and IL-6, expression of adhesive molecules and fibroblast proliferation. Release of TNF-α is mediated by a specific metalloproteinase (TNF-α convertase). After secretion, TNF-α binds as a soluble ligand to two cell-bound transmembrane TNF receptors, namely TNFR1 and TNFR2[19]. Chronic inflammation in Crohn's disease can be attributed mainly to the production of proinflammatory cytokines, especially TNF-α. This cytokine is considerably increased in the histologically normal as well as inflamed large bowel mucosa of patients with Crohn's disease. It has been described that thalidomide, a drug with well-known anti-TNF-α action, significantly reduces colonic inflammation induced by iodoacetamide, probably via the inhibition of TNF-α[20]. This experiment is another paradigm of amelioration of colitis by a drug with inhibitory influence of TNF-α.

An important finding of this study is the increased tissue levels of tissue MDA in the untreated rats and the reduced tissue levels of MDA in the groups treated with 5 and 10 mg/kg BW. However, the animals treated with 15 mg/kg BW did not show any statistically significant difference from the untreated ones. There is no obvious explanation for that, though it seems that the reduction of MDA levels could not be a phenomenon related to quantity of the reactive elements. We must emphasize that the results of the 5 and 10 mg/kg BW administration of infliximab on MDA levels were quite similar with the results concerning the corresponding histological lesions. We suppose that the reduction in lipid peroxidation and cellular damage originating from oxidative stress following the administration of infliximab is an important factor contributing to amelioration of experimental colitis. A growing number of data suggests that in experimentally-induced colitis, the colon may be subjected to considerable oxidative stress[21]. Oxidative stress leads to the extension and propagation of crypt abscesses either through direct membrane disruption by lipid peroxidation or through generation of secondary toxic oxidants such as chloramines. Subsequently, chemotactic products of lipid peroxidation provide positive feedback to accelerate the inflammatory/oxidative process[22]. Colonic mucosa may be overwhelmed during active inflammation resulting in intestinal inflammation due to the inability of the mucosa to ameliorate the generating stress because of the small amount of antioxidant enzymes contained in it. It could be possible that colonic injury and dysfunction observed in inflammatory bowel disease are due to the elaboration of these reactive species. Infliximab may have antioxidant properties as well, as it can be suggested by the significant reduction of MDA levels observed in all treated groups of animals though this could simply be the consequence of its anti-inflammatory action.

Another point of interest of this study is the fact that subcutaneous administration of the drug resulted in the reduction of inflammation and tissue damage. The recommended route of administration of infliximab is the IV route. The beneficial effect observed in this model suggests that the other routes of administration of the drug could be effective in human beings as well.

In conclusion, the results of the present study suggest that subcutaneous administration of infliximab reduces the inflammatory activity, as well as tissue TNF-α and MDA levels in chemical colitis in rats. Moreover, infliximab at a dose of 5 mg/kg BW achieves better histological results and produces higher reduction of the levels of TNF-α than at a dose 10 mg/kg BW. Finally, infliximab at a dose of 5 mg/kg BW produces higher reduction of tissue MDA levels than at a dose of 15 mg/kg BW. The administration of infliximab in rats with chemical colitis supports the clinical observations that the dose of 5mg/kg BW produces better results than the dose of 10 or 15 mg/kg BW. The possible antioxidant properties of infliximab must be further investigated both from clinical and experimental points of view.

REFERENCES

1 **Nassif A,** Longo WE, Mazuski JE, Vernava AM, Kaminski DL. **Role of cytokines and platelet-activating factor in** inflammatory bowel disease. Implications for therapy. *Dis Colon Rectum* 1996; **39**: 217-223

2 **Pavlick KP,** Laroux FS, Fuseler J, Wolf RE, Gray L, Hoffman J, Grisham MB. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* 2002; **33**: 311-322

3 **Kruidenier L,** Verspaget HW. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease: radicals or ridiculous? *Aliment Pharmacol Ther* 2002; **16**: 1997-2015

4 **Holtmann MH,** Schuchmann M, Zeller G, Galle PR, Neurath MF. **The emerging distinct role of TNF-receptor 2 (p80)** signaling in chronic inflammatory disorders. *Arch Immunol Ther Exp (Warsz)*. 2002; **50**: 279-288

5 **Targan SR,** Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; **337**: 1029-1035

6 **Rutgeerts P,** D'Haens G, Targan S, Vasiliauskas E, Hanauer

SB, Present DH, Mayer L, Van Hogezand RA, Braakman T, DeWoody KL, Schaible TF, Van Deventer SJ. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999; **117**: 761-769

7 **Chey WY**. Infliximab for patients with refractory ulcerative colitis. *Inflamm Bowel Dis* 2001; **7** Suppl 1:S30-S33

8 **Kohn A**, Prantera C, Pera A, Cosintino R, Sostegni R, Daperno M. Anti-tumor necrosis factor alpha (infliximab) in the treatment of severe ulcerative colitis: result of an open study on 13 patients. *Dig Liver Dis* 2002; **34:** 626-630

9 **Actis GC,** Bruno M, Pinna-Pintor M, Rossini FP, Rizzetto M. Infliximab for treatment of steroid-refractory ulcerative colitis. *Dig Liver Dis* 2002; **34:** 631-634

10 **Su C,** Salzberg BA, Lewis JD, Deren JJ, Kornbluth A, Katzka DA, Stein RB, Adler DR, Lichtenstein GR. Efficacy of anti-tumor necrosis factor therapy in patients with ulcerative colitis. *Am J Gastroenterol* 2002; **97**: 2577-2584

11 **Probert CS,** Hearing SD, Schreiber S, Kuhbacher T, Ghosh S, Arnott ID, Forbes A. Infliximab in moderately severe glucocorticoid resistant ulcerative colitis: a randomised controlled trial. *Gut* 2003; **52**: 998-1002

12 **Andreadou I,** Papalois A, Triantafillidis JK, Demonakou M, Govosdis V, Vidali M, Anagnostakis E, Kourounakis PN. Beneficial effect of a novel non-steroidal anti-inflammatory agent with basic character and antioxidant properties on experimental colitis in rats. *Eur J Pharmacol* 2002; **441**: 209-214

13 **Jenkins D,** Balsitis M, Gallivan S, Dixon MF, Gilmour HM, Shepherd NA, Theodossi A, Williams GT. Guidelines for the initial biopsy diagnosis of suspected chronic idiopathic inflammatory bowel disease. The British Society of Gastroenterology Initiative. *J Clin Pathol* 1997; **50:** 93-105

14 **Robinson JW**, Mirkovitch V, Winistorfer B, Saegesser F. Response of the intestinal mucosa to ischaemia. *Gut* 1981; **22**: 512-527

15 **Burns BJ,** Brandt LJ. Intestinal ischemia. *Gastroenterol Clin North Am* 2003; **32:** 1127-1143

16 **Geboes K,** Riddell R, Ost A, Jensfelt B, Persson T, Lofberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. *Gut* 2000; **47**: 404-409

17 **Wooddruff TM,** Arumugam TV, Shiels IA, Reid RC, Fairlie DP, Taylor SM. A potent human C5a receptor antagonist protects aginst disease pathology in a rat model of inflammatory bowel disease. *J Immunol* 2003; **171**: 5514-5520

18 **Hehlgans T,** Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* 2005; **115**: 1-20

19 **Mizoguchi E**, Mizoguchi A, Takedatsu H, Cario E, de Jong YP, Ooi CJ, Xavier RJ, Terhorst C, Podolsky DK, Bhan AK. Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice. *Gastroenterology* 2002; **122**: 134-144

20 **Kenet G,** Wardi J, Avni Y, Aeed H, Shirin H, Zaidel L, Hershkoviz R, Bruck R. Amelioration of experimental colitis by thalidomide. *Isr Med Assoc J* 2001; **3**: 644-648.

21 **Thiele GM,** Worrall S, Tuma DJ, Klassen LW, Wyatt TA, Nagata N. The chemistry and biological effects of malondialdehyde-acetaldehyde adducts. Alcohol Clin Exp Res 2001; **25**: 218S-224S.

22 **Yamada T,** Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. *Klin Wochenschr* 1991; **69**: 988-994.

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• RAPID COMMUNICATION •

Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection

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Abstract

AIM: To determine the predictive factors for hepatocellular carcinoma (HCC) development in patients after spontaneous or therapeutic HBeAg seroconversion.

METHODS: In 48 patients who seroconverted to anti-HBe positive during follow-up, the background factors for HCC development were analyzed.

RESULTS: HCC was developed in six patients during follow-up (average follow-up after HBeAg seroconversion: 10.9±5.4 years). The incidence of HCC evaluated by Kaplan–Meier analysis was significantly higher in patients with abnormal aspartate aminotransferase (AST> 40 IU/L) level, lower platelet counts (PLT<10×10^4/μL), lower albumin level (Alb<30 g/L), positive HBV-DNA or older age at seroconversion (>40 years). However, lower platelet count was the only predictive factor for HCC development shown by multivariate proportional-hazard analysis.

CONCLUSION: Active hepatitis or advanced hepatitis at HBeAg seroconversion or progressive hepatitis even after HBeAg seroconversion would be the risk factors for HCC development. These predictive factors should be taken into account in determining the frequency of biochemical study or imaging studies for HCC surveillance.

Key words: HBeAg seroconversion; Hepatocellular carcinoma; Predictive factors

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INTRODUCTION

Hepatitis B virus

Hepatitis B virus (HBV) in Japan, where genotype C is dominant, is generally infected during delivery or early childhood with immature immune systems, and those children become HBV carriers before HBV vaccine was introduced. In adolescents, hepatitis e antigen (HBeAg) seroconversion to its antibody (anti-HBe) often occurs after multiple exacerbations, which coincide with subsidence of hepatic inflammatory activities[1,2]. The failure of seroconversion usually results in chronic progressive liver diseases and hepatocellular carcinoma (HCC). The calculated annual incidence of acute exacerbation was two times higher in patients with positive HBeAg or HBV-DNA than those without these markers[3]. The patients who have recurrent episodes of acute exacerbations with bridging hepatic necrosis are more likely to develop cirrhosis[4]. These observations suggest that HBeAg seroconversion to anti-HBe confers favorable long-term outcomes. On the other hand, most HCC occurred in HBeAg-negative patients with undetectable HBV-DNA[5,6], suggesting that all HBV patients should be carefully followed-up for HCC even after HBeAg seroconversion. However, there seems to be some predictive factors for HCC in our clinical experiences. Previous reports demonstrated the natural history of anti-HBe positive patients. However, they included patients with spontaneous reactivation, which may be the major cause of progressive hepatic damage[7,8]. To our knowledge, there are no reports about incidences of HCC that selected only patients who seroconverted to anti-HBe during follow-up. Therefore, we focused on anti-HBe-positive patients without any reactivation of HBeAg and tried to determine the risk factors for HCC development to select high risk group for HCC.

MATERIALS AND METHODS

Three hundred and thirty-one HBsAg-positive patients were followed-up in our hospital between 1990 and 2004. Inclusion criteria in this study were: (1) follow-up until death or October 2004; (2) sufficient data to evaluate; and (3) careful follow-up for HCC with evaluation of both tumor markers (alpha-fetoprotein or des-gamma-carboxyprothrombin) and image studies (USG or CT) every 3-6 mo. HBeAg serocoversion was determined if anti-HBe completely became positive and HBeAg was never recovered retrospectively. We excluded the cases that showed fluctuation of HBeAg or anti-HBe levels.

None of the patients had any other viral infections, such as hepatitis C virus (HCV) or human immunodeficiency virus (HIV). Patients were followed-up by biochemical studies and image studies for at least 3-6 mo intervals. The biochemical tests were measured using routine automated techniques. HBsAg, HBeAg, and anti-HBe were assayed using commercial enzyme immunoassay kits (Abbott Japan, Tokyo). HBV-DNA was determined by a transcription-mediated amplification (TMA) method. The detection sensitivity of this assay is 3.7 LGE/mL. All HCC were initially found by USG or CT and confirmed by CT during abdominal angiography.

Statistical analysis

Fisher's exact test or the χ^2 test was used to compare patients' backgrounds. Kaplan–Meier analysis and log-rank test were performed to test a cumulative incidence of HCC in each group. Multivariate proportional-hazards survival analysis was used to determine the important risk factors for HCC. $P<0.05$ was considered statistically significant.

RESULTS

One hundred and thirty-four out of the 331 HBV patients were enrolled in this study. Forty six patients (M:F = 34:12) were HBeAg-positive and 88 (M:F = 64:24) patients were anti-HBe-positive. There were no patients who had positive tests for both HBeAg and anti-HBe. Regarding the patients' backgrounds, age, sex, platelet counts, albumin level and AST level were similar in both HBeAg-positive and anti-HBe-positive patients. However, ALT level was significantly higher in HBeAg-positive patients as compared with anti-HBe-positive patients (P = 0.001, Table 1). HCC developed in 15 HBeAg-positive patients and in 11 anti-HBe-positive patients during follow-up. The average age for the appearance of HCC was similar in both groups (55±8 years in HBeAg-positive and 53±12 years in anti-HBe-positive patients). The cumulative incidence of HCC was significantly higher in HBeAg-positive patients than in anti-HBe-positive patients as previously reported (P = 0.016, Figure 1). However, the incidence of HCC was found to be increased with age even in patients with anti-HBe, especially in those who were over 70 years. Out of 88 patients with anti-HBe, the time of HBeAg seroconversion was observed in 48 patients. Other 40 patients already showed anti-HBe positive when the patients first presented. The current study focused on these 48 patients. The average age of 48 patients was 50

Table 1 Patients' characteristics

Feature	HBeAg	Anti-HBe	*P*
n	46	88	NS
Age	53±12	54±14	NS
Male sex	34 (74%)	64 (73%)	NS
Platelet ($\times10^4/\mu L$)	14.3±6.6	18.7±6.4	NS
Alb (g/dL)	4.0±0.5	4.1±0.5	*NS*
AST (IU/L)	56±33	41±36	NS
ALT (IU/L)	62±57	36±27	0.001

NS: not significant.

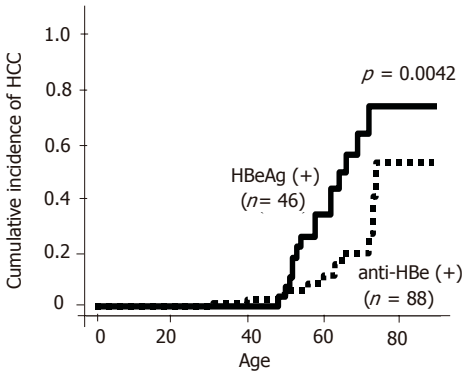

Figure 1 Cumulative incidence of HCC in patients with HBeAg or anti-HBe (Kaplan –Meier analysis and log-rank test). The incidence of HCC in both groups was increasing after the age of 50. The incidence of HCC was significantly higher in the patients with HBeAg positive (solid line) than those with anti-HBe positive (dot line) (P=0.0042).

±14 years (M:F = 34:14). The average age at HBeAg seroconversion was 40±12 years (range, 16-67 years). HBeAg seroconversion occurred spontaneously in 75% (36/48), by IFN in 18.8% (9/48) and by propagermanium in 6.2% (3/48) cases. HBV-DNA was detectable by TMA methods in 27.1% (13/48) patients. The average follow-up periods after HBeAg seroconversion were 10.9±5.4 years (range, 1-25 years). Six patients developed HCC during follow-up. All six patients were male and the average age of HCC development was 57±13 years (14.0±5.1 years after HBeAg seroconversion). The incidence of HCC evaluated by Kaplan–Meier analysis was significantly higher in patients with abnormal AST (>40 IU/L) level (P = 0.007, Figure 2A), suggesting that active hepatitis even after seroconversion may be one of the risk factors for HCC. However, there were no differences in ALT levels (Figure 2B). Low platelet count ($<10\times10^4/\mu L$) ($P<0.0001$) or low albumin level (Alb<30 g/L) (P = 0.009) was another risk factor for HCC, suggesting that HCC may develop in patients with advanced liver diseases at HBeAg seroconversion or with progressive liver diseases even after seroconversion (Figures 2C and D). Positive HBV-DNA (P = 0.014) increased the incidence of HCC as well (Figure 2E). The incidence of HCC tended to be non-significantly higher in the patients with HBeAg seroconversion by therapy as compared with the spontaneous seroconversion (Figure 3A). The cumulative incidence of HCC was significantly higher in the patients with HBeAg seroconversion after 40 years of age (Figure 3B). Multivariate proportional-hazards analysis demonstrated that low platelet counts ($<10\times10^4/\mu L$) was the only predictive factor for HCC (P = 0.032, hazard ratio = 21.6), whereas serum albumin and AST levels and HBV-DNA positivity could not be determined as predictive factors for HCC (Table 2). Furthermore, the incidence of HCC was not observed in the patients with both normal platelet counts ($>10\times10^4/\mu L$) and normal AST level (<40 IU/L) during follow-up in our study. In contrast, the incidence of HCC was annually increasing after HBeAg seroconversion in patients with either lower platelet counts ($<10\times10^4/\mu L$) or abnormal AST level (>40 IU/L) (Figure 4).

DISCUSSION

The present study demonstrated that low platelet counts ($<10\times10^4/\mu L$), low albumin level (<30 g/L), abnormal AST level (>40 IU/L), older age at HBeAg seroconversion (>40 years) and positive HBV-DNA were the predictive factors for HCC after HBeAg seroconversion. Multivariate proportional-hazard analysis demonstrated that platelet count was the only significant risk factor. These observations suggested that active hepatitis or progression of liver disease even after HBeAg seroconversion or advanced hepatitis at HBeAg seroconversion would be the predictive factors for HCC. In other words, patients without any of these factors after HBeAg seroconversion would be at low risk for HCC development.

In patients with chronic hepatitis C virus (HCV) infection, we should focus on patients with advanced

Table 2 Predictve Features of HCC

Variable	Hazard ratio	95%CI	*P*
Platelet counts ($<10^5$ *vs* $\geq10^5$/mL)	21.6	1.3 – 354.8	0.032
Alb (< 3.0 *vs* ≥ 3.0 g/dL)	0.4	0.1 – 3.4	0.376
AST (> 40 *vs* ≤ 40 IU/L)	3.6	0.2 – 51.3	0.351
HBV-DNA (+ *vs* -)	0.1	0.1 – 10.4	0.912

Multivariate proportional-hazards analysis.

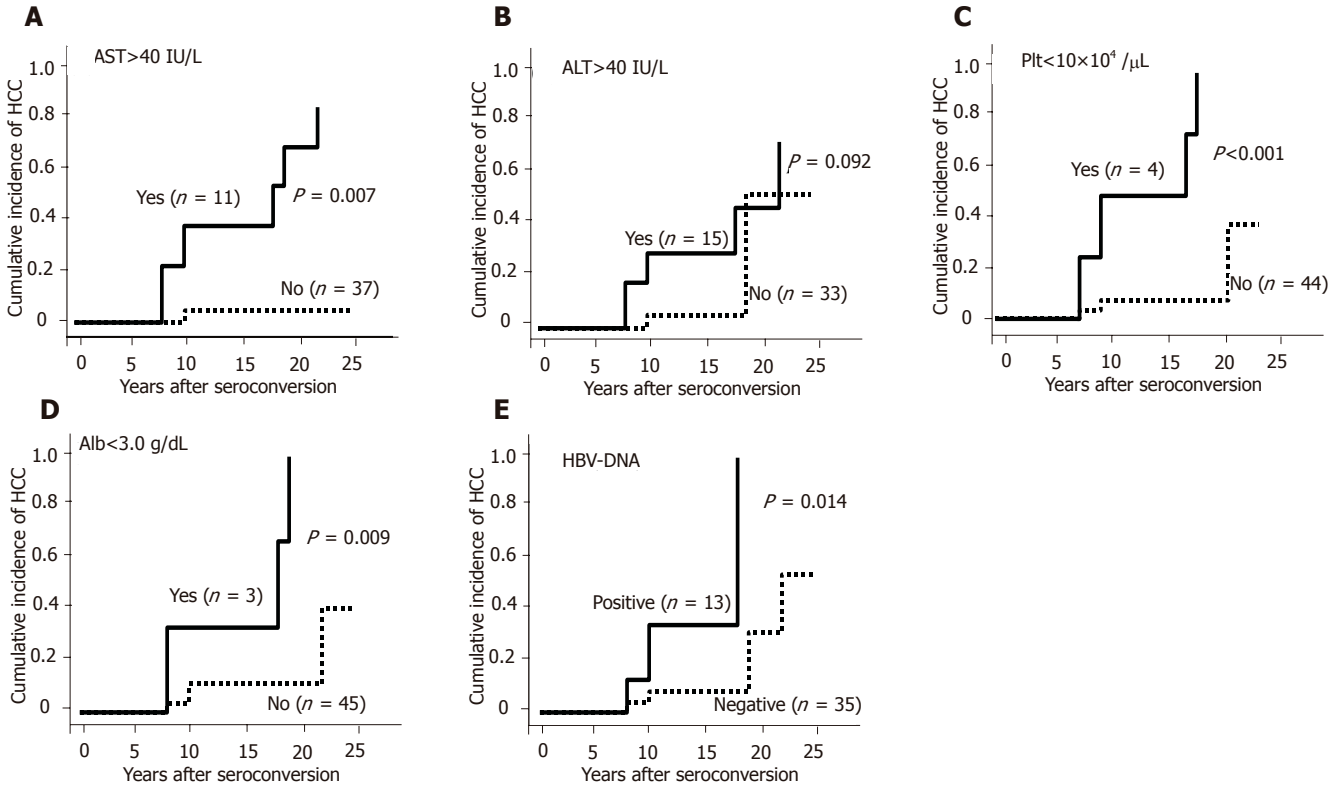

Figure 2 Cumulative incidence of HCC in patients with different parameters (Kaplan–Meier analysis and log-rank test). **A:** AST level (>40 or ≤40 IU/L); **B:** ALT level (>40 or ≤40 IU/L); **C:** platelet counts ($<10\times10^4$ or $\geq10\times10^4/\mu L$): **D:** albumin level (<30 or ≥30 g/L); **E:** HBV-DNA (positive or negative).

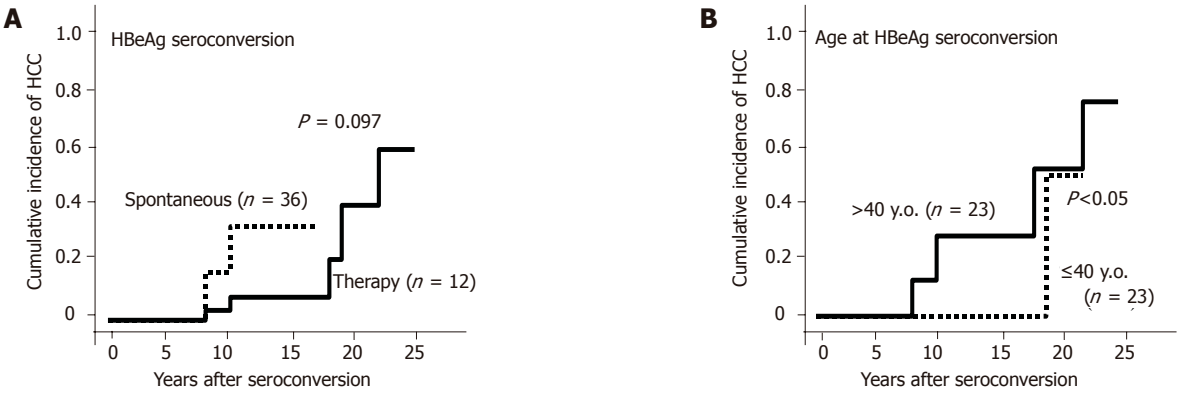

Figure 3 Cumulative incidence of HCC in patients after seroconversion (Kaplan–Meier analysis and log-rank test). **A:** Spontaneous or post-therapeutic seroconversion; **B:** age at seroconversion (>40 or ≤40 years).

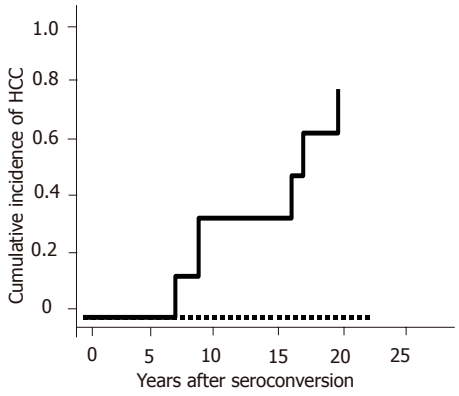

Figure 4 Cumulative incidence of HCC in patients with or without risk factors (Kaplan–Meier analysis and log-rank test). Solid line: either platelet counts<10×10^4/μL or AST>40 IU/L; dot line: both platelet counts≥10×10^4/μL, and AST<40 IU/L.

chronic hepatitis or cirrhosis as high risk group for HCC development and follow-up with tight observation for biochemical studies, tumor markers and image studies since HCC is usually accompanied with the progression of liver diseases. In patients with chronic HBV infection, however, HCC sometimes develops not only in liver cirrhosis, but even in mild hepatitis[9,10]. So, it is more difficult to select high risk patients for HCC in chronic HBV infection than that in HCV infection. Of course, positive HBeAg or reactivation after HBeAg seroconversion with active hepatitis is one of the risk factors for cirrhosis[7,8], HCC[3,11] or survival[12]. However, the cumulative incidence of HCC in our study was high even in anti-HBe-positive patients, especially in older patients (Figure 1). Therefore, we conducted this study to determine the predictive factors for HCC in patients with anti-HBe.

We found that the cumulative incidence of HCC was significantly higher in patients with abnormal AST (Figure 2A), suggesting that active hepatitis even after HBeAg seroconversion was one of the risk factors. This result was consistent with previous reports that progression to cirrhosis or HCC occurred in patients with HBeAg negative hepatitis without reversion to HBeAg[11,13]. Furthermore, the incidence of HCC was significantly reduced in HBeAg-negative chronic hepatitis or cirrhotic patients who maintained virological response by lamivudine[14] or interferon (IFN)[15,16]. These previous reports support our observation that therapy for transaminase control may reduce the risk of HCC development in our patients with abnormal AST level. On the other hand, the cumulative incidence of HCC was not statistically different in ALT levels. We, therefore, hypothesized that ALT elevation might be observed in patients with fatty liver as well as necroinflammatory liver disease.

Other risk factors for HCC development in anti-HBe-positive patients were lower platelet counts and low albumin level. Interestingly, multivariate proportional-hazards analysis showed that low platelet counts (<10×10^4/μL) was the only risk factor for HCC development, suggesting that HCC may develop in patients with progressive liver disease even after HBeAg seroconversion or advanced liver diseases before HBeAg seroconversion. Since multiple exacerbations usually occurred before HBeAg seroconversion, necroinflammation occurs more frequently in patients with delayed HBeAg seroconversion, which results in progression to liver cirrhosis or HCC[13]. Our results show that the cumulative incidence of HCC was significantly higher in patients with delayed HBeAg seroconversion (older than 40 years) are consistent with the previous reports. Furthermore, the cumulative incidence of HCC in our study was annually increasing in the patients with either low platelet counts (<10×10^4/μL) or abnormal AST (>40 IU/L) after HBeAg seroconversion, whereas no HCC was observed in patients with both normal platelet counts (≥10×10^4/μL) and normal AST (<40 IU/L) during follow-up (median follow-up period: 11±5 years).

In this study, we observed a relatively higher incidence of HCC in patients with anti-HBe as compared with the previous reports. This might be because our study was not a prospective study and we might have lost the patients with relative low risk of active hepatitis or fibrosis during follow-up. It is possible that there might be a bias, in which the patients with relative high risk (high AST or ALT) were accumulated in our study. Prospective and large-scale studies need to determine the predictive factors for HCC in patients with anti-HBe.

In conclusion, in anti-HBe-positive patients, lower platelet counts, lower albumin, abnormal AST and positive HBV-DNA are the predictive factors for HCC. In other words, anti-HBe-positive patients without any of these factors are at low risk for HCC. In the follow-up of anti-HBe-positive patients, those predictive factors, especially platelet counts, should be taken into account for determining the frequency of biochemical study or imaging studies. Moreover, to reduce the risk for HCC development, we should induce HBeAg seroconversion before progression to advanced liver diseases and control AST level within the normal range using lamivudine or IFN. These results may be supported by our clinical experiences. However, to our knowledge, there are no reports to clarify the high risk group in anti-HBe positive patients with known time of HBeAg seroconversion.

REFERENCES

1 **Hoofnagle JH**, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; **94:** 744-748
2 **Realdi G**, Alberri M, Rugge M, Borrolotti F, Rigoli AM, Tremolada F, Ruol A. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. *Gastroenterology* 1980; **79:** 195-199
3 **Liaw YF**, Lin DY, Chen TJ, Chu CM. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Liver* 1989; **9:** 235-241
4 **Chu CM**, Natural history of chronic hepatitis B virus infection in adults emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. *J Gastroenterol Hepatol* 2000; **15:** E25-30

5 **Lok AS**, Prevention of hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2004; **127:** S303-S309
6 **Yuen MF**, Lai CL. Natural history of chronic hepatitis B virus infection. *J Gastroenterol Hepatol* 2000; **15:** E20-24
7 **Fattovich G**, Brollo L, Alberti A, Realdi G, Pontisso P, Giustina G, Ruol A. Spontaneous reactivation of hepatitis B virus infection in patients with chronic type B hepatitis. *Liver* 1990; **10:** 141-146
8 **Takano S**, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21:** 230-235
9 **Di Bisceglie AM**, Rustgi VK, Hoofnagle JH, Dusheiko GM, Lotze MT. NIH conference. Hepatocellular carcinoma. *Ann Intern Med* 1988; **108:** 390-401
10 **Takano S**, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21:** 650-655
11 **Hsu YS**, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35:** 1522-1527
12 **Niederau C**, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. *N Eng J Med* 1996; **334:** 1422-1427
13 **Chu CM**, Hung SJ, Lin J, Tai DI, Liaw YF. Natural history of hepatitis B e antigen to antibody seroconversion in patients with normal serum aminotransferase levels. *Am J Med* 2004; **116:** 829-834
14 **Marco VD**, Marzano A, Lampertico P, Andreone P, Santantonio T, Almasio PL, Rizzetto M, Craxi A for the Italian Association for the Study of the Liver (AISF) Lamivudine Study Group. Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. *Hepatology* 2004; **40:** 883-891
15 **Marco VD**, Iacono OL, Camma C, Vaccaro A, Giunta M, Martorana G, Fuschi P, Almasio PL, Craxi A. The long-term course of chronic hepatitis B. *Hepatology* 1999; **30:** 257-264
16 **Paratheodoridis GV**, Manesis E, Hadziyannis SJ. The long-term outcome of interferon-a treated and untreated patients with HBeAg-negative chronic hepatitis B. *J Hepatol* 2001; **34:** 306-313

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• RAPID COMMUNICATION •

Evaluation of liver tissue by polymerase chain reaction for hepatitis B virus in patients with negative viremia

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Abstract

AIM: To assess the clinical significance of Hepatitis B virus (HBV) DNA localization in the liver tissue of patients with positive HBsAg and negative viremia.

METHODS: HBV virological parameters of 33 HBsAg positive chronic hepatitis patients, including seromarkers and HBV DNA amplification in both sera and liver biopsies, were evaluated.

RESULTS: Ten patients had negative viremia and positive HBV DNA in their liver biopsies. Most of them had HBeAg-negative/HBeAb-positive chronic hepatitis. Their liver biochemical and histopathological profiles were different from the viremic patients. Their disease pattern was designated as "hepatitis B *in situ*".

CONCLUSION: Hepatitis B *in situ* is a consequential entity which can be missed in clinical practice. It is a new clinical pattern of chronic HBV infection that considers HBV in liver biopsy and adds a new indication for antiviral therapy.

Key words: Hepatitis B *in situ*; Antiviral therapy; HBV DNA; Chronic hepatitis

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INTRODUCTION

Hepatitis B virus is a virus that infects 350 million people worldwide with a clinical spectrum of acute hepatitis, the healthy carrier state, cirrhosis, and hepatocellular carcinoma. The outcome of infection is the result of complicated viral-host interactions. As in other infections with non-cytopathic viruses, the immune response is thought to play a crucial role in disease pathogenesis. However, there are increasing evidences that varieties of viral mechanisms, some depending on the function of virally encoded proteins, have a profound impact on the infected hepatocytes, the liver microenvironment and host antiviral responses[1].

Occult HBV infection is characterized by undetectable HBsAg. Serum HBV DNA level is usually less than 10^4 copies/mL in these patients. Diagnosis requires a sensitive HBV-DNA assay[2]. To our knowledge, no data are available about the amount of HBV genomes in the liver of patients with chronic HBV infection. However, it has been reported that cases with suppressed HBV activity, despite the very low level of viremia, maintain a relatively high amount of intrahepatic viral genomes. This virus reservoir is likely involved in HBV reactivation, which is usually observed after stopping lamivudine treatment[3].

The detection of HBV genomes in nested PCR-based assays appears to be the most reliable methods for monitoring infection and assessing response to antiviral treatment[4]. The majority of patients with HBsAg in serum have HBV DNA present in serum as well. Some patients have HBsAg but no HBV DNA in serum. These are chronic carriers with normal transaminases[5]. On the other hand, HBV DNA can be detected in serum and liver by PCR in patients who have lost their HBsAg. In addition, hepatic HBV DNA can be detected in cirrhotic liver in the absence of serum HBV DNA[5,6]. HBV DNA can be treated as a sign of HBV activity[7], without ignoring the facts that HBV DNA levels fluctuate in patients with chronic HBV infection and that the correlation between serum HBV DNA levels and histologic activity is poor[8].

In this study, we evaluated the clinical significance of the localization of HBV in liver tissue of chronic HBV patients with negative viremia and elevated ALT.

PATIENTS AND METHODS

This prospective study included chronic liver disease patients who attended the out-patient clinic of the

Endemic Medicine Department, Cairo University during the period between September 2002 and July 2003. All patients with data suggestive of liver affection were evaluated, and those with positive HBsAg and elevated ALT were selected as the candidates for this study.

Exclusion criteria were HCV infection, advanced liver disease, alcohol consumption, autoimmune hepatitis, drug-induced hepatitis, hepatic schistosomiasis, evidence of acute hepatitis in the last 6 mo, presence of HDVAb, hepatocellular carcinoma, and advanced co-morbid conditions such as cardiac or renal disease.

Selected patients were subjected to clinical evaluation and laboratory investigations including viral serologic tests using enzyme immunoassay technique applying the Abbott System®. These assays included the detection of HBsAg, HBsAb, HBcAb (total), HBeAg, HBeAb, and HCVAb (total) by 3rd generation ELISA assay (Sorin®, Italy). Abdominal ultrasonography and liver biopsy were performed. The modified HAI score was used to stage and grade liver disease[9]. For the detection of hepatic total HBV DNA (integration plus CCC form), part of the liver sample was incubated at 56 °C for 3 h with 270 μL of phosphate buffer saline containing 160 μg of proteinase K (Promega, Madison, WI, USA) and 30 μL of 10 buffer. Serum HBV DNA was extracted by incubation at 56 °C for 3 h with 270 μL of serum plus 30 μL of 10×buffer containing 160 μg proteinase K. DNA extraction was purified by phenol: chloroform (300 μL, 1:1 V/V) extraction, ethanol/0.2 mol/L sodium acetate precipitation. Qualitative PCR testing for HBV in serum and liver tissue was carried out using nested PCR technique[10]. Total genomic DNA was extracted from serum or liver tissue using phenol/chloroform extraction after proteinase K digestion. Purified DNA (1–2 μg) was utilized as a template in the polymerase chain reaction using two rounds of amplification with nested primers as follows:
Outer primers: B1 5' AAGGTCTTACATAAGAGGAC 3' (nt 1 644–1 663), B2 5' CTAACATTGAGATTCCCGAG ATTGAGA 3' (nt 2 458–2 432);
Inner primers: B3 5' GGCTGTAGGCATAAATTGGTC-TG 3' (nt 1 781–1 803), B4 5' TTGCCTGAGTGCAGTA-TGGT 3' (nt 2 075–2 056).

The PCR reactions were performed in 50 μL reaction volume containing 10 μmol/L dNTPs, 50 pmol from each primer, and 2 U of Taq. The amplification protocol for the two nested rounds consisted of 35 cycles, each cycle contained a denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 2 min, followed by a single cycle with final extension at 72 °C for 7 min. Positive samples infected with HBV DNA produced a amplified product of 295 bp in length.

Statistical analysis

Patients' data were tabulated, and then processed using the software SPSS for Windows 9 version 10.0®. Quantitative variables were expressed as mean±SD and compared using Student's *t* test. Qualitative variables were compared using Fisher's exact test. A $P<0.05$ was considered statistically significant.

RESULTS

Out of the 670 patients with chronic liver disease, 44 were seropositive for HBsAg. Eleven of the forty-four HBsAg seropositive patients were excluded because of normal liver enzymes. The study was then conducted on the remaining 33 patients (26 males and 7 females; mean age 32.8±12.0 years, range 18–60 years). Fifteen patients were originally from urban areas and 18 from rural areas (10 from Nile Delta, and 8 from Upper Egypt).

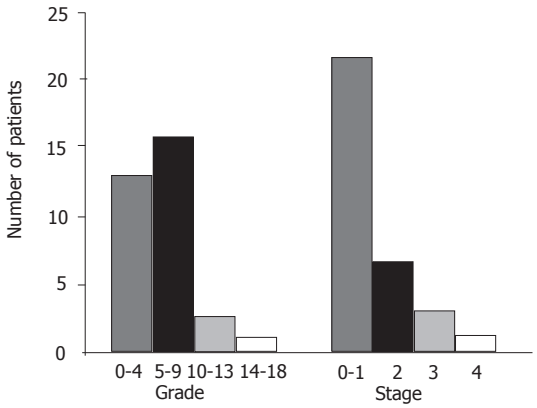

Figure 1 Histopathological grading and staging

According to the modified HAI grading and staging, histopathological findings are shown in Figure 1. Ground glass pattern of the hepatocytes was detected in 21 patients, steatosis was found in 14 patients, while cholestasis was detected in two cases.

HBV markers were spread into Poynard's table of classification of HBV infected patients[10]. Seventeen patients were fit with the "chronic carrier, pre-core mutant type", and six with the "chronic carrier, wild type" definitions. Ten patients remained uncategorized, showing a unique pattern (Table 1).

These 10 patients (group 1) were compared to the remaining 23 chronic hepatitis B patients (group 2) as shown in Tables 2 and 3. No statistically significant differences were observed regarding the liver biochemical or histological parameters. In this study, we found five possible permutations of the HBV seromarkers, which were grouped according to the HBV DNA results (Table 4). Among the HBcAb-negative patients, three patients were also negative for HBeAg and HBeAb. HBV DNA was detectable in

Table 1 Characteristics of patients uncategorized in Poynard (2000) classification

	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb *IgG*	HBcAb *IgM*	DNA serum	DNA liver	Symptom	ALT	HAI/fibrosis	No. of patients
Uncategorized	+	+/–	–/+	+/–	+	–	–	+	+/–	+	+	10

Table 2 Hepatic biochemical tests in group I *vs* group II patients

	Group I (n = 10)	Group II (n = 23)	P values
Serum bilirubin (mg/dL)	1.36±1.31	1.44±2.1	0.9
AST (IU/L)	63.9±24.1	82.0±51.3	0.3
ALT (IU/L)	62.6±27.4		0.2
Serum albumin (g/dL)	3.9±0.50	4.0±0.57	0.4
INR	1.25±0.22	1.23±0.14	0.7

Table 3 Hepatic histopathologic parameters in group I *vs* group II patients

		Group I (n = 10)	Group II (n = 23)	P values
Grading	0–9	8	20	0.4
	10–18	2	3	
Staging	0–2	9	19	0.9
	3–6	1	4	

Table 4 HBV seromarker pattern according to HBV DNA testing

Marker pattern	n	HBV DNA status			
		+ve serum +ve liver	–ve serum –ve liver	+ve serum –ve liver	–ve serum +ve liver
cAb+eAg–eAb+	18	7	1	3	7
cAb+eAg+eAb–	7	5	–	1	1
cAb–eAg–/+eAb–/+	5	2	2	1	–
cAb+eAg–eAb–	2	1	–	–	1
cAb+eAg+eAb+	1	–	–	–	1
Total	33	15	3	5	10

cAb: hepatitis B core antibody; eAg: hepatitis B envelope (e) antigen; eAb: hepatitis B antibody against envelope (e) antigen.

six patients with positive HBsAb (four with detectable viral DNA in both serum and liver tissue, while two with detectable viral DNA only in liver tissue). Although four of the HBeAg-negative cases had higher HAI score (10–18) compared to none of the HBeAg-positive chronic hepatitis cases, the two groups showed no significant difference in either biochemical or histological parameters.

DISCUSSION

HBV infection varies widely from high (>8%, e.g., Africa, Asia, and the Western Pacific) to intermediate (2–7%, e.g., Southern and Eastern Europe) to low (<2%, e.g., Western Europe, North America, and Australia) prevalence areas. Egypt lies in the intermediate prevalence region[10]. HBV DNA is a good marker of the level of viremia and can be correlated with serum transaminase levels. The majority of patients with HBsAg in serum have HBV DNA. However, some patients are positive for HBV DNA in serum even in the absence of HBsAg, while some others have HBsAg but not HBV DNA in serum[5]. The presence of HBV genome is associated with ongoing necroinflammation[8]. In the presence of HBeAg, the diagnosis of replicating chronic HBV can be made whatever the viral load is. In contrast, the interpretation of HBV DNA quantification is difficult in HBeAg-negative/HBeAb-positive patients (pre-core mutant HBV) who generally have lower replication levels than HBeAg-positive patients. Active HBV replication is associated with a significant risk of progression to chronic hepatitis B complications[11]. The presence of HBV DNA is the single most important indication to start antiviral therapy[12]. This work aimed at evaluating the clinical significance of localizing HBV DNA in the liver in case of negative viremia. Patients with HBsAg and elevated ALT were selected among patients who presented at the outpatient clinic of the Tropical Medicine Department, suffering from a variety of chronic liver diseases.

Of 670 patients, the prevalence of HBsAg-positive patients among those with chronic liver disease was 6.6% (44/670). This figure represented both the carriage rate and the infection rate among chronic liver disease patients, whatever the causes were. The prevalence of HBV chronic hepatitis among chronic liver disease patients was 4.9% (33/670). The prevalences of HBV infection in Egypt have been found to be variable among different studies, ranging from 3.2% to 21%[13–19], depending on the number of patients. The higher figures studied the prevalence among cirrhotic patients, the lowest figures were found among school children in certain villages. Thirty-three patients eligible for the present study were considered to have chronic hepatitis B. NASH was not totally excluded. Fourteen liver biopsies showed prominent steatosis. The prevalence of steatosis in Egypt is expected to be quite high. It is known that 35% of the Egyptians are considered obese[20]. Moreover, high comorbid factors are recorded among Egyptians, such as diabetes, pollution, etc. It is worth mentioning that none of our 33 patients had a BMI >29 kg/m^2, and we had two diabetic patients.

In this study, the histopathological results showed that most of our patients had relatively mild disease, with an HAI score between 0 and 9, and a fibrosis score between 0 and 2. Conversely, the HBsAb-positive patients had a higher HAI score as compared with the HBsAb-negative patients. This might be explained by a stronger reaction to HBV infection in the former subgroup. The co-existence of both HBsAg and HBsAb has been previously described, and it is thought that HBsAb in such a situation is unable to neutralize the virus[21]. It has also been postulated that infection with multiple types of HBV is another explanation for this finding[22]. Most of our patients (78.8%) were HBeAg-negative chronic hepatitis B. It is known that the core promoter mutants are predominant in Egypt[23]. Compared to the HBeAg-negative chronic hepatitis B, the HBeAg-positive patients had a milder disease histopathologically, which was in agreement with previous reports[23–25]. We observed that the detection of HBV viremia was more in HBeAg-positive patients, which is expected, because of the formerly documented strong correlation between HBV DNA and HBeAg[26]. Characteristically, five patients (15.2%) were negative for both HBeAg and HBeAb, and five patients were negative for HBcAb. Three patients were negative for all seromarkers except HBsAg. These unusual patterns of serological profile can be explained either by mutations or viral DNA integration. Absence of cytotoxic T-lymphocyte (CTL) recognition of epitopes in HBeAg, HBsAg, and other viral proteins contributes to viral persistence. Failure of antigen presentation to CD4 helper T cells, failure of CD4

proliferating responses, immune modulation by secreted proteins, viral mutation, and integration of viral DNA into the cellular genome are all mechanisms involved in viral persistence[27]. Three patients had undetectable HBV DNA in either serum or liver tissue. This might be explained by an enhanced immunological response to HBV DNA, and extraordinarily low viral DNA copy number[28], or by the integration phenomenon affecting the primer orientation used in PCR technique[29]. Another explanation stands on HBV infection being a dynamic process, so that some of these patients might have been in the immune clearance phase[23]. Five patients had negative HBV DNA in the liver tissue, but positive in serum. This can be explained by the liver being a vast size organ, and hence, substantial viremia can result even if each cell produces only a few numbers of virions per day. This hypothesis has been proposed by Branch *et al*.[31] on HCV infection. In our opinion, it can also be applied on HBV infection if the viral genome is present in a very low level to be detected by biopsy. Ten patients (30.3%) did not fulfill any of the clinical criteria identified in literature. They could not classically fit in the Poynard's 2002 classification. They had one common serological profile, which is, detection of HBV DNA in the liver tissues but not in the sera (negative viremia). The disease burden as evidenced by hepatic biochemical profile and the spectrum of HAI and fibrosis was not different in this group of patients from those with the classic chronic HBV infection. This group has been named in this study as "hepatitis B *in situ*". Seven out of the eighteen patients, showing a classic unequivocal pattern of HBeAg-negative chronic hepatitis, had hepatitis B *in situ*. This implies that the reliance on mere testing of serum HBV DNA would have missed viral replication in such condition. On the other hand, HBeAg-positive hepatitis showed viremia in most cases.

In conclusion, a significant number of patients with chronic hepatitis B fail to show viremia, yet HBV DNA can be detected in their liver tissues. Hepatitis B *in situ* is a term used to describe the detection of total HBV DNA (Integrated plus CCC form) in liver tissue of patients with positive HBsAg and negative viremia. It is particularly frequent in the HBeAg-negative variety of chronic hepatitis B. The bearing of "hepatitis B *in situ*" on the liver is no less than the viremic form; serious sequelae are thus liable to take place. The recognition of this form of the disease may have its impact on the choice of therapy, since such patients should not be denied the chance to receive antiviral treatment.

REFERENCES

1 **Rapicetta M**, Ferrari C, Levrero M. Viral determinants and host immune responses in the pathogenesis of HBV infection. *J Med Virol* 2002; **67:** 454-457

2 **Fong TL**, Di Bisceglie AM, Gerber MA, Waggoner JG, Hoofnagle JH. Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. *Hepatology* 1993; **18:** 1313-1318

3 **Cacciola I**, Pollicino T, Squadrito G, Cerenzia G, Villari D, de Franchis R, Santantonio T, Brancatelli S, Colucci G, Raimondo G. Quantification of intrahepatic hepatitis B virus (HBV) DNA in patients with chronic HBV infection. *Hepatology* 2000; **31:** 507-512

4 **Pawlotsky JM**, Bastie A, Hezode C, Lonjon I, Darthuy F, Remire J, Dhumeaux D. Routine detection and quantification of hepatitis B virus DNA in clinical laboratories: performance of three commercial assays. *J Virol Methods* 2000; **85:** 11-21

5 **Kaneko S**, Miller RH, Di Bisceglie AM, Feinstone SM, Hoofnagle JH, Purcell RH. Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. *Gastroenterology* 1990; **99:** 799-804

6 **Lee CZ**, Huang GT, Yang PM, Sheu JC, Lai MY, Chen DS. Correlation of HBV DNA levels in serum and liver of chronic hepatitis B patients with cirrhosis. *Liver* 2002; **22:** 130-135

7 **Meng Q**, Wong C, Rangachari A, Tamatsukuri S, Sasaki M, Fiss E, Cheng L, Ramankutty T, Clarke D, Yawata H, Sakakura Y, Hirose T, Impraim C. Automated multiplex assay system for simultaneous detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type 1 RNA. *J Clin Microbiol* 2001; **39:** 2937-2945

8 **Chu CJ**, Lok AS. Clinical utility in quantifying serum HBV DNA levels using PCR assays. *J Hepatol* 2002; **36:** 549-551

9 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22:** 696-699

10 **De Castro L**, Niel C, Gomes SA. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. *BMC Microbiol* 2001; **1:** 10

11 **Poynard T**. Hepatitis B. In Hepatitis B and C: management and treatment. 1st ed. Mobipocket ebooks. 2002: 63 http://www.ebookmall.com/ebook/88983-ebook.htm

12 **Pawlotsky JM**. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002; **122:** 1554-1568

13 **Tsai NC**. Practical management of chronic hepatitis B infection. *Semin Liver Dis* 2004; **24 Suppl 1:** 71-76

14 **Sherif MM**, Abou-Aita BA, Abou-Elew MH, el-Kafrawi AO. Hepatitis B virus infection in upper and lower Egypt. *J Med Virol* 1985; **15:** 129-135

15 **Kamel MA**, Miller FD, el Masry AG, Zakaria S, Khattab M, Essmat G, Ghaffar YA. The epidemiology of Schistosoma mansoni, hepatitis B and hepatitis C infection in Egypt. *Ann Trop Med Parasitol* 1994; **88:** 501-509

16 **Attia MA**. Prevalence of hepatitis B and C in Egypt and Africa. *Antivir Ther* 1998; **3:** 1-9

17 **Andre F**. Hepatitis B epidemiology in Asia, the Middle East and Africa. *Vaccine* 2000; **18 Suppl 1:** S20-S22

18 **Angelico M**, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, De Santis A, Nagi A, Amin G, Capocaccia L, Callea F, Rapicetta M, Badr G, Rocchi G. Chronic liver disease in the Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. *J Hepatol* 1997; **26:** 236-243

19 **Zaki A**, Bassili A, Amin G, Aref T, Kandil M, Abou Basha LM. Morbidity of schistosomiasis mansoni in rural Alexandria, Egypt. *J Egypt Soc Parasitol* 2003; **33:** 695-710

20 **Reda AA**, Arafa MA, Youssry AA, Wandan EH, Ab de Ati M, Daebees H. Epidemiologic evaluation of the immunity against hepatitis B in Alexandria, Egypt. *Eur J Epidemiol* 2003; **18:** 1007-1011

21 http://www.tribuneindia.com/2000/20000917/world.htm#6

22 **Park LC**, Maruyama T, Kamiya N, Goto M, Kuma H, Hamasaki N. Mutation detection in the drug-resistant hepatitis B virus polymerase gene using nanostructured reverse micelles. *Anal Sci* 2004; **20:** 1609-1611

23 **Saudy N**, Sugauchi F, Tanaka Y, Suzuki S, Aal AA, Zaid MA, Agha S, Mizokami M. Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. *J Med Virol* 2003; **70:** 529-536

24 **Fattovich G**. Natural history and prognosis of hepatitis B. *Semin Liver Dis* 2003; **23:** 47-58

25 **Hadziyannis SJ**, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2001; **34:** 617-624
26 **Yalcin K**, Degertekin H, Yildiz F, Celik Y. Markers of disease activity in chronic hepatitis B virus infection. *Clin Invest Med* 2003; **26:** 27-34
27 **Zhang L**, Zhu J, Ma Y, Zhang H. Nucleic acid quantifying assay of hepatitis B virus. *Zhonghua Ganzangbing Zazhi* 2002; **10:** 49-51
28 **Carman WF**, Thomas HC, Zuckermann AJ. Molecular variants of hepatitis B. In: viral hepatitis. Zuckermann A, Thomas HC, eds. 2nd ed. *Churchill Livingstone*. 1998; **Section III:** 141-172
29 **Kuhns M**, McNamara A, Mason A, Campbell C, Perrillo R. Serum and liver hepatitis B virus DNA in chronic hepatitis B after sustained loss of surface antigen. *Gastroenterology* 1992; **103:** 1649-1656
30 **Balderas-Renteria I**, Munoz-Espinosa LE, Dector-Carrillo MA, Martinez-Martinez FJ, Barrera-Saldana HA. Detection of hepatitis B virus in seropositive and seronegative patients with chronic liver disease using DNA amplification by PCR. *Arch Med Res 2002*; **33:** 566-571
31 **Branch AD**, Seeff LB. Afterword: HCV in the decades ahead. *Semin Liver Dis* 2000; **20:** 233-237

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• *RAPID COMMUNICATION* •

Effect of transjugular intrahepatic portosystemic shunt on pulmonary gas exchange in patients with portal hypertension and hepatopulmonary syndrome

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Abstract

AIM: To assess the impact of transjugular intrahepatic portosystemic shunt (TIPS) on pulmonary gas exchange and to evaluate the use of TIPS for the treatment of hepatopulmonary syndrome (HPS).

METHODS: Seven patients, three of them with advanced HPS, in whom detailed pulmonary function tests were performed before and after TIPS placement at the University of Alabama Hospital and at the Hospital Clinic, Barcelona, were considered.

RESULTS: TIPS patency was confirmed by hemodynamic evaluation. No changes in arterial blood gases were observed in the overall subset of patients. Transient arterial oxygenation improvement was observed in only one HPS patient, early after TIPS, but this was not sustained 4 mo later.

CONCLUSION: TIPS neither improved nor worsened pulmonary gas exchange in patients with portal hypertension. This data does not support the use of TIPS as a specific treatment for HPS. However, it does reinforce the view that TIPS can be safely performed for the treatment of other complications of portal hypertension in patients with HPS.

Key words: Portal hypertension; Transjugular intrahepatic portosystemic shunt; Pulmonary gas exchange; Hepatopulmonary syndrome

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) has been used increasingly in patients with cirrhosis complicated by bleeding varices and refractory ascites[1]. Recently, TIPS has also been proposed as a potential treatment for pulmonary vasodilatation associated with cirrhosis, the so-called hepatopulmonary syndrome (HPS)[2-7]. However, both hemodynamic and metabolic effects of TIPS have been widely studied, little data exists on the effects of TIPS on pulmonary gas exchange in patients with portal hypertension.

HPS occurs when intrapulmonary vasodilatation causes abnormal pulmonary gas exchange in the setting of liver disease[8]. The abnormalities in pulmonary gas exchange result from microvascular dilatation and involve varying degrees of ventilation-perfusion imbalance, oxygen diffusion limitation (diffusion-perfusion defect), and increased intrapulmonary shunting[9]. Although the natural history of HPS is poorly understood, pulmonary gas exchange abnormalities are generally progressive, even in the setting of clinically stable hepatic dysfunction[10]. Currently, liver transplantation is the only therapeutic approach that can resolve HPS. However, perioperative mortality remains high (16-38% within 1 year) and appears to be greatest in those with more advanced HPS[11,12].

HPS has been described most commonly in patients

with cirrhosis of the liver, although it has also been reported in patients with severe acute hepatitis and in non-cirrhotic portal hypertension[13-15]. These findings suggest that portal hypertension plays an important role in the development of intrapulmonary vasodilatation and in the occurrence of HPS. TIPS, by correcting portal hypertension[1], could improve HPS. Indeed, TIPS may be an attractive alternative therapeutic option in selected patients with advanced HPS who are not transplant candidates or in whom liver function is well preserved, particularly if HPS is of sufficient severity to increase transplant mortality or if the waiting time for transplantation is expected to be prolonged. However, the small number of case reports published to date have had variable clinical features and results, and the majority TIPS placement was performed for an acute indication other than HPS[2–7]. Therefore, it is difficult to determine if TIPS specifically improves HPS. In addition, TIPS is known to further exacerbate the hyperdynamic circulation present in patients with cirrhosis and portal hypertension, a fact that might trigger or increase pulmonary vasodilatation and adversely effect pulmonary gas exchange[16-19].

Accordingly, in the present study, we aimed to assess the impact of TIPS on pulmonary gas exchange in patients with portal hypertension and to evaluate the use of TIPS for the treatment of HPS.

MATERIALS AND METHODS

Patients submitted to TIPS treatment at the University of Alabama Hospital and at the Hospital Clínic, Barcelona, were reviewed and the characteristics of patients in whom pulmonary function was evaluated in a period of less than 6 mo before TIPS placement and no more than 1 year after, were included. In three of these patients, TIPS was placed specifically for the treatment of HPS. The diagnosis of HPS was made on the basis of established criteria: the presence of an increase of alveolar-arterial oxygen difference ($AaPO_2$ >15 mmHg); with or without hypoxemia; and evidence of pulmonary vascular dilatation by means of contrast-enhanced echocardiography and/or nuclear isotope lung perfusion scanning, in the context of liver disease[8-10]. The presence of mild intrinsic cardiopulmonary disease was not considered as an exclusion criterion for the diagnosis of HPS[20,21].

Before TIPS placement, all patients underwent measurements of forced spirometry, plethysmography, and single-breath carbon monoxide diffusing capacity (DL_{CO}) after correcting appropriately for hemoglobin concentration. Arterial blood gases were collected while breathing room air in the upright position. The $AaPO_2$ was calculated according to the alveolar gas standard equation using 0.8 as the standard respiratory exchange ratio. Arterial blood gases were repeated after a 20-min period of 100% oxygen breathing in an upright position in patients with HPS.

The TIPS procedure was similar in both institutions and has been previously described[1]. After TIPS, patients underwent close follow-up with US-Doppler and hemodynamic evaluation of TIPS patency. Clinical and biochemical evolution, pulmonary gas exchange analysis, and systemic and pulmonary hemodynamics after TIPS placement were recorded for at least 1 mo following TIPS.

RESULTS

Pulmonary gas exchange was assessed before and after TIPS placement in seven patients. Three patients had HPS and the specific indication for TIPS in each of these patients was treatment of HPS. The remaining four patients had no HPS, but one of them had mild hypoxemia due to a moderate obstructive ventilatory pattern, and the indication for TIPS in these patients was variceal bleeding in three and refractory ascites in one. Demographic and clinical data are summarized in Table 1. TIPS substantially reduced the portal pressure gradient (PPG) (from 15±2 to 5±2 mmHg). In each case, the PPG immediately after TIPS was below 12 mmHg. In addition, during follow-up, TIPS patency, defined as a PPG <12 mmHg, was confirmed by hemodynamic evaluation. The clinical course of pulmonary function in patients without HPS was stable during all the study period. Pulmonary function tests and gas exchange data before and at least 3 mo after TIPS placement in patients with normal gas exchange prior to placement are shown in Table 2. As shown, TIPS neither influenced arterial blood gases nor pulmonary function tests in these patients.

Individual data of patients with HPS are shown in Table 3. All patients had advanced HPS as evidenced by PaO_2<60 mmHg with low $PaCO_2$ values; only Patient #2 responded favorably to 100% oxygen administration. Patients #1 and #2 had a mild restrictive ventilatory pattern possibly related to a coexistent diffuse interstitial

Table 1 Demographic and clinical characteristics (mean±SD, *n*)

	HPS (*n* = 3)	Non-HPS (*n* = 4)
Sex (M/F)	2/1	4/–
Age (yr)	50±22	48±10
Smoking (*n*)	1	4
Current	-	2
Past	1	2
Etiology of chronic liver disease (*n*)		
Hepatitis C	1	1
Alcohol abuse	1	3
Idiopathic portal hypertension	1	
Child–Pugh score A/B/C (*n*)	1/2/–	1/2/1
Presence of esophageal varices	3	4
Gastrointestinal bleeding	2	3
Ascites	1	1
Hepatic encephalopathy	–	–
Cutaneous spider nevi	3	3
Concomitant respiratory symptoms		
Digital clubbing	3	–
Dyspnea	3	1
Cyanosis	3	–
Indication of TIPS		
Variceal bleeding	–	3
Refractory ascites	–	1
Hepatopulmonary syndrome	3	–

TIPS: transjugular intrahepatic portosystemic shunt ; Query: please check the change made.

lung disease (Patient #1) and unilateral pleural fibrosis (Patient #2), respectively. Pulmonary angiography excluded the presence of arterio-venous communications. Ventilation-perfusion (V_A/Q) studies in these two patients, using the multiple inert gas elimination technique[22], was consistent with HPS, showing increased intrapulmonary shunt (43% and 11% of cardiac output) with a mild to moderate degree of V_A/Q mismatch, characterized by areas with low V_A/Q units (6.4% and 0.1% of cardiac output). All three patients exhibited a very severely reduced diffusing capacity (<25% predicted). Transient improvement in arterial oxygenation was observed in only one patient early after TIPS placement (Patient #1) but this was not maintained 4 mo later despite TIPS patency. In the other two patients, no improvement in gas exchange was observed early after TIPS and progressive deterioration in arterial oxygen saturation was evident at 4 mo in Patient #2 and at 4 wk in Patient #3 after TIPS. Patient #1 was excluded for liver transplantation because of the potential coexistence of diffuse interstitial pulmonary disease and severe hypoxemia with a poor response to 100% oxygen breathing. He died of progressive deterioration of pulmonary, hepatic, and renal function. Patient #2 was transplanted, 8 mo after TIPS placement. At the time of liver transplantation, he had persistent severe oxygenation abnormalities. Ten months after orthotopic liver transplant (OLT), pulmonary gas exchange was markedly improved with normal arterial oxygenation and contrast-enhanced echocardiography confirmed resolution of intrapulmonary vasodilatation. Patient #3 was excluded for liver transplantation due to severe hypoxemia. Subsequently, she developed progressive hypoxemia 4 wk after TIPS and died of multisystem organ failure one week later.

Table 2 Pulmonary gas exchange before and after TIPS placement in patients without HPS (mean±SD)

	Pre-TIPS	Post-TIPS
PaO_2 (mmHg)	95±16	95±27
$PaCO_2$ (mmHg)	29±6	31±7
$AaPO_2$ (mmHg)	18±12	18±20
FEV1 (% pred)	72±9	74±5
FVC (% pred)	76±6	80±2
FEV1/FVC	75±10	76±8
DLCO (mL/(min×mmHg))	22±4	20±5
DLCO (% pred)	67±13	63±9

PaO_2: partial pressure of arterial oxygen; $PaCO_2$: partial pressure of arterial carbon dioxide; $AaPO_2$: alveolar to arterial oxygen partial pressure gradient; FEV1: forced expiratory volume at 1 s; FVC: forced vital capacity; FEV1/FVC: ratio of forced expiratory volume at 1 s to forced vital capacity; DL_{CO}: single-breath carbon monoxide diffusing capacity.

DISCUSSION

Portal hypertension is characteristically associated with a hyperkinetic circulation reflected by an increased cardiac output and splanchnic and peripheral vasodilatation[23]. A number of mechanisms contribute to these hemodynamic changes including increased bioavailability of vasodilators, such as nitric oxide[23]. HPS is most commonly observed in the setting of portal hypertension and likely appears to result from a disequilibrium between vasodilator and vasoconstrictor factors in the pulmonary microcirculation. Increased production of nitric oxide has also been proposed as one major determinant of pulmonary vascular dilatation in human HPS[24]. However, whether the same mechanisms that drive the splanchnic and peripheral vasodilatation are operative in the pulmonary microvasculature in HPS is still unknown.

Since HPS is associated with the presence of portal hypertension, portal pressure reduction through TIPS placement may be a useful therapeutic alternative. Till date, six case reports have evaluated the effects of TIPS on gas exchange in HPS[2–7]. Surprisingly, five demonstrated some

Table 3 Pulmonary gas exchange and hemodynamics before and after TIPS placement in patients with HPS

	Pre-TIPS			Post-TIPS <1 mo			Post-TIPS >4 mo		
Patients	# 1	# 2	# 3	# 1	# 2	# 3	# 1	# 2	# 3
FEV1 (% pred)	69	77	100						
FVC (% pred)	66	77	113						
FEV1/FVC	77	80	82						
TLC (% pred)	68	70	83						
DLCO (% pred)	21.3	24	8						
PaO_2 (mmHg)	32	59	28	58	61	31	33	34	–
$PaCO_2$ (mmHg)	27	28	35	31	27	38	26	34	–
$AaPO_2$ (mmHg)	88	54	78	53	69	71	87	73	–
PaO_2 100% O_2 breathing (mmHg)	64	606	86	–	–	–	–	–	–
PAP (mmHg)	14.5	9	18	–	8	22	20	8	–
PVR (dyn.s/cm5)	49	31	111	–	21	82	38	18	–
SVR (dyn.s/cm5)	773	415	1 174	–	400	922	549	560	–
QT (L/min)	9.0	10.4	6.47	–	11.2	7.8	10.5	11.3	–
PP (mmHg)	22	16	19	14	11	22	18.5	11	–
IVCP (mmHg)	5	4	3	7	2.5	19	8	2.5	–
PPG (mmHg)	17	12	16	7	8.5	3	10.5	8.5	–

FEV_1: forced expiratory volume at 1 s; FVC: forced vital capacity; TLC: total lung capacity; DL_{CO}: single-breath carbon monoxide diffusing capacity; PaO_2: partial pressure of arterial oxygen; $PaCO_2$: partial pressure of arterial carbon dioxide; $AaPO_2$: alveolar to arterial oxygen partial pressure gradient; PAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; SVR: systemic vascular resistance; Q_T: cardiac output; PP: portal pressure; IVCP: inferior vena cava pressure; PPG: portocaval pressure gradient.

degree of improvement in oxygenation. However, short-term (<1 mo) duration of follow-up in two patients[2,3] and the presence of coexistent hepatic hydrothorax in the third one[4] limit the evaluation of the utility of TIPS for hypoxemia in these cases. In two other reports[5,6], with a longer follow-up (>7 mo), arterial desaturation improved after TIPS and in one of these, a 11 year-old female with biliary atresia, gas exchange improvement was associated with a remarkable reduction in intrapulmonary shunt[6]. In the sixth report[7], TIPS failed to improve arterial hypoxemia in one patient. Altogether these reports suggest that portal pressure reduction *per se* does not consistently improve pulmonary gas exchange in HPS.

Portal pressure reduction after TIPS is also associated with an exacerbation of the hyperdynamic circulatory state, an effect that persists for at least 3 mo following TIPS placement[16-19]. During this time, pulmonary vascular resistance may also decline[17,18,25]. The mechanisms by which TIPS accentuate the hyperdynamic circulation are not well understood but may involve porto-systemic shunting of vasoactive substances through the TIPS which alter NO production[17,26-28]. The net result could enhance pulmonary vasodilation, thereby resulting in the development and/or worsening of HPS. In two of our patients with HPS who underwent TIPS, we confirmed obviously decreased systemic and pulmonary vascular resistance early after placement. Despite these hemodynamic changes, arterial oxygenation did not significantly deteriorate. In addition, in our four other patients without HPS who underwent successful TIPS, no changes in gas exchange were shown following TIPS placement. These findings complement and extend the findings of prior reports and support that the exacerbation of the hyperdynamic state following TIPS placement is not associated with arterial oxygenation worsening.

The portal pressure gradient (PPG) was reduced below 10 mmHg in each of our patients who underwent TIPS for HPS. Despite this, none of them had a sustained improvement in arterial blood gases. One patient did have a transient improvement in arterial oxygenation early after TIPS placement. Conceivably, one explanation for this improvement could be improved ventilation-perfusion matching induced by a rise in cardiac output resulting in increased blood flow, selective redistribution to the upper lobes of the lung, where vasodilatation is typically less severe in HPS. A similar mechanism may have been operative in the study by Selim *et al.*[4] where oxygenation improved after TIPS in association with a rise in cardiac output despite significant persistent intrapulmonary shunting. Alternatively, the increased cardiac output may improve PaO_2 through increased mixed venous PO_2, other things being equal. Our study and patients were different from prior case reports in that our group represented patients who specifically underwent TIPS for HPS. In four of the six prior reports, another acute indication for TIPS was present, including variceal bleeding and hepatic hydrothorax. The presence and resolution of other major complications of portal hypertension could have influenced favorably arterial blood gases in these cases. In addition, our patients had more severe gas exchange abnormalities than those in prior studies and it is unknown whether or not the severity of pulmonary vasodilatation may modulate the response to TIPS. Finally, our findings are clearly different from the report by Paramesh *et al.*[7], where TIPS placement resulted in a sustained and dramatic improvement in both pulmonary vasodilatation and arterial oxygenation. However, this case involved a child with biliary atresia and whether pathophysiologic mechanisms and responses to portal decompression in this setting are applicable to adults with other causes of liver disease is unknown[7].

In summary, our findings suggest that treatment with TIPS in patients with portal hypertension has no deleterious effects on pulmonary gas exchange, despite coexisting exacerbation of the hyperdynamic circulatory state. However, portal decompression with TIPS as a specific therapy for HPS is ineffective. This data supports the use of TIPS in patients with HPS for the treatment of other accepted indications for TIPS, but not as a specific therapy to improve arterial oxygenation defects in HPS.

REFERENCES

1 **Casado M**, Bosch J, Garcia-Pagan JC, Bru C, Banares R, Bandi JC, Escorsell A, Rodriguez-Laiz JM, Gilabert R, Feu F, Schorlemer C, Echenagusia A, Rodes J. Clinical events after transjugular intrahepatic portosystemic shunt: correlation with hemodynamic findings. *Gastroenterology* 1998; **114**: 1296-1303

2 **Riegler JL**, Lang KA, Johnson SP, Westerman JH. Transjugular intrahepatic portosystemic shunt improves oxygenation in hepatopulmonary syndrome. *Gastroenterology* 1995; **109**: 978-983

3 **Lasch HM**, Fried MW, Zacks SL, Odell P, Johnson MW, Gerber DA, Sandhu FS, Fair JH, Shrestha R. Use of transjugular intrahepatic portosystemic shunt as a bridge to liver transplantation in a patient with severe hepatopulmonary syndrome. *Liver Transpl* 2001; **7**: 147-149

4 **Selim KM**, Akriviadis EA, Zuckerman E, Chen D, Reynolds TB. Transjugular intrahepatic portosystemic shunt: a successful treatment for hepatopulmonary syndrome. *Am J Gastroenterol* 1998; **93**: 455-458

5 **Allgaier HP**, Haag K, Ochs A, Hausentein KH, Jeserich M, Krause T, Heilmann C, Gerok W, Rossle M. Hepato-pulmonary syndrome: succesful treatment by transjugular intrahepatic portosystemic shunt (TIPS) (letter). *J Hepatoly* 1995; **23**: 102-105

6 **Corley DA**, Scharschmidt B, Bass N, Somberg K, Gold W. Lack of efficacy of TIPS for hepatopulmonary syndrome *Gastroenterology* 1997; **113**: 728-730

7 **Paramesh AS**, Husain SZ, Shneider B, Guller J, Tokat I, Gondolesi GE, Moyer S, Emre S. Improvement of hepatopulmonary syndrome after transjugular portosystemic shunting : case report and review of literature. *Pediatric transplant* 2003;**7**: 157-162

8 **Rodriguez-Roisin R**, Krowka MJ, Herve P, Fallon MB. Pulmonary-Hepatic vascular Disorders (PHD). *Eur Respir J* 2004; **24**: 861-880

9 **Fallon MB**, Abrams GA. Pulmonary dysfunction in chronic liver disease. *Hepatology* 2000; **32**: 859-865

10 **Martínez GP**, Barbera JA, Visa J, Rimola A, Pare JC, Roca J, Navasa M, Rodes J, Rodriguez-Roisin R. Hepatopulmonary syndrome in candidates for liver transplantation. *J Hepatol* 2001; **34**: 651-657

11 **Arguedas MR**, Abrams GA, Krowka MJ, Fallon MB. Prospective evaluation of outcomes and predictors of mortality

in patients with hepatopulmonary syndrome undergoing liver transplantation. *Hepatology* 2003; **37**: 192-197

12 **Krowka MJ**, Porayko MK, Plevak DJ, Pappas C, Steers JL, Krom RAF, Wiesner RH. Hepatopulmonary syndrome with progressive hypoxemia as an indication for liver transplantation: case reports and literature review. *Mayo Clin Proc* 1997; **72**: 44-53

13 **Babbs C**, Warners TW, Haboubi NY. Non cirrhotic portal hypertension with hypoxemia. *Gut* 1988; **29**:129-131.

14 **De BK**, Sen S, Biswas PK, Mandal SK, Das D, Das U, Guru S, Bandyopadhyay K. Occurrence of hepatopulmonary syndrome in Budd-Chiari syndrome and the role of venous decompression. *Gastroenterology* 2002; **122**: 897-903

15 **Abrams GA**, Fallon MB. The hepatopulmonary syndrome. *Clin Liver Dis* 1997; **1**: 185-200

16 **Azoulay D**, Castaing D, Dennison A, Martino W, Eyraud D, Bismuth H. Transjugular intrahepatic portosystemic shunt worsens the hyperdynamic circulatory state of the cirrhotic patient: preliminary report of a prospective study. *Hepatology* 1994; **19**: 129-132

17 **Rodriguez-Laiz JM**, Banares R, Echenagusia A, Casado M, Camunez F, Perez-Roldan F, de Diego A, Cos E, Clemente G. Effects of transjugular intrahepatic portasystemic shunt (TIPS) on splanchnic and systemic hemodynamics, and hepatic function in patients with portal hypertension. Preliminary results. *Dig Dis Sci* 1995; **40**: 2121-2127

18 **Van Der Linden P**, Le Moine O, Ghysels M, Ortinez M, Deviere J. Pulmonary hypertension after transjugular intrahepatic portosystemic shunt: effects on right ventricular function. *Hepatology* 1996; **23**: 982-987

19 **Francois E**, Garcia-Pagan JC, Bru C, Feu F, Gilabert R, Escorsell A, Bosch J, Rodes J. Effects of percutaneous intrahepatic portosystemic shunt on splanchnic and systemic hemodynamics in patients with portal hypertension. *Gastroenterol Hepatol* 1997; **20**: 11-14

20 **Martinez-Pallí G**, Barbera JA, Navasa M, Roca J, Visa J, Rodriguez-Roisin R. Hepatopulmonary syndrome associated with cardiorespiratory disease. *J Hepatol* 1999; **30**: 882-889

21 **Krowka MJ**. Caveats concerning hepatopulmonary syndrome. *J Hepatol* 2001; **34**: 756-758

22 **Roca J**, Wagner PD. Contribution of multiple inert gas elimination technique to pulmonary medicine. 1. Principles and information content of the multiple inert gas elimination technique. *Thorax* 1994; **49**: 815-824

23 **Bosch J**, García-Pagán JC. Complications of cirrhosis. I. Portal hypertension. *J Hepatol* 2000; **32S**: 141-156

24 **Cremona G**, Higenbottam TW, Mayoral V, Alexander G, Demoncheaux E, Borland C, Roe P, Jones GJ. Elevated exhaled nitric oxide in patients with hepatopulmonary syndrome. *Eur Respir J* 1995; **8**: 1883-1885

25 **Lotterer E**, Wengert A, Fleig WE. Transjugular intrahepatic portosystemic shunt: short term and lshort-term effects on hepatic and systemic hemodynamics in patients with cirrhosis. *Hepatology* 1999; **29**: 632-639

26 **Bernadich C**, Bandi JC, Melin P, Bosch J. Effects of F-180, a new selective vasoconstrictor peptide, compared with terlipressin and vasopressin on systemic and splanchnic hemodynamics in a rat model of portal hypertension. *Hepatology* 1997; **26**: 262-267

27 **Jalan R**, Damink SO, Deutz N, Redhead D, Lee A. Increased cerebral and peripheral vasodilation, and whole body nitric oxide (NO) production after insertion of a transjugular intrahepatic portosystemic stent-shunt (TIPSS) in patient with cirrhosis. *J Hepatol* 2002; **36**: A178:55

28 Colombato LA, Spahr L, Martinet JP, Dufresne MP, Lafortune M, Fenyves D, Pomier-Layrargues G. Haemodynamic adaptation two months after transjugular intrahepatic portosystemic shunt (TIPS) in cirrhotic patients. *Gut* 1996; **39**: 600-604

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• *RAPID COMMUNICATION* •

Incidence and localization of lymphoid follicles in early colorectal neoplasms

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Abstract

AIM: To investigate the incidence and localizations of lymphoid follicles (LFs) in early colorectal neoplasms in human beings.

METHODS: From July 1992 to September 1999, a total of 1 324 early colorectal neoplasms were removed endoscopically or surgically at our hospital; 1 031 (77.9%) were available for analysis in this study. Localization of LFs was defined histologically: as submucosal LFs, if located under the muscularis mucosa; and as intramucosal LFs, if located across or over the muscularis mucosa.

RESULTS: Histologically, the materials included 903 intramucosal neoplasms and 128 submucosal cancers. Overall incidence of LFs was 27.2% (280/1 031). The incidence of LFs was significantly higher in females (33.6% *vs* 24.9%, $P = 0.0064$), the right-sided colon (32.2% *vs* 25.6%, $P = 0.0403$) and in flat or depressed type lesions (34.6% *vs* 25.2%, $P<0.0001$) as compared to males, left-sided colon and protruding type lesions, respectively. The incidences of intramucosal neoplasms and submucosal cancers were 24.3% and 43.8%, respectively ($P<0.0001$). Localizations of LFs (intramucosal LF/submucosal LF) in depressed, flat, and protruding types were 1/24, 14/36, and 131/74, respectively.

CONCLUSION: The incidence of LFs in early human colorectal neoplasms significantly differs by gender, location, macroscopic type, and histology. Moreover, localization significantly differs by macroscopic type.

Key words: Lymphoid follicle; Early colorectal neoplasm; Carcinogenesis

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INTRODUCTION

Several reports have investigated the association between lymphoid aggregates and colonic tumors in rodents[1-4]. The results indicate that colonic crypts overlying lymphoid follicles (LFs) show a significantly higher proliferative activity. These results also showed that the risk of carcinoma increased in the colonic mucosa on LFs compared to mucosa without LFs. Consequently, it was considered that factors from LFs promote carcinogenesis in the epithelium in rodents. Rubio *et al*.[5] also investigated the incidence of LFs in early stage adenomas and adenocarcinomas in Swedish and Japanese patients, showing that 38% of 174 consecutive non-polypoid adenomas and flat incipient adenocarcinomas had subjacent LFs. Moreover, they observed a higher incidence of LFs in non-polypoid neoplasms as compared to polypoid neoplasms in rats[6].

Recently, advances in endoscopic techniques and equipment have enabled much smaller flat and depressed colorectal neoplasms to be detected[7-11]. We have also microscopically encountered LFs and found that their incidence and localization tended to be different according to their macroscopic features in early colorectal neoplasms. In this study, we, therefore, aimed to investigate the incidence and localization of LFs revealed in early human colorectal neoplasms.

MATERIALS AND METHODS

Subjects

From July 1992 to September 1999, a total of 1 324 early colorectal neoplasms, including adenoma with high-grade

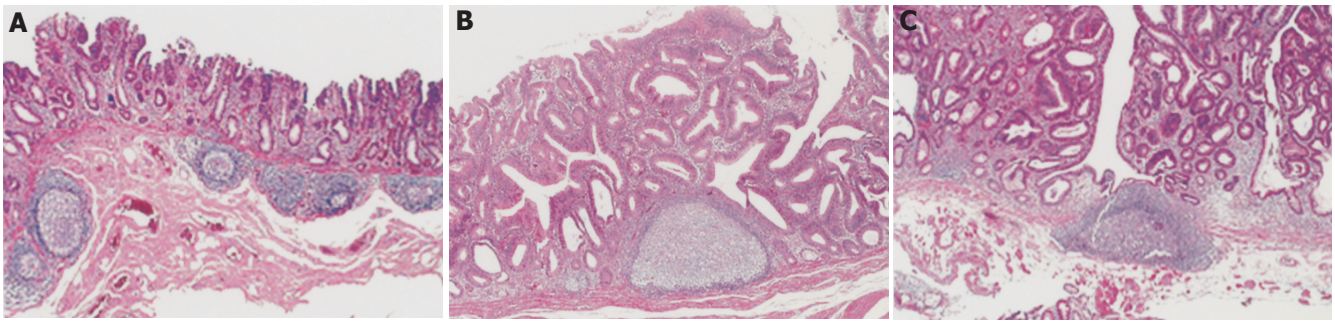

Figure 1 Localizations of LFs in early colorectal neoplasms. **A:** Submucosal LF in intramucosal carcinoma; **B:** intramucosal LFs in adenoma with moderate atypia; **C**: intermucosal LFs in adenoma with moderate atypia.

atypia, intramucosal cancer and submucosal invasive cancer, were removed endoscopically or surgically in our hospital. Of the 1 324 lesions, 293 (22.1%) were excluded from this study because the histology had been damaged by endoscopic procedures and they were judged inappropriate for analysis. The lesions of patients with familial polyposis, hereditary non-polyposis colorectal cancer, and inflammatory bowel disease were also excluded.

Location

The locations of the lesions were categorized into two groups at the splenic flexure: right-sided colon (including the cecum, ascending colon, and transverse colon); and left-sided colon (including descending colon, sigmoid colon, and rectum).

Macroscopic type

Macroscopically, colorectal lesions were classified into three groups according to the criteria of the Japanese Research Society for Cancer of the Colon and Rectum (JRSCCR): depressed (D) type; flat (F) type; and protruding (P) type.

Evaluation

Histological examinations of the available lesions were performed with hematoxylin-and-eosin (HE) staining. The pathological definitions of the lesions were as established by the JRSCCR. We defined a lymphoglandular complex with an enlarged germinal center as a LF. The localizations of LFs were classified into two groups: LFs located beyond the muscularis mucosa were defined as "submucosal LFs" (Figure 1A); and LFs located across or above the muscularis mucosa were defined as "intramucosal LFs" (Figures 1B and C). The presence and localization of LFs were investigated histologically.

Statistical analysis

Using χ^2 test and Fisher's exact probability test, we compared the clinicopathological characteristics and the incidence and localization of LFs in early human colorectal neoplasms. Correlations between lesion size and the presence or absence of LFs in each macroscopic type were evaluated by using Spearman's rank correlation. A value of $P<0.05$ was considered statistically significant.

RESULTS

The details of the clinicopathological characteristics of the subjects are shown in Table 1. A total of 1 031 lesions, including 903 intramucosal neoplasms and 128 submucosal cancers, were investigated histologically. Endoscopically, 60 were depressed type, 157 flat type, and 814 protruding type.

Incidence of LFs

LFs were present in 280 of the 1 031 lesions, an overall incidence of 27.2%. The incidence of LFs was significantly higher in females (89/265, 33.6%) compared to males (191/766, 24.9%) ($P = 0.0064$). The incidence of LFs was markedly higher in the right-sided colon (32.2%, 79/166) than in the left-sided colon (25.6%, 201/786, $P = 0.0403$). In addition, the incidence of LFs in submucosal cancers (43.8%, 56/128) was significantly higher than in intramucosal neoplasms (24.3%, 224/903, $P<0.0001$). The incidences of LFs in submucosal cancers with or without lymph node metastasis were 8.9% (5/56) and 8.3% (6/72), respectively, showing no significant correlation between the presence of LFs and lymph node metastasis in submucosal cancers. LFs were present in 41.7% (25/60) of D type, 31.8% (50/157) of F type, and 25.2% (205/814) of P type lesions. The incidence of LFs in F and D type (34.6%, 75/217) was significantly higher than in P type (25.2%, 205/814) ($P<0.0001$, Table 1).

Localization of LFs

Of the 280 lesions with LFs, 146 were classified as intramucosal and 134 as submucosal. In addition, the localizations of LFs (intramucosal/submucosal) were also different according to macroscopic types: 1/24 in D, 14/36 in F, and 131/74 in P types (Table 2). The D and F type lesions harboring LFs also showed a significantly higher incidence of submucosal LFs as compared to the P type lesions ($P<0.0001$).

Correlation between the presence of LFs and tumor size

The mean tumor sizes of the early colorectal neoplasms with or without LFs in each macroscopic type were 12.3±6.3 and 8.0±5.7 mm in D type, 14.8±10.6 and 8.6±5.1 mm in F type, and 17.9±12.0 and 12.8±8.3 mm in P type, respectively,

Table 1 Correlations between clinicopathological characteristics and the presence or absence of lymphoid follicles

		LF[1] present (%)	LF absent (%)	*P*
Total cases	1 031	280 (27.2)	51 (72.8)	
Gender				
Male	766	191 (24.9)	575 (75.1)	
Female	265	89 (33.6)	176 (66.4)	0.0064
Location				
Right - sided colon	245	79 (32.2)	166 (67.8)	
Left - sided colon	786	201 (25.6)	585 (74.4)	0.0403
Size (mean)(mm)				
Depressed + Flat	10.3 ± 3.4	13.9 ± 6.3	8.5 ± 5.3	
Protruding	14.1 ± 9.6	17.9 ± 12.0	12.8 ± 8.3	0.0095
Macroscopic type				
Depressed + Flat	217	75 (34.6)	142 (65.4)	
Protruding	814	205 (25.2)	609 (74.8)	0.0133
Histology				
Intramucosal neoplasias	903	224 (24.8)	679 (75.2)	
Submucosal cancer	128	56 (43.8)	72 (56.2)	<0.0001

[1]Lymphoid follicle.

Table 2 Localizations of the lymphoid follicles in each macroscopic types

	Intramucosal LF[1] (%)	Submucosal LF (%)	Total
Depressed	1 (4)	24 (96)	25
Flat	14 (28)	36 (72)	50
Protruding	131 (64)	74 (36)	205

[1]Lymphoid follicle

which showed that the mean tumor size in each macroscopic type with LFs was obviously larger compared to the macroscopic type without LFs ($P<0.0001$), and the mean tumor sizes of D and F types with LFs were markedly smaller than that of P type ($P = 0.0095$).

DISCUSSION

We believe that this study is the first report to describe the incidence and localization of LFs subjacent to early colorectal neoplasms in human beings. Rubio *et al*.[5,6] concluded that there appeared to be a genuine association between LFs and non-polypoid adenomas, not only in rodents but also in human beings; however, they did not mention depressed type lesions. Recently, with advances in endoscopic instruments and techniques, concerns have arisen about depressed and flat type early colorectal neoplasms that have increasingly been found worldwide[7-11]. To clarify the characteristics of these depressed and flat type lesions, we investigated the incidence of LFs not only of protruding, but also of depressed and flat type lesions.

Our present results showed that the incidence of LFs differed according to the macroscopic features of early colorectal neoplasms, being especially high in depressed type lesions (41.7%). We also observed that the localizations of LFs seen in early colorectal neoplasms were different according to macroscopic type: 96% (24/25) of depressed and 72% (36/50) of flat lesions-associated LFs were located under the muscularis mucosa (submucosal LF), while 36% (74/205) of protruding lesions harboring LFs were located over or across the muscularis mucosa (intramucosal LF). To the best of our knowledge, no other reports on the localization of LFs in early colorectal neoplasms have been published as yet.

Depressed lesions are known to demonstrate invasive tendencies despite their smaller size[7-9]. Furthermore, flat lesions are reported to be 10 times more likely to contain high-grade dysplasia than protruding ones[12]. In this study, the depressed or flat lesions were found to have a significantly higher incidence of LFs and submucosal LFs compared to the protruding lesions. Based on reports of the aggressive growth patterns of depressed and flat lesions, we speculate that the differences in incidence and location of LFs between flat or depressed and protruding lesions might reflect the host physical defense against neoplasms in human beings; that the submucosal LFs might prevent depressed or flat lesions from downward growth; and that LFs might act against the upward growth of protruding lesions. However, the results from experimental colon cancer studies indicated that aggregates of LFs might promote the development of adenocarcinomas[1-4]. On the other hand, studies in experimental animals have also shown that the intestinal lymphoid system plays an important role in the immunologic defense mechanisms; that is, antigenic stimuli result in germinal center formation, subsequently antibody production, and finally enlargement of the follicles[13]. *In vivo*, the presence of tumor-infiltrating lymphocytes is associated with improved prognosis in colorectal cancers, as does the presence of high level DNA microsatellite instability[14-15]. Carcinomas with lymphoid stroma in various organs are also reported to be associated with better prognosis[16]. Thus, these results suggest LFs in early colorectal neoplasms play an important role in defense rather than promotion.

In our study, the incidence of LFs was markedly higher in submucosal invasive cancers than in intramucosal lesions, and it was also more frequently observed in depressed or flat lesions and in larger sizes of each macroscopic type. We observed significant correlations

among the incidence of LFs and the invasive tendencies and size of early colorectal lesions. Mortality rates for colorectal cancer in Japan tend to be lower in females than males. Our results also showed a significant higher incidence of LFs in females compared to males (33.6% *vs* 24.9%). Taking these results together, we suggest that LFs in early colorectal neoplasms are signs of a possible early physical defense event against neoplastic cells.

Submucosal cancers are reported to show lymph node metastasis in 3.6–16.2%[17-20]. However, in this study, we could not find a significant difference between the presence of LFs and lymph node metastasis in submucosal cancers. This might be due to the small numbers of submucosal cancers (8.6%, 11/128) harboring lymph node metastasis. Another explanation is that LFs may have defense only against the process of tumor invasion but not that of metastasis.

In conclusion, significant differences exist in the incidence and localization of LFs in early colorectal neoplasms in human beings. We suggest that LFs in early colorectal neoplasms might be considered as a sign of the host physical defense against neoplastic cells. Further studies, including experimental and clinical analyses, will be necessary to confirm this phenomenon.

REFERENCES

1 **Nauss KM**, Locniskar M, Pavlina T, Newberne PM. Morphology and distribution of 1,2-dimethylhydrazine dihydrochloride-induced colon tumors and their relationship to gut-associated lymphoid tissue in the rat. *J Natl Cancer Inst* 1984; **73**: 915-924

2 **Hardman WE**, Cameron IL. Colonoic crypts located over lymphoid nodules of 1,2-dimethylhydrazine-treated rats are hyperplastic and at high risk of forming adenocarcinoma. *Carcinogenesis* 1994; **15**: 2353-2361

3 **Ward JM**. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab Invest* 1974; **30**: 505-513

4 **Martin MS**, Hammann A, Matin F. Gut-associated lymphoid tissue and 1,2-dimethylhydrazine intestinal tumors in the rat: a histological immunoenzymatic study. *Int J Cancer* 1986; **38**: 75-80

5 **Rubio CA**, Kumagai J, Kanamori T, Nakamura K. Apoptosis in flat neoplasias of the colorectal mucosa. *In Vivo* 1995; **9:** 173-176

6 **Rubio CA**, Shetye J, Jaramillo E. Non-polypoid adenomas of the colon are associated with subjacent lymphoid nodules. An experimental study in rats. *Scand J Gastroenterol* 1999; **34**: 504-508

7 **Kudo S**, Tamura S, Hirota S, Sano Y, Yamano H, Serizawa M, Fukuoka T, Mitsuoka H, Nakajima T, Kusaka H. The problem of de novo colorectal carcinoma. *Eur J Cancer* 1995; **31A:** 1118-1120

8 **Kudo S**, Tamura S, Nakajima T, Hirota S, Asano M, Ito O, Kusaka H. Depressed type of colorectal cancer. *Endoscopy* 1995; **27**: 54-57

9 **Fujii T**, Rembacken BJ, Dixon MF, Yoshida S, Axon AT. Flat adenomas in the United Kingdom: are treatable cancers being missed? *Endoscopy* 1998; **30**: 437-443

10 **Hart AR**, Kudo S, Mackay EH, Mayberry JF, Atkin WS. Flat adenomas exist in asymptomatic people: important implications for colorectal cancer screening programmes. *Gut* 1998; **43:** 229-231

11 **Rembacken BJ**, Fujii T, Cairns A, Dixon MF, Yoshida S, Chalmers DM, Axon AT. Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK. *Lancet* 2000; **355:** 1211-1214

12 **Wolber RA**, Owen DA. Flat adenoma of the colon. *Hum Pathol* 1991; **34**: 981-986

13 **O'Leary AD**, Sweeney EC. Lymphoglandular complexes of the colon: structure and distribution. *Histopathology* 1986; **10:** 267-283

14 **Ropponen KM**, Eskelinen MJ, Lipponen PK, Alhava E, Kosma VM. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *J Pathol* 1997; **182:** 318-324

15 **Michael-Robinson JM**, Biemer-Huttman A-E, Purdie DM, Walsh MD, Simms LA, Biden KG, Young JP, Leggett BA, Jass JR, Radford-Smith GL. Tumor infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. *Gut* 2001; **48:** 360-366

16 **Minamoto T**, Mai M, Watanabe K, Ooi A, Kitamura T, Takahashi Y, Ueda H, Ogino T, Nakanishi I. Medullary carcinoma with lymphocytic infiltration of the stomach – Clinicopathologic study of 27 cases and immunohistochemical analysis of the subpopulations of infiltrating lymphocytes in the tumor. *Cancer* 1990; **66:** 945-952

17 **Kyzer S**, Begin LR, Gordon PH, Mitmaker B. The care of patients with colorectal polyps that contain invasive adenocarcinoma. Endoscopic polypectomy or colectomy? *Cancer* 1992; **70:** 2044-2050

18 **Minamoto T**, Mai M, Ogino T, Sawaguchi K, Ohta T, Fujimoto T, Takahashi Y. Early invasive colorectal carcinomas metastatic to the lymph node with attention to their nonpolypoid development. *Am J Gastroenterol* 1993; **88**: 1035-1039

19 **Tanaka S**, Yokota T, Saito D, Okamoto S, Oguro Y, Yoshida S. Clinicopathologic features of early rectal carcinoma and indications for endoscopic treatment. *Dis Colon Rectum* 1995; **38**: 959-963

20 **Tanaka S**, Haruma K, Teixeira CR, Tatsuta S, Ohtsu N, Hiraga Y, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F. Endoscopic treatment of submucosal invasive colorectal carcinoma with special reference to risk factors for lymph node metastasis. *J Gastroenterol* 1995; **30:** 710-717

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• RAPID COMMUNICATION •

MSX2 overexpression inhibits gemcitabine-induced caspase-3 activity in pancreatic cancer cells

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Abstract

AIM: To evaluate the effect of MSX2 on gemcitabine-induced caspase-3 activation in pancreatic cancer cell line Panc-1.

METHODS: Using V5-tagged MSX2 expression vector, stable transfectant of MSX2 was generated from Panc-1 cells (Px14 cells). Cell viability under gemcitabine administration was determined by MTT assay relative to control cell line (empty-vector transfected Panc-1 cells; P-3EV cells). Hoechst staining was used for the detection of apoptotic cell. Activation of caspase-3 was assessed using Western blotting analysis and direct measurement of caspase-3 specific activities.

RESULTS: MSX2 overexpression in Panc-1 cells resulted in decreased gemcitabine-induced caspase-3 activation and increased cell viability under gemcitabine treatment in Px14 cells.

CONCLUSION: MSX2 exerts repressive effects on gemcitabine-induced apoptotic pathway. This novel apoptosis-regulating function of MSX2 may provide a new therapeutic target for pancreatic cancer.

Key words: MSX2; Caspase-3; Gemcitabine

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INTRODUCTION

Pancreatic cancer is known as a cancer with poor prognosis. Surgical resection of pancreatic cancer is available only in 15-20% of all patients[1], while medical approaches, such as chemotherapy or radiation, have no cure. The resistance of pancreatic cancer to chemotherapeutic agents is one of the serious problems in clinical situation. Gemcitabine (2',2'-difluoro-2'-deoxycytidine) is a widely used chemotherapeutic agent for unresectable pancreatic cancer treatment, and its administration triggers apoptosis in pancreatic cancer cell line[2]. On the other hand, previous report showed that acquired gemcitabine resistance was accompanied by altered expression of apoptosis regulatory genes[3]. The mechanisms how cancer cells evade apoptotic signals are beginning to come to light, but its upstream regulators are not fully understood as yet.

We have previously demonstrated that overexpression of homeobox gene MSX2 accelerated proliferation of pancreatic cancer cell lines, combined with epithelial-mesenchymal transition (EMT)-like phenotypic changes *in vitro* (Satoh *et al*., submitted). In addition, forced expression of MSX2 increased anchorage-independent growth of pancreatic cancer cells, indicating enhanced aggressive biological behavior. Recent studies have demonstrated that MSX2 is a downstream target of the Wnt signaling pathway in several cancer cell lines[4,5]. The upregulation of Wnt signaling is reported in various cancer cell lines, and its target genes are closely related to cancer cell growth and survival[6–9]. At this point of view, we hypothesized that MSX2 might affect apoptotic signaling pathway, which leads to the chemoresistance of pancreatic cancer cells.

In this study, we generated MSX2-overexpressing pancreatic cancer cell line Px14, and this cell line revealed resistance to the gemcitabine treatment. When cells were treated with gemcitabine, the caspase-3 activation was significantly upregulated in empty vector-transfected control cells, but not in Px14 cells compared to basal control cells. Taken together, MSX2 might act as a negative regulator of apoptosis in this cell line. This new upstream regulator of apoptotic signaling pathway may provide a novel therapeutic target of chemotherapy-resistant pancreatic cancer.

MATERIALS AND METHODS

Cell line and cell culture

Panc-1, a human pancreatic cancer cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 100 mL/L fetal bovine serum (FBS), and incubated at 37 °C in a humidified atmosphere containing 50 mL/L CO_2 in air.

Chemical substances

Gemcitabine (Gemzar; Eli Lilly Co., Indianapolis, IN, USA) was dissolved in phosphate buffered saline (PBS) at various concentrations of 0.1, 1, 10, 100 μg/mL and 1 mg/mL as stock solutions. In cell culture, vehicle and these stock solutions were used in 1:100 dilutions.

Plasmids, gene transduction and expression

Expression vector, pcDNA3.1-MSX2V5, was generated as described previously (Satoh *et al*. submitted). Panc-1 cells were plated in six-well plates and cultured until reaching a subconfluent state. Cells were transfected with 1 μg of pcDNA3.1-V5His (empty vector) or pcDNA3.1-MSX2V5 using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland) in normal growth medium. Three days later, cells were plated on 10-cm dishes and cultured until reaching a confluent state. After G418 selection, clones were subjected to Western blot analysis with a specific anti-V5 antibody (Invitrogen, Carlsbad, CA, USA).

Cell proliferation and cell viability assays

For cell proliferation assay, 6 000 cells were seeded per well in triplicate in 96-well plates in normal growth media. After 24-h (d1) and 72-h (d3) incubation, cell proliferation assay was performed using cell proliferation ELISA, BrdU (5-bromo-2-deoxyuridine) kit (Roche Diagnostics) according to the manufacturer's instructions. Cell proliferation ratio at d3 was normalized by that of d1. For cell viability assay, 10 000 cells were seeded per well in triplicate in 96-well plates in normal growth media. After 24-h incubation, cells were incubated with gemcitabine at various concentrations. After 48-h incubation, cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with MTT solution at a concentration of 0.5 mg/mL for 2 h, and then solubilized in dimethylsulfoxide (DMSO). Color reaction was measured by a spectrometer at a wavelength of 570 nm. Each experiment was repeated at least twice.

Detection of apoptotic cells

Apoptosis was determined by staining with Hoechst. Cells were plated at 1×10^4 on culture slide (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to adhere to the slide overnight. Gemcitabine (1 μg/mL) was added and incubated for an additional 24 h. The cells were fixed with 40 g/L paraformaldehyde for 20 min, followed by Hoechst 33342 (Calbiochem-Novabiochem, La Jolla, CA, USA) staining in these cells for 30 min. Then the cells were analyzed under a fluorescence microscope (Leica, Cambridge, UK). Between the incubations, the specimens were washed thrice with PBS. Chromatin condensation, nuclear shrinkage, and nucleosomal fragmentation were considered to be morphological markers of apoptosis.

Measurement of caspase-3 activities

APOCYTO Caspase-3 Colorimetric Assay Kit (MBL, Nagoya, Japan) was used for the detection of caspase-3 activity following the manufacturer's protocol. The subconfluent state cells in 10-cm dishes were harvested after 24-h incubation with gemcitabine (1 μg/mL), and subjected to caspase-3 activity detection.

Western blotting analysis

Cells were lysed by the addition of lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris–HCl, 10 mL/L Nonidet P40 and 5 g/L sodium deoxycholate. Cell lysates were cleared by centrifugation at 16 000 *g* at 4 °C for 15 min. Cleared lysates were boiled for 5 min at 100 °C after the addition of 5× sample loading buffer containing 1 mol/L Tris–HCl (pH 6.8), sodium dodecyl sulfate, glycerol, and bromophenol blue. Samples were electrophoresed at 200 V on 125 g/L polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 50 g/L nonfat dry milk, and then incubated with primary antibodies, such as anti-caspase-3 antibody (BD610322), anti-v5 antibody (R960-25, Invitrogen) and anti-α-tubulin antibody (sc-8035, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-mouse antibody (NA931, Amersham, Buckinghamshire, UK) was used as secondary antibody. Reactive bands were detected using ECL™ Western Blotting Detection Reagents (Amersham).

Statistical analysis

The unpaired *t*-test and one-way ANOVA were used for statistical comparison. Calculations were made with the help of Microsoft Excel computer software (Microsoft, Redmond, WA, USA). $P<0.05$ was considered statistically significant.

RESULTS

Forced MSX2 expression in human pancreatic cancer cell line Panc-1

To evaluate whether MSX2 expression correlates with gemcitabine resistance in pancreatic cancer cells, we generated MSX2 stable transfectant cell line. As described previously (Hamada *et al*., submitted), V5-tagged MSX2 expression vector was transfected in Panc-1 cells. MSX2-expressing clone (Px14) was selected and subjected to further analysis (Figure 1A). Panc-1 cells transfected with empty vector were maintained with the culture media containing G418, and used as control cell line (P-3EV). BrdU incorporation assay showed no significant difference in cell growth between two cell lines, but there was a tendency of slightly increased proliferation ratio in Px14 cells ($P = 0.074$, Figure 1B) relative to control cells (P-3EV cells), which was compatible to our previous report (Satoh *et al*., submitted).

Decrease of gemcitabine sensitivity in Panc-1 cells by MSX2 overexpression

Gemcitabine administration at a concentration of 10 μg/mL

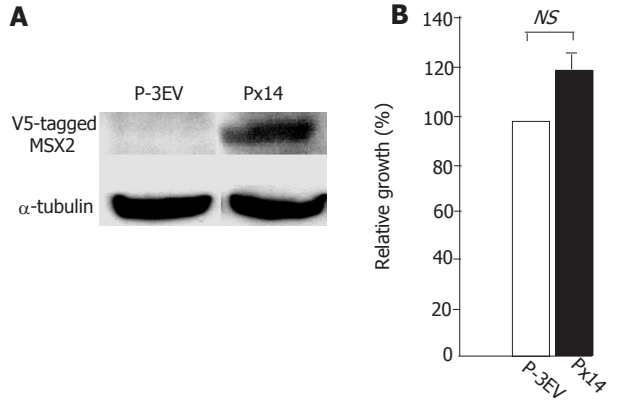

Figure 1 A: Western blotting analysis of V5-tagged MSX2 expression in Px14 cells; **B:** detection of cell proliferation of P-3EV and Px14 by BrdU incorporation assay. No significant difference was observed in these two cell lines, but slight increase of proliferation ratio was observed in Px14 cells (unpaired *t*-test, $n = 3$).

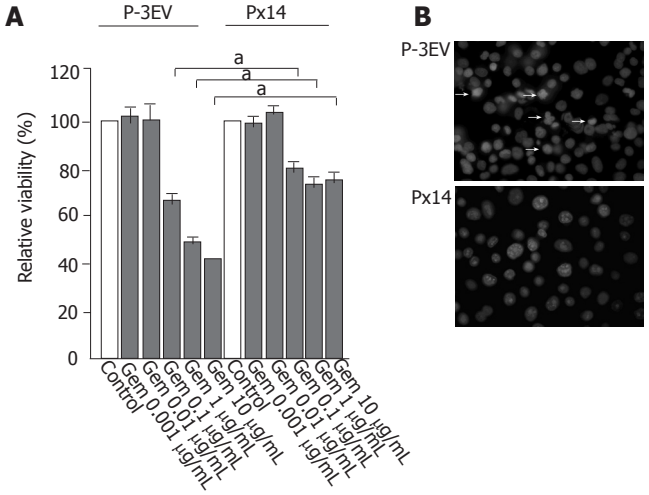

Figure 2 A: Cell viability of P-3EV and Px14 cell lines after 24 h of gemcitabine treatment at various concentrations. Gemcitabine reduced cell viability in a dose-dependent manner. At a concentration of 0.1 μg/mL or above, Px14 cells exhibited higher viability relative to P-3EV cells (one-way ANOVA, $n = 3$, $^aP<0.05$ *vs* others); **B:** Hoechst staining of P-3EV and Px14 cell lines. After 24 h of gemcitabine treatment at a concentration of 1 μg/mL, apoptotic changes (nuclear chromatin condensation and nuclear fragmentation) were obviously observed in P-3EV cells (arrow), but not in Px14 cells.

for 48 h decreased the viability of P-3EV cells by 60%, whereas that of Px14 cells only by 30% (Figure 2A). Px14 cells also depicted gemcitabine resistance at concentrations of 1 and 0.1 μg/mL (Figure 2A). Since Px14 cells were thought to be resistant to gemcitabine cytotoxicity, we analyzed gemcitabine-induced morphological alteration in P-3EV cells and Px14 cells. Gemcitabine-treated P-3EV cells at a concentration of 1 μg/mL for 24 h showed obvious apoptotic characteristics such as nuclear fragmentation and chromatin condensation, while these morphological changes were unremarkable in Px14 cells (Figure 2B).

Gemcitabine-induced caspase-3 activation in P-3EV cells and Px14 cells

Gemcitabine treatment led P-3EV cells to apoptotic state,

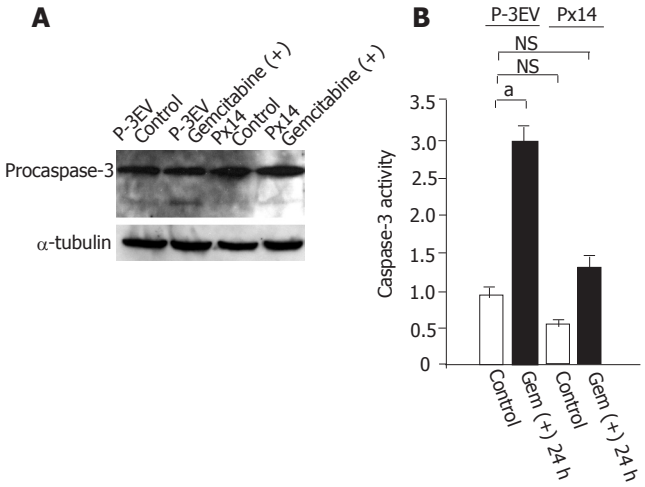

Figure 3 A: Western blotting analysis for caspase-3 activation. Cells were harvested 24 h after gemcitabine (1 g/mL) or vehicle administration. In P-3EV cells, active caspase-3 (cleaved form) was detected after gemcitabine treatment (arrow), whereas in Px14 cells, cleaved form of caspase-3 was undetectable with or without gemcitabine; **B:** direct caspase-3 activity measurements by APOCYTO Caspase-3 Colorimetric Assay Kit. Gemcitabine (1 μg/mL) treatment significantly enhanced caspase-3 specific activity in P-3EV cells, but not in Px14 cells. No significant differences in caspase-3 activities were detected between vehicle-treated P-3EV cells and Px14 cells, even after gemcitabine treatment (one-way ANOVA, $n = 3$, $^aP<0.05$ *vs* others).

thus we examined caspase-3 activation in P-3EV and Px14 cells. Gemcitabine administration at a concentration of 1 μg/mL clearly induced active (cleaved) form of caspase-3 in P-3EV cells, but not in Px14 cells (Figure 3A). Caspase-3 activation is a critical event in apoptosis induction; therefore, we hypothesized that gemcitabine resistance in Px14 cells might be due to suppressed caspase-3 activity. To evaluate this hypothesis, we conducted direct measurements of caspase-3 activities in P-3EV cells and Px14 cells, with or without gemcitabine treatment. The basal activities of caspase-3 in these cells were not significantly different (Figure 3B). However, gemcitabine treatment at a concentration of 1 μg/mL significantly increased caspase-3 activity in P-3EV cells but not in Px14 cells as compared with its basal activity in control cells.

DISCUSSION

Even in pancreatic cancer cells, apoptotic signal does exist, but the signal is considered to be overwhelmed by inhibitor of apoptosis[10]. In our experiment, gemcitabine treatment induced apoptotic changes in P-3EV cells, whereas this effect was attenuated by MSX2 overexpression. Studies have shown that the ability of evasion from apoptosis is one of the critical steps for tumor progression. For example, interference of X-linked inhibitor of apoptosis (XIAP) expression in MDA-MB-231 mammary cancer cell line increases sensitivity to several chemotherapeutic agents[11]. The expression level of survivin in pancreatic cancer tissue is significantly associated with the reduction of the apoptotic index of tumor cells[12] and is also reported to be correlated with the prognosis of patients[13],

indicating that anti-apoptotic effectors directly affect clinical outcomes.

MSX2 overexpression enhanced cell proliferation in pancreatic cancer cell line BxPC-3 (Satoh K *et al.*, submitted), and tended to stimulate the Panc-1 cell growth in the current study, indicating that MSX2 enhances the proliferation effect rather than the pro-apoptotic effect in pancreatic cancer cells. Association of MSX2 with cell proliferation has also been shown in facial mesenchyme[14] and in skeletogenic mesenchyme[15], suggesting that the downstream targets of this homeobox gene may include regulators of cell proliferation. In addition, MSX2 itself and/or BMP-induced MSX2 has shown to lead the pancreatic cancer cells to epithelial-mesenchymal transition (EMT) state (Satoh *et al.*, Hamada *et al.*, submitted), indicating the enhancement of malignant phenotype of pancreatic cancer cells by MSX2. In this study, we clearly demonstrated that forced expression of MSX2 in pancreatic cancer cells produced the resistance to gemcitabine-induced apoptosis via the suppression of caspase-3 activity, which is an additional new aspect of MSX2 to accelerate the pancreatic cancer cell malignancy. Although further detailed investigations would be required to clarify how MSX2 inhibits caspase-3 activation in pancreatic cancer cells, the involvement of this gene in the development of gemcitabine resistance provides us some clues for further understanding of the cancerous cell nature and a novel therapeutic target.

REFERENCES

1 **Li D,** Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; **363:** 1049-1057

2 **Chandler NM**, Canete JJ, Callery MP. Caspase-3 drives apoptosis in pancreatic cancer cells after treatment with gemcitabine. *J Gastrointest Surg* 2004; **8:** 1072-1078

3 **Schniewind B**, Christgen M, Kurdow R, Haye S, Kremer B, Kalthoff H, Ungefroren H. Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. *Int J Cancer* 2004; **109:** 182-188

4 **Hussein SM**, Duff EK, Sirard C. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. *J Biol Chem* 2003; **278:** 48805-48814

5 **Schwartz DR**, Wu R, Kardia SL, Levin AM, Huang CC, Shedden KA, Kuick R, Misek DE, Hanash SM, Taylor JM, Reed H, Hendrix N, Zhai Y, Fearon ER, Cho KR. Novel candidate targets of beta-catenin/T-cell factor signaling identified by gene expression profiling of ovarian endometrioid adenocarcinomas. *Cancer Res* 2003; **63:** 2913-2922

6 **Tetsu O**, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398:** 422-426

7 **Shtutman M**, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* 1999; **96:** 5522-5527

8 **He TC**, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281:** 1509-1512

9 **Zhang T**, Otevrel T, Gao Z, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Res* 2001; **61:** 8664-8667

10 **Yang L**, Cao Z, Yan H, Wood WC. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. *Cancer Res* 2003; **63:** 6815-6824

11 **McManus DC**, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S, Morris SJ, Durkin JP, Lacasse EC. Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene* 2004; **23:** 8105-8117

12 **Satoh K**, Kaneko, K, Hirota, M, Masamune, A, Satoh, A, and Shimosegawa, T. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* 2001; **92:** 271-278

13 **Kami K**, Doi R, Koizumi M, Toyoda E, Mori T, Ito D, Fujimoto K, Wada M, Miyatake S, Imamura M. Survivin expression is a prognostic marker in pancreatic cancer patients. *Surgery* 2004; **136**: 443-448

14 **Ashique AM**, Fu K, Richman JM. Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. *Development* 2002; **129**: 4647-4660

15 **Ishii M**, Merrill AE, Chan YS, Gitelman I, Rice DP, Sucov HM, Maxson RE Jr. Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. *Development* 2003; **130**: 6131-6142

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• *RAPID COMMUNICATION* •

Association of *Helicobacter pylori Ig*A antibodies with the risk of peptic ulcer disease and gastric cancer

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Abstract

AIM: To compare the prevalence of *Helicobacter pylori (H pylori) Ig*G and *Ig*A antibodies between adult subjects, with defined gastric diseases, nondefined gastric disorders and those representing the population.

METHODS: Data on *H pylori Ig*G and *Ig*A antibodies, determined by enzyme immunoassay, were analyzed in 3 252 subjects with DGD including 482 patients with gastric ulcer, 882 patients with duodenal ulcer, 1 525 patients with chronic gastritis only and 363 subjects with subsequent gastric cancer, 19 145 patients with NoDg and 4 854 POPUL subjects. The age-adjusted prevalences were calculated for 1- and 20-year age cohorts.

RESULTS: The prevalences of *Ig*G antibodies were equally high (89-96%) in all 20-year age cohorts of the DGD groups, whereas the prevalences of *Ig*G antibodies were lower and increased by age in the POPUL and NoDg groups. The prevalences of *Ig*A antibodies were also higher in the DGD groups; among them CA (84-89%) and GU groups (78-91%) showed significantly higher prevalences than DU (68-77%) and CG patients (59-74%) (OR 2.49, 95%CI 1.86-3.34 between the GU and DU groups). In the CA, GU, and DU groups, the *Ig*A prevalences showed only minor variation according to age, while they increased by age in the CG, POPUL, and NoDg groups ($P \leqslant 0.0001$). The *Ig*A response, but not the *Ig*G response, was associated with an increased risk of CA (OR 2.41, 95%CI 1.79-3.53) and GU (OR 2.57, 95%CI 1.95-3.39) in comparison with CG patients.

CONCLUSION: An *Ig*A antibody response during *H pylori* infection is significantly more common in CA and GU patients as compared with CG patients.

Key words: *Helicobacter pylori*; *Ig*A antibodies; Gastric cancer; Gastric ulcer; Duodenal ulcer; Chronic gastritis

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INTRODUCTION

Helicobacter pylori (*H pylori*), the causative agent of chronic gastritis[1], is also the most important risk factor for peptic ulcer disease[25] and distal gastric cancer[4,5]. The presence of *H pylori* antibodies signify this chronic infection and their prevalence increases with age in all populations, mainly due to the birth of cohort phenomenon[6,7]. The optimal serological tests for *Ig*G antibodies to *H pylori* show a sensitivity and a specificity of over 95%[8-10]. Antibodies of the *Ig*A class are usually detected in combination with elevated *Ig*G antibodies in approximately two-thirds of infected subjects[8,11,12]. They are diagnostically useful in the 2-7% of *H pylori* patients who do not have elevated *Ig*G levels[7,8,12-15]. *Ig*A antibodies have been shown to be a sensitive indicator of an increased risk for gastric cancer[14]. In this context, it may be important that subjects with CagA antibodies have more often *H pylori* antibodies of the *Ig*A class as compared with those who are CagA antibody-negative[15], since CagA-positive infections have been associated with an increased risk of both peptic ulcer disease and gastric cancer[16,17].

In the present study, we analyzed the prevalences of *H pylori* antibodies determined in our laboratory from 1986 to 2000 in clinical samples taken from patients with endoscopically verified or undefined gastric disorders and

in samples collected from the Finnish population.

MATERIALS AND METHODS

Study subjects

Serum samples for this study were obtained from 1986 to 2000 from the following patient groups: 3 252 patients with defined gastric diseases (DGD), including 482 patients with an endoscopically confirmed gastric ulcer (GU) (mean age 60.79 years, SD±12.59 years), 882 patients with an endoscopically confirmed duodenal ulcer (DU) (mean age 53.80 years, SD±13.64 years), 1 525 patients with a histologically verified chronic gastritis (CG) (mean age 50.58 years, SD±15.95 years) and 363 subjects with subsequent gastric cancer (CA) (mean age at the time of the serum sampling 57.23 years, SD±10.91 years). Sera from GU, DU, and CG patients were collected on the day of the endoscopy, those from CA patients between 2 wk to 24 years before the diagnosis of cancer was made (reported in part earlier[5,14]). In the GU, DU, and CG groups, patients who had prior successful eradication therapy were excluded from the study. In addition, serum samples were obtained from 4 854 subjects participating in a population study in Vammala, Finland (POPUL) (mean age 41.73 years, SD±20.60 years), reported in part earlier[7] and from 19 145 patients whose sera were sent by general practitioners, Municipal Health Centers or Hospitals to our diagnostic laboratory for *H pylori* antibody tests without any information on possible gastric disorders (NoDg) (mean age 51.47 years, SD±16.97 years).

Ethics

The study was approved by the Ethics Committee for Epidemiology and Public Health of the Helsinki and Uusimaa Hospital district.

Laboratory assessment

H pylori *Ig*G and *Ig*A antibody titers were determined by in-house enzyme immunoassays[8,10].The antigen used was an acid glycine extract from *H pylori* strain NCTC 11637. During the study period, the sensitivity and specificity of the *Ig*G test were 95-99% and 93-97%, respectively, and those of the *Ig*A test were 64-67% and 92-98%, respectively, as determined in patients in whom the presence of *H pylori* infection had been verified by culture and histology of gastric biopsies[8,10].

Statistical analysis

The trend in changes in the prevalences of *Ig*G and *Ig*A antibodies by age was studied using the linear trend test. The comparisons of prevalences of *Ig*A and *Ig*G antibodies between the groups were analyzed using the logistic regression model adjusting for age based on 1-year age cohorts. For an overview, the prevalences were determined for 20-year age-adjusted cohorts (15-34, 35-54, 55-74, and 75-94 years), each including at least 50 subjects. The association of *Ig*A and *Ig*G responses with the risk of serious complications (CA, GU, and DU) was analyzed using a logistic regression model by comparing the number of subjects in each antibody response and complication category to that in CG patients, who are regarded to present the basic disease caused by *H pylori*. Statistical analyses were carried out using the SPSS 12.0 software package (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

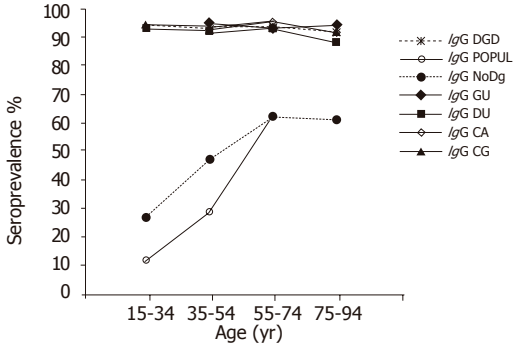

Figure 1 Prevalence of *H pylori* *Ig*G antibodies by 20-year age cohorts in the Finnish population and patients with different gastric disorders. (Only cohorts including at least 50 subjects are shown).

RESULT

Of the 27 251 subjects, 13 939 (51.2%) were positive for *H pylori* antibodies. Of the antibody-positive subjects, 61.8% were positive for both *Ig*G and *Ig*A antibodies, 34.9% for *Ig*G antibodies only and 3.3% for *Ig*A antibodies only.

IgG antibody prevalences

Among the subjects in the DGD groups, 88.6-95.7% had *H pylori* antibodies of the *Ig*G class in all 20-year age cohorts (Figure 1). In contrast, among the subjects representing the POPUL and NoDg groups, significantly lower seroprevalences were observed (OR 19.73, 95%CI 16.15-24.10 and OR 14.11, 95%CI 12.28-16.21, respectively) (Figure 1). Furthermore, the prevalence was seen to increase by age from 12% in the youngest cohort to 63% in the 55-74-year-old cohort in the POPUL group ($P<0.0001$; trend test), and from 27% to 62%, respectively, in the NoDg patients ($P<0.0001$; trend test). The prevalence of *Ig*G antibodies was significantly higher in the NoDg patients than in the POPUL group (OR 2.18, 95%CI 2.00-2.36) (Figure 1).

Within the DGD group, the prevalences did not differ between the GU, DU, CG, and CA groups, nor did they show any significant variation by age (trend test).

IgA antibody prevalences

The prevalence of *Ig*A antibodies group in all age cohorts was significantly higher in the DGD group than in the POPUL (OR 9.61; 95%CI 8.20-11.26) and NoDg groups (OR 5.00; 95%CI 4.59-5.44) (Figure 2). Within the DGD group, the highest prevalences were found in the GU and CA groups in all 20-year cohorts (77.7-90.7% and 84.3-88.6%, respectively) (Figure 2) without a significant difference between these two groups (OR 1.09, 95%CI

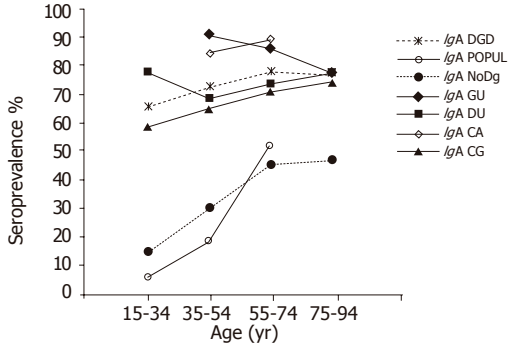

Figure 2 Prevalence of *H pylori IgA* antibodies by 20-year age cohorts in the Finnish population and patients with different gastric disorders. (Only cohorts including at least 50 subjects are shown.)

0.75-1.58). Although the GU patients showed a small decrease of *Ig*A-positive subjects by increasing age ($P = 0.016$; trend test), the prevalence was markedly higher than in DU (68.4-77.4%, OR 2.49; 95%CI 1.86-3.34) and CG patients (58.7-74.2%, OR 2.57, 95%CI 1.95-3.39). In the DU patients, the *Ig*A prevalence showed no significant trend by age (trend test), whereas a significantly increased trend by age was found in CG patients ($P = 0.0001$; trend test); the overall prevalences did not differ significantly between these two groups (OR 1.13; 95%CI 0.95-1.35) (Figure 2, Table 1).

In the subjects representing the POPUL and NoDg groups, the prevalence of *Ig*A antibodies increased by age from the lowest rates (6.5% and 15.1%, respectively) to significantly higher rates in the 55-74-year-old cohorts (52.1% and 45.6%, respectively; $P<0.0001$; trend test) (Figure 2). The overall prevalence of *Ig*A antibodies was higher in the NoDg patients than that in the POPUL group (OR 1.93, 95%CI 1.73-2.10).

Association of IgG and IgA responses with the risk of CA, GU, and DU in comparison with CG

*Ig*A response was more common in CA and GU groups as compared with CG patients (OR 2.41, 95%CI 1.79-3.53 and OR 2.57, 95%CI 1.95-3.39, respectively); however, this difference was not significant in DU patients (Table 1). The number of *Ig*G responders in CA and GU groups did not differ significantly as compared with the CG patients (Table 1), whereas it was even slightly lower in DU patients as compared with the CG patients (OR 0.72, 95%CI 0.55-0.99).

DISCUSSION

In the present study, we analyzed, according to age cohorts, a large body of serological data collected during a 15-year period. DGD subjects with gastric disorders known to be associated with *H pylori* infection showed a high and rather a constant prevalence of *H pylori Ig*G antibodies in all the 20-year age cohorts. Based on the prevalence of *Ig*A antibodies, the DGD group could be divided into two categories: in GU-CA-category, the age-adjusted *Ig*A prevalences ranged from 78% to 91%; whereas in DU-CG-category, the age-adjusted *Ig*A prevalences remained significantly lower. With the exception of CG patients, the *Ig*A antibody rates also remained rather constant throughout the age range. In contrast, the infected subjects in the POPUL and NoDg groups showed significantly lower *Ig*G and *Ig*A rates than those in the DGD group and that increased significantly by age. The CG patients formed a special intermediate group with overall *Ig*G and *Ig*A antibody prevalences at the same level as those of the DU patients, but with a significantly increasing trend by age in the prevalence of *Ig*A antibodies.

Table 1 Association of *H pylori Ig*A and *Ig*G antibodies with the risk of CA, GU or DU in comparison to CG

Subjects	*Ig*A		*Ig*G	
with	OR	95%CI	OR	95%CI
CG	1		1	
CA	2.41	1.79–3.53	1.28	0.81–2.02
GU	2.57	1.95–3.39	0.69	0.46–1.03
DU	1.13	0.95–1.35	0.72	0.55–0.99

The importance of the *Ig*A response increases when considered in connection with our earlier findings showing the association of *H pylori* antibodies of the *Ig*A class with a CagA-positive infection[15], as well as with other reports showing an increased risk of peptic ulcer disease and gastric cancer in CagA-positive infection[16,17]. The present results imply that an *Ig*A response during *H pylori* infection might be regarded as an indicator of an increased risk not only for gastric cancer[14] but also for gastric ulcer disease. In these comparisons, that we carried out using the data from patients with chronic gastritis as baseline values, we found that the higher *Ig*A response rate seen in DU patients did not reach significance.

By using the data obtained in prospective gastric cancer studies, we wanted to avoid the bias caused by severe atrophic gastritis, regarded as a precancerous process[18]. Severe atrophic gastritis may progress to a disease stage when *Helicobacters* first gradually decrease in number, then disappear and finally also *Helicobacter* antibodies, the longest lasting indicators of the infection, fall to a normal level[19]. In particular, in elderly subjects with non-cardia cancer, there may be several individuals who at the time of diagnosis may have lost all direct indicators of their burnt out *Helicobacter* infection.

Our large materials and the high sensitivity and specificity of our antibody tests also gave an opportunity to compare the prevalence of *H pylori* antibodies between the POPUL and NoDg groups. It is tempting to speculate that the higher *H pylori* prevalence in the two youngest cohorts (by 15% and 18% units in antibodies of *Ig*G class in the order of increasing age) in NoDg patients would preferentially reflect the strength of gastric symptoms driving patients to clinical consultations.

In conclusion, irrespective of age, practically all DGD subjects have *H pylori Ig*G antibody. The prevalence of *Ig*A antibodies highest in CA and GU patients, second highest in DU and CG patients, and lowest in the NoDg patients and POPUL subjects. An *Ig*A response is associated with serious sequelae of *H pylori* infection.

REFERENCES

1 **Marshall BJ**, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Med J Aust* 1985; **142**: 436-439
2 **Rauws EA,** Tytgat GN. Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. *Lancet* 1990; **335**: 1233-1235
3 **Marshall BJ.** *Helicobacter pylori*. *Am J Gastroenterol* 1994; **89**: S116-128
4 Infection with *Helicobacter pylori*. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 177-240
5 Helicobacter and Cancer Collaborative Group. Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut* 2001; **49**: 347-353
6 **Talley NJ,** Noack KB. The woldwide prevalence of *Helicobacter pylori*: Asymptomatic infection and clical states associated with infection in adults. In: Goodwin CS, Worsley BW, eds. *Helicobacter pylori: Biology and clinical practice*. Boca Raton, Florida: CRC Press Inc. 1993; 63-83
7 **Kosunen TU,** Aromaa A, Knekt P, Salomaa A, Rautelin H, Lohi P, Heinonen OP. Helicobacter antibodies in 1973 and 1994 in the adult population of Vammala, Finland. *Epidemiol Infect* 1997; **119**: 29-34
8 **Kosunen, TU**, Seppälä K, Sarna S, Sipponen P. Diagnostic value of decreasing IgG, IgA, and IgM antibody titres after eradication of *Helicobacter pylori*. *Lancet* 1992; **339**: 893-895
9 **Feldman RA,** Deeks JJ, Evans SJ. Multi-laboratory comparison of eight commercially available *Helicobacter pylori* serology kits. *Helicobacter pylori* Serology Study Group. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 428-433
10 **Oksanen A,** Veijola L, Sipponen P, Schauman KO, Rautelin H. Evaluation of Pyloriset Screen, a rapid whole-blood diagnostic test for *Helicobacter pylori* infection. *J Clin Microbiol* 1998; **36**: 955-957
11 **Kosunen TU,** Höök J, Rautelin HI, Myllylä G. Age-dependent increase of *Campylobacter pylori* antibodies in blood donors. *Scand J Gastroenterol* 1989; **24**: 110-114
12 **Andersen LP,** Rosenstock SJ, Bonnevie O, Jorgensen T. Seroprevalence of immunoglobulin G, M, and A antibodies to *Helicobacter pylori* in an unselected Danish population. *Am J Epidemiol* 1996; **143**: 1157-1164
13 **Jaskowski TD**, Martins TB, Hill HR, Litwin CM. Immunoglobulin A antibodies to *Helicobacter pylori*. *J Clin Microbiol* 1997; **35**: 2999-3000
14 **Aromaa A,** Kosunen TU, Knekt P, Maatela J, Teppo L, Heinonen OP, Harkonen M, Hakama MK. Circulating anti-Helicobacter pylori immunoglobulin A antibodies and low serum pepsinogen I level are associated with increased risk of gastric cancer. *Am J Epidemiol* 1996; **144**: 142-149
15 **Rautelin HI,** Oksanen AM, Karttunen RA, Seppala KM, Virtamo JR, Aromaa AJ, Kosunen TU. Association of CagA-positive infection with Helicobacter pylori antibodies of IgA class. *Ann Med* 2000; **32**: 652-656
16 **Cover TL,** Glupczynski Y, Lage AP, Burette A, Tummuru MK, Perez-Perez GI, Blaser MJ. Serologic detection of infection with cagA$^+$ *Helicobacter pylori* strains. *J Clin Microbiol* 1995; **33**: 1496-1500
17 **Parsonnet J,** Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut* 1997; **40**: 297-301
18 **Correa P,** Haenszel W, Cuello C, Zavala D, Fontham E, Zarama G, Tannenbaum S, Collazos T, Ruiz B. Gastric precancerous process in a high risk population: cohort follow-up. *Cancer Research* 1990; **50**: 4737-4740
19 **Kokkola A,** Kosunen TU, Puolakkainen P, Sipponen P, Harkonen M, Laxen F, Virtamo J, Haapiainen R, Rautelin H. Spontaneous disappearance of Helicobacter pylori antibodies in patients with advanced atrophic corpus gastritis. *APMIS* 2003; **111**: 619-624

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• *RAPID COMMUNICATION* •

Polymorphisms in sulfotransferase 1A1 and glutathione *S*-transferase P1 genes in relation to colorectal cancer risk and patients' survival

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Abstract

AIM: To examine whether polymorphisms in SULT1A1 and GSTP1 genes contribute to colorectal cancer development and whether they are associated with clinicopathological variables are not well identified.

METHODS: We examined the genotypes of 125 colorectal cancer patients and 666 healthy controls in a Swedish population by using PCR-restriction fragment length polymorphism (RFLP).

RESULTS: SULT1A1 *2/*2 genotype (OR = 2.49, 95%CI = 1.48-4.19, P = 0.0002) and *2 allele (OR = 1.56, 95%CI = 1.16-2.10, P = 0.002) had an effect on colorectal cancer susceptibility, while GSTP1 genotype was without effect. However, GSTP1 G-type predicted a worse prognosis in the patients independently of gender, age, Dukes' stage, growth pattern, and differentiation (P = 0.03).

CONCLUSION: Polymorphism in SULT1A1 may predispose to colorectal cancer and GSTP1 may be a biological indicator of prognosis in the patients.

Key words: GSTP1; SULT1A1; Survival; Colorectal cancer; PCR-RFLP

Sun XF, Ahmadi A, Arbman G, Wallin Å, Asklid D, Zhang H. Polymorphisms in sulfotransferase 1A1 and glutathione *S*-transferase P1 genes in relation to colorectal cancer risk and patients' survival. *World J Gastroenterol* 2005; 11(43): 6875-6879
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INTRODUCTION

Sulfotransferase 1A1 (SULT1A1), a major sulfotransferase enzyme in human beings, is an important component in the detoxification pathway of numerous xenobiotics. The enzyme also plays an important role in the metabolism and bioactivation of many dietary and environmental mutagens, including heterocyclic amines implicated in carcinogenesis of colorectal and other cancers[1]. *SULT1A1* is polymorphic with the most common variant allele, *SULT1A1**2, where a G→A change occurs at nucleotide 638, resulting in an arg213→his213 change. This allele codes for an allozyme with low enzyme activity and stability compared to the *SULT1A1**1 variant. Therefore, *SULT1A1* genotype may influence susceptibility to mutagens following exposure to heterocyclic amines and other environmental toxins[1]. However, regarding the results of the *SULT1A1* polymorphism in relation to colorectal cancer risk was inconsistent[2-4].

Glutathione *S*-transferase P1 (GSTP1) plays a central role in the inactivation of toxic and carcinogenenic electrophiles[5]. The A to G polymorphism at nucleotide 313 in the *GSTP1* gene results in an isoleucine to valine change at residue 105, which reduces the catalytic activity of the enzyme. The polymorphism of lower activity allele of *GSTP1* was related to several types of cancers including bladder, testicular, and lung cancer, but not to colorectal cancer[5-10].

There was no study on the polymorphisms of *SULT1A1* and *GSTP1* in patients with colorectal cancer in comparison with healthy controls in Sweden; we, therefore, investigated the polymorphisms in this population in order to clarify whether the polymorphisms were related to colorectal cancer risk, and further to analyze whether the polymorphisms had any clinicopathological significance. We analyzed genomic DNA of normal colorectal mucosa from 125 colorectal cancer patients and of blood samples from 666 healthy controls by using PCR-restriction-fragment length polymorphism (RFLP).

MATERIALS AND METHODS

Materials

Normal colorectal mucosal samples and colorectal cancer tissue were obtained from 125 patients with primary colorectal adenocarcinomas diagnosed at the Department of Pathology, Vrinnevi Hospital, Norrköping, Sweden, during the period between 1990 and 2001. All patients were Caucasians. Patients' gender, age, tumor site, Dukes' stage and the grade of differentiation were obtained from their surgical and pathological records and reviewed by two authors. Normal colorectal mucosa was taken from the distant resection margin and the corresponding tumor tissue from the colorectal cancer. The patients with colon cancers in Dukes' stage C/advanced tumor received chemotherapy (the influence on survival was less than 20%), and some patients with rectal cancers received adjuvant preoperative radiotherapy (the treatment has a marginal influence on survival). The patients were followed up until the end of October 2003, and 32 deaths due to the cancer had been registered.

Control population comprised 666 individuals from the same residential area as the patients. The individuals had neither gastrointestinal diseases nor history of tumors. DNA samples were extracted from the peripheral leukocytes.

Methods

DNA extraction Genomic DNA was isolated from frozen normal colorectal mucosa and tumor from colorectal cancer patients by a modified proteinase K digestion and phenol/chloroform extraction technique. In brief, the tissue (about 200 mg) was cut into small pieces and dissolved in a cell lysis buffer (40 μL of 20% SDS, 40 μL of 10 mg/mL proteinase K, 400 μL TEN buffer, 0.1 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.4). Samples were kept in a shaking water bath at 55 °C overnight. The digestive step was repeated by the addition of half of the volume of the cell lysis solution (above), until the sample solution turned completely clear. DNA was extracted with phenol, phenol/chloroform and chloroform, precipitated with ice-cold ethanol, washed with 70% ethanol, and redissolved in double distilled water. The amount of purified DNA was measured by a DU 640 spectrophotometer (Beckman, Fullerton, USA), and the DNA concentration was calculated. Genomic DNA from the controls was extracted from peripheral leukocytes by means of the Wizard Genome Purification Kit (Promega Inc., Madison, USA) according to the manufacturer's instructions.

PCR-RFLP Two sets of primers were used respectively for determining the polymorphisms of the *SULT1A1* (A1VIIF: GTT GGC TCT GCA GGG TCT CTA GGA GAG and 1A1VIIR: CCC AAA CCC CCG TAC TGG CCA GCA CCC) and *GSTP1* (P105F: ACC CCA GGG CTC TAT GGG AA and P105R: TGA GGG CAC AAG AAG CCC CT). PCR reactions were performed in a total volume 20-80 μL of a solution containing PCR buffer (2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.4, 1.2 μL DMSO (only for SULT1A1)), 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Uppsala, Sweden) and 0.4-1.0 U of Taq DNA polymerase (Sigma Chemical, St. Louis, USA). The reaction started with 94 °C for 3 min, followed by 35-39 cycles consisting of denaturation (at 94 °C for 0.5-3 min), annealing (SULT1A1: at 63 °C for 1 min and GSTP1: 55 °C for 0.5 min), and extension (at 70-72 °C for 0.5-5 min). PCR products were checked by electrophoresis on a 1.5-2% agarose gel (Invitrogen Life Technologies, Groningen, The Netherlands) with 0.2 μg/mL ethidium bromide. A negative control (PCR without template) was included in each set of PCR reactions. All PCR reactions were repeated at least three times for confirmation and to ensure reproducibility.

Hae II (New England BioLabs) was applied to identify *SULT1A1* genotypes[11] and Alw26I (New England Biolabs, Hertfordshire, UK) for *GSTP1* genotypes[8]. The PCR products were digested with the restriction enzymes and separated in a 3.5% agarose gel or in a 4% of 3:1 NuSieve GTG agarose (BioWhittaker Molecular Applications, Rockland, ME, USA)/agarose, stained with ethidium bromide to visualize the bands.

Genotyping and grouping Genotyping and grouping of genotypes in the present study were based on their effect on carcinogenic metabolism and commonly used classifications in previous other studies. For *SULT1A1,* three genotypes of *1/*1, *1/*2, and *2/*2 were classified[2,11-13]. To analyze the relationship of *SULT1A1* genotype with clinicopathological factors, we combined *SULT1A1**1/* with *1/*2 as one group *vs* *SULT1A1**2/*2 group. The polymorphisms of *GSTP1* were confirmed to be A/A, A/G, and G/G. *GSTP1* A type was referred to A/A, and G type was referred to both A/G and G/G[6,14,15].

Statistical analysis

The χ^2 method was used to test the frequencies of genotypes/allele in colorectal cancer patients with the control population and various clinicopathological variables. McNemar's method was used for testing the differences of the genotype/allele frequency in normal colorectal mucosa and tumor tissue. The odds ratio (OR), an estimate of the relative risk, with 95% confidence intervals (CI) was computed to assess the relationship of the genotypes/allele to the risk of colorectal cancer. Cox's Proportional Hazard Model was used to test the relationship between the polymorphisms and the survival of patients. Survival curves were computed according to the Kaplan-Meier method. All *P* values cited were two-sided and *P* values <0.05 were judged as statistically significant.

RESULTS

We examined the polymorphisms of *SULT1A1* in 109 colorectal cancer patients and 666 healthy individuals. As shown in Table 1, the frequencies of *SULT1A1* *2/*2

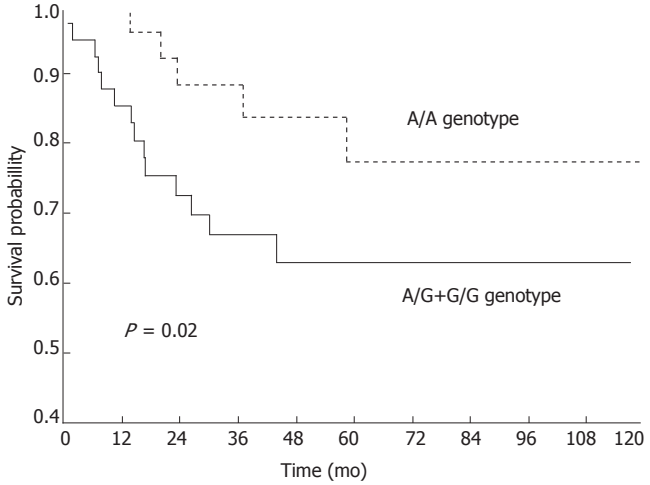

Figure 1 Colorectal cancer patients with G-type genotype (A/G+G/G) of the *GSTP1* gene had a worse prognosis than those with A/A genotype (A/A) ($P = 0.02$).

Table 1 Polymorphisms of SULT1A1 and GSTP1 in colorectal cancer patients and healthy controls

Gene	Patients (%)	Controls (%)	Odds ratio (95%CI)	*P*
SULT1A1				
*1/*1	43 (39)	266 (40)	1.0	
*1/*2	27 (25)	303 (45)	0.55 (0.32–0.94)	
*2/*2	39 (36)	97 (15)	2.49 (1.48–4.19)	0.0002[1]
Allele *1	113 (52)	835 (63)	1.0	
Allele *2	105 (48)	497 (37)	1.56 (1.16–2.10)	0.002
GSTP1				
A/A	59 (47)	127 (50)	1.0	
A/G	51 (41)	101 (40)	1.09 (0.67–1.76)	
G/G	15 (12)	27 (10)	1.20 (0.56–2.55)	0.63[2]
A allele	169 (68)	355 (70)	1.0	
G allele	81(32)	155 (30)	1.10 (0.78–1.54)	0.57

[1]SULT1A1: *2/*2 *vs* *1/*1+*1/*2, [2]GSTP1: G type (G/G+A/G) *vs* A type (A/A).

genotype (36% *vs* 15%, OR = 2.49, 95%CI = 1.48-4.19, $P = 0.0002$) and *2 allele (48% *vs* 37%, OR = 1.56, 95% CI = 1.16-2.10, $P = 0.002$) in the patients were significantly higher than in the controls. The *SULT1A1*2/*2* was still related to the risk for developing colorectal cancer when we did age-matched analyses of case-control, age ≤70 ($P = 0.04$), ≤80 ($P<0.0001$) and >70 years ($P<0.0001$). The status did not change when the tests were performed in different tumor sites (proximal or distal) in comparison with the control group ($P>0.05$).

The genotypes and allele frequency of the *GSTP1* gene were determined in 125 patients and 255 healthy controls. *GSTP1* G type was referred to both A/G and G/G. As shown in Table 1, there was no significant difference between the patients and controls based on G type (53% *vs* 50%, OR = 0.90, 95%CI = 0.57-1.41, $P = 0.63$) or allele frequency (32% *vs* 30%, OR = 1.10, 95%CI = 0.78-1.54, $P = 0.57$), neither in the subgroups of the age and tumor location ($P>0.05$).

The patients with *GSTP1* G-type genotype had a worse prognosis than those with *GSTP1* A (A/A) type genotype ($P = 0.02$, Figure 1). Even in multivariate analysis, the genotype was still related to survival, independent of gender, age, tumor location, Dukes' stage, growth pattern, and differentiation ($P = 0.03$, Table 2). Besides, there was no relationship of genotype/allele frequency with any of the clinicopathological variables studied in the patients ($P>0.05$).

Table 2 Multivariate analysis of GSTP1, gender, age, site, Dukes' stage, growth pattern, and histological type in relation to survival in colorectal cancer

Variable	Number	Cancer death Rate ratio	95%CI	*P*
GSTP1 genotype				
A/A	14	1.0	–	0.03
A/G+G/G	26	5.3	1.26–19.53	
Gender				0.09
Male	25	1.0	–	
Female	15	0.2	0.05–1.18	
Age				0.37
≤70	15	1.0	–	
>70	25	1.9	0.51–7.94	
Tumor location				1.00
Colon	21	1.0	–	
Rectum	19	1.0	0.26–3.98	
Dukes' stage				1.16
A+B	19	1.0	–	
C+D	21	2.7	0.69–10.72	
Growth pattern				0.08
Expansive	24	1.0	–	
Infiltration	16	4.1	0.85–19.45	
Histological type				0.02
Non-mucinous	27	1.0	–	
Mucinous	13	7.1	1.39–32.07	

In order to check whether there was a difference between normal colorectal mucosa and tumor regarding the two polymorphisms, we isolated DNA from the tumors in the same patients and analyzed the genotype/allele frequencies of *SULT1A1* and *GSTP1* polymorphisms by using the same protocol. Neither genotype nor allele frequency of the polymorphisms in the tumors differed from that of the polymorphisms determined in normal mucosal samples ($P>0.05$).

DISCUSSION

In the present study, we demonstrated that the patients with colorectal cancer, comparing with the controls, had significantly higher frequencies of *SULT1A1*2/*2* genotype (36% *vs* 15%) and *2 allele (48% *vs* 37%), indicating that *SULT1A1*2/*2* allele was related to colorectal cancer risk. Our results supported previous findings in colorectal, esophagus, breast and lung cancer studies, where *SULT1A1* *2/*2 genotype was associated with an increased risk for the tumor development[2,16-18]. It has been observed that a G to A transition at nucleotide 638 in *SULT1A1* gene causes an Arg213 to His substitution associated with a low SULT activity. Thus, these results suggest that low activity of SULT1A1*2 allozyme lacks a protection against dietary and/or environmental chemicals involved in the carcinogenesis of colorectal cancer.

It has been demonstrated that individuals with *GSTP1* G/G alleles had a lower catalytic activity compared with individuals with *GSTP1* A/A. An intermediate activity was reported for heterozygotes[19,20]. In the present study, the frequencies of genotype and allele in the control (50% for A/A, 40% for A/G, 10% for G/G, and 70% for A allele) and in the patients (47%, 41%, 12%, and 68%, respectively) were similar to the frequencies raised by other studies in Caucasian controls (40-52%, 39-48%, 7-11%, and 67%, respectively) and colorectal cancer patients (37-49%, 42-55%, 8-12%, and 70%, respectively)[7,8,10,14,21]. We failed to find any evidence supporting an association between the allelic variants of *GSTP1* and susceptibility to colorectal cancer or certain clinicopathological factors including gender, age, tumor location, Dukes' stage and differentiation, which is supportive to previous findings[6,7,9,21].

Interestingly, we found that patients with *GSTP1* G-type had a worse prognosis than the patients with *GSTP1* A-type, even after adjustment for gender, age, tumor location, Dukes' stage, and differentiation. A study in breast cancer found that a significantly higher proportion of breast cancer patients with a *GSTP1* G-type had more frequency of p53 mutations and loss of heterozygosity at the TPp53 gene locus, compared with *GSTP1* A-genotype[22]. It has been widely accepted that altered p53 predicts a poor prognosis in breast cancer patients, although there is not a direct evidence of *GSTP1* in relation to survival in their study. It seems that *GSTP1*, through the A→G polymorphism, may reduce its effect on the inactivation of toxic and carcinogenic electrophiles[5]. In contrast, Stoehlmacher *et al*.[21] demonstrated that the *GSTP1* A→G polymorphism was associated in a dose-dependent fashion with increased survival of patients with advanced colorectal cancers receiving 5-FU/oxaliplatin chemotherapy. They suggest that the effect of certain chemotherapeutic drugs might be altered when enzymes that could enhance the elimination of these drugs show a reduced activity. The opposite results related to survival in colorectal cancer patients may be due to different characteristics of patients included in the two studies. We included all Dukes' stages with a proper stratification (A = 12, B = 46, C = 34, and D = 17); among them, the cases with colon cancers in Dukes' stage C or advanced tumors received chemotherapy, and some patients with rectal cancer received adjuvant preoperative radiotherapy. All patients in their study had a metastasis and received 5-FU/oxaliplatin combination chemotherapy. Furthermore, we had a longer follow-up period than theirs (median month: 33 *vs* 10.9). Comparing with their data, we have more Caucasians (100% *vs* 72%) and older patients (median: 73 *vs* 60 years).

In conclusion, our results suggest that *SULT1A1* may predispose to colorectal cancer, and *GSTP1* may be used as a prognostic factor to predict the patients' survival.

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REFERENCES

1 **Glatt H**. Sulfotransferases in the bioactivation of xenobiotics. *Chem Biol Interact* 2000; **129:** 141-170
2 Rommelspacher H. Unravelling genetic modifiers of anxiety and depressiveness in psychiatric disorders. Pharmacogenetics 2001; **11:** 679-685
3 **Wong CF**, Liyou N, Leggett B, Young J, Johnson A, McManus ME. Association of the SULT1A1 R213H polymorphism with colorectal cancer. *Clin Exp Pharmacol Physiol* 2002; **29:** 754-758
4 **Nowell S**, Coles B, Sinha R, . MacLeod S, Luke Ratnasinghe D, Stotts C, Kadlubar FF, Ambrosone CB, Lang NP. Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. *Mutat Res* 2002; **506-503:** 175-185
5 **Hengstler JG**, Arand M, Herrero ME, Oesch,F. Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. Recent Results Cancer Res 1998; **154:** 47-85
6 **Katoh T,** Kaneko S, Takasawa S, Nagata N, Inatomi H, Ikemura K, Itoh H, Matsumoto T, Kawamoto T, Bell DA. Human glutathione S-transferase P1 polymorphism and susceptibility to smoking related epithelial cancer; oral, lung, gastric, colorectal and urothelial cancer. Pharmacogenetics 1999; **9:** 165-169
7 **Loktionov A**, Watson MA, Gunter M, Stebbings WS, Speakman CT, Bingham SA. Glutathione-S-transferase gene polymorphisms in colorectal cancer patients: interaction between GSTM1 and GSTM3 allele variants as a risk-modulating factor. *Carcinogenesis* 2001; **22:** 1053-1060
8 **Harries LW**, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis* 1997; **18:** 641-644
9 **Harris MJ**, Coggan M, Langton L, Wilson SR, Board PG. Polymorphism of the Pi class glutathione S-transferase in normal populations and cancer patients. *Pharmacogenetics* 1998; **8:** 27-31
10 **Welfare M**, Monesola Adeokun A, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1, and GSTT1 and susceptibility to colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 1999; **8:** 289-292
11 **Coughtrie MW**, Gilissen RA, Shek B, Strange RC, Fryer AA, Jones PW, Bamber DE. Phenol sulphotransferase SULT1A1 polymorphism: molecular diagnosis and allele frequencies in Caucasian and African populations. *Biochem J* 1999; **337**(pt 1)**:** 45-49
12 **Seth P**, Lunetta KL, Bell DW, Gray H, Nasser SM, Rhei E, Kaelin CM, Iglehart DJ, Marks JR, Garber JE, Haber DA, Polyak K. Phenol sulfotransferases: hormonal regulation, polymorphism, and age of onset of breast cancer. *Cancer Res* 2000; **60:** 6859-6863
13 **Stone J,** Wade JA, Cauch-Dudek K, Ng C, Lindor KD, Heathcote EJ. Human leukocyte antigen Class II associations in serum antimitochondrial antibodies (AMA)-positive and AMA-negative primary biliary cirrhosis. J Hepatol **2002; 35:** 137-142
14 **Ryberg D**, Skaug V, Hewer A, Phillips DH, Harries LW, Wolf CR, Ogreid D, Ulvik A, Vu P, Haugen A. Genotypes of glutathione transferase M1 and P1 and their significance for lung DNA adduct levels and cancer risk. *Carcinogenesis* 1997; **18:** 1285-1289
15 **Yoshioka M**, Katoh T, Nakano M, Takasawa S, Nagata N, Itoh H. Glutathione S-transferase (GST) M1, T1, P1, N-acetyltransferase (NAT) 1 and 2 genetic polymorphisms and

susceptibility to colorectal cancer. *J UOFH* 1999; **21:** 133-147
16 **Wu MT**, Wang YT, Ho CK, Wu DC, Lee YC, Hsu HK, Kao EL, Lee JM. SULT1A1 polymorphism and esophageal cancer in males. *Int J Cancer* 2003; **103:** 101-104
17 **Han DF**, Zhou X, Hu MB, Wang CH, Xie W, Tan XD, Zheng F, Liu F. Sulfotransferase 1A1 (SULT1A1) polymorphism and breast cancer risk in Chinese women. *Toxicol Lett* 2004; **150:** 167-177
18 **Liang G,** Miao X, Zhou Y, Tan W, Lin D. A functional polymorphism in the SULT1A1 gene (G638A) is associated with risk of lung cancer in relation to tobacco smoking. *Carcinogenesis* 2004; **25**: 773-778
19 **Watson MA**, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis* 1998; **19:** 275-280
20 **Srivastava SK**, Singhal SS, Hu X, Awasthi YC, Zimniak P, Singh SV. Differential catalytic efficiency of allelic variants of human glutathione S-transferase Pi in catalyzing the glutathione conjugation of thiotepa. *Arch Biochem Biophys* 1999; **366:** 89-94
21 **Stoehlmacher J**, Park DJ, Zhang W, Groshen S, Tsao-Wei DD, Yu MC, Lenz HJ. Association between glutathione S-transferase P1, T1, and M1 genetic polymorphism and survival of patients with metastatic colorectal cancer. *J Natl Cancer Inst* 2002; **94:** 936-942
22 **Nedelcheva Kristensen V**, Andersen TI, Erikstein B, Geitvik G, Skovlund E, Nesland JM, Borresen-Dale AL. Single tube multiplex polymerase chain reaction genotype analysis of GSTM1, GSTT1 and GSTP1: relation of genotypes to TP53 tumor status and clinicopathological variables in breast cancer patients. *Pharmacogenetics* 1998; **8:** 441-447

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• *RAPID COMMUNICATION* •

Immunoproteomics of membrane proteins of *Shigella flexneri* 2a 2457T

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Abstract

AIM: To screen the immunogenic membrane proteins of *Shigella flexneri* 2a 2457T.

METHODS: The routine two-dimensional polyacrylamide gel electrophoresis (2-DE) and Western blotting were combined to screen immunogenic proteins of *S. flexneri* 2a 2457T. Serum was gained from rabbits immunized with the same bacteria. Immunogenic spots were cut out from the polyacrylamide gel and digested by trypsin in-gel. Matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS) was performed to determine the molecular weight of peptides. Electrospray ionization (ESI-MS/MS) was performed to determine the sequences of the interesting peptides.

RESULTS: A total of 20 spots were successfully identified from Coomassie brilliant blue stained gels representing 13 protein entries, 5 known antigens and 8 novel antigens. A hypothetical protein (YaeT) was detected, which might be a candidate target of vaccine.

CONCLUSION: Membrane proteins of *S. flexneri* 2a 2457T were successfully observed by 2-DE. Several known and novel antigens were identified by mass spectrum.

Key words: *Shigella flexneri* 2a 2457T; Immunoproteomics; Membrane proteins

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INTRODUCTION

The genus *Shigella* spp. is a group of Gram-negative enteric bacilli which cause bacillary dysentery in human beings, accounting for 20% of the 4.6 million diarrhea-associated deaths among children[1]. Though the LPS can induce a good immune response in human beings, the role of proteins (especially the membrane proteins) in conferring immunity to shigellosis is at best speculative. Considering outer membrane proteins of *Shigella* spp. function as a dynamic interface between the cell and its surroundings, it is possible to develop new antigens from them. Due to the methodology limitations of protein separation and identification, it is difficult to identify the immunogenic proteins in bands on 1-D gel. With the improvement of 2-DE in recent years much valuable information is available and immunoproteomics has been built around 2-DE and routine immunologic technologies.

S. flexneri 2a is the dominant serotype causing shigellosis in China. Our laboratory has finished a two-dimensional electrophoresis reference map and a proteomic database of *S. flexneri* 2a 2457T[2], but only a few of membrane proteins can be identified in that database. In order to develop new protective antigens against *S. flexneri* and to understand their immune mechanism, we applied immunoproteomic technologies in screening new antigens of *S. flexneri* 2a 2457T.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. flexneri 2a 2457T was aerobically cultured in LB overnight at 37 °C. Overnight cultures were diluted 1:100 and shaken at 250 r/min. Growth was stopped at the early stationary phase at an A_{600} of 3.3.

Membrane protein preparation

Cells were harvested and centrifuged for 15 min at 2 000 r/min (Sigma 3K12, No. 12150; St. Louis, MO, USA) at 4 °C. The pellet was washed thrice for 10 min at 2 000 r/min with low-

salt washing buffer (3 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 68 mmol/L NaCl, 9 mmol/L NaH_2PO_4)[3]. Proteins were extracted using the ReadyPrep™ protein extraction kit (Membrane I) (BioRad, USA). Integral membrane proteins were separated from hydrophilic proteins using the nonionic detergent Triton X-114.

Two-dimensional electrophoresis

Eighteen-centimeter immobilized pH gradient (IPG) strips (pH ranges, 4-7) (Amersham Pharmacia Biotech, Sweden) were used. Isoelectric focusing (IEF) was conducted for 60 000 Vh (IPGphor, Amersham Pharmacia Biotech). Vertical slab SDS-PAGE (12.5%) was run at 30 mA/gel for the second dimension. Gels were stained with Colloidal Coomassie Blue[4]. Image analysis was performed with Image-Master 2D Elite Version 3.1.

Preparation of antisera against 2457T

S. flexneri 2a 2457T was aerobically cultured in LB overnight at 37 °C. Rabbits were immunized six times with culture solution intravenously at intervals of 5 d. The doses were (5, 7.5, 10, 15, 20, 20)×10^8 CFU, respectively. Eight days after the last immunization, blood was collected from the tested animals and the sera were separated. Antibody titers 1:5 120 was measured by microaggalutination test and ELISA.

Immunoblot assay

After two-dimensional electrophoresis, the gels were electroblotted onto Hybond™ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech) using a semi-dry transfer unit (Hoefer™ TE 77, Amersham Pharmacia Biotech, Sweden). Before immunodetection, the membranes were stained for 10 min with 5 g/L Ponceau S in 10 mL/L acetic acid and the positions of some selected spots were marked by clean needles. Western blotting was performed as previously described[5]. Then antigen-antibody complexes were detected with peroxidase-labeled goat anti-rabbit IgGs and substrate.

In-gel protein digestion and MALDI-TOF-MS protein identification

In-gel protein digestion was performed as previously described[6]. All MALDI-MS measurements were performed on a Bruker Reflex. III MALDI-TOF-MS (Bruker Daltonik, Bremen, Germany) operating in reflectron mode.

Nanospray ESI-MS/MS

The peptide solution after in-gel protein digestion was desalted with ZipTip C18™ pipette tips (Millipore, Bedford, MA, USA). Electrospray ionization (ESI-MS/MS) was carried out with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF2) (Micromass, Manchester, UK)[2].

Peptide mass fingerprinting

Peptide mass fingerprinting searches were performed using the program Mascot developed by Matrix Science Ltd (http://www.matrixscience.com). For protein identification, peptide mass searches against the database of 2457T by Mascot licensed in-house and the searches against the NCBInr database with free access on the internet were done. A peptide mass accuracy of 0.3 Da was defined.

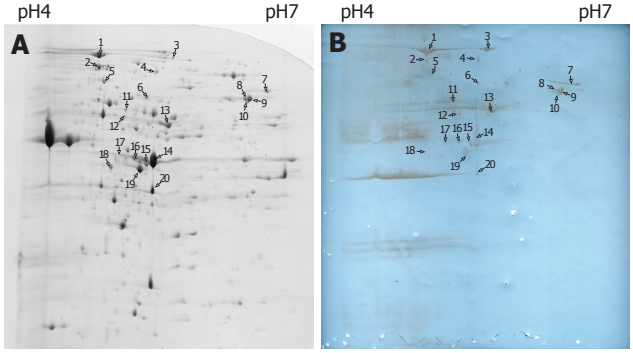

Figure 1 A: Two-dimensional electrophoresis profile of *S. flexneri* 2a 2457T membrane proteins, stained with Colloidal Coomassie Blue; **B:** Western blot of membrane proteins of *S. flexneri* 2a 2457T. Gel equal to Figure 1A was electroblotted onto nitrocellulose membrane using a semi-dry transfer unit.

RESULTS

The sample was prepared on the basis of the separation of membrane proteins by temperature-dependent phase partitioning using Triton X-114 detergent. Proteins anchored to the membrane or containing one or two transmembrane domains were efficiently partitioned to the detergent-rich phase. In order to solubilize the protein thoroughly, thiourea was used. In pH 4-7 gradient 2-DE map, 148 spots were cut and 111 spots were successfully identified by MALDI-TOF-MS presenting 82 protein entries. Twenty-five proteins were not observed/identified in our previous work[2]. The majority of these 25 proteins (data not shown) were hydrophobic and associated with the membrane. The relative abundance of membrane-associated proteins identified in this study was higher than that in our previous study[2].

On the basis of the established immunoproteomic map of soluble proteins of *S. flexneri* 2a 2457T (unpublished), we described a group of spots in a 2-DE map of immunogenic proteins from hydrophobic proteins in this study. Five hundred micrograms of protein sample was used to perform the 2-DE. One of the parallel gels was electroblotted onto nitrocellulose membrane and the other was stained with Coomassie brilliant blue G-250. We successfully identified 20 immunoreactive spots from Coomassie brilliant blue stained gels using sera from immunized rabbits, which represented 13 protein entries, 5 known antigens and 8 novel antigens. The 20 spots were marked on the 2-D gel and corresponding blotting membrane (Figure 1). Table 1 lists all the identified proteins. ESI-MS/MS was used to confirm the protein marked as spot 1. Figure 2 shows the result of ESI-MS/MS identification.

Table 1 List of immunoreactive proteins of membrane proteins

Spot ID	Gene symbol	Protein common name	NCBI GI identifier	Cellular role
1	YaeT	Hypothetical protein	gi|30061734	Cell envelope
2	DnaK	Chaperone Hsp70; autoregulated heat shock protein	gi|30061584	Protein fate
3	ClpB	Heat shock protein	gi|30063993	Protein fate
4/14/16/17/ 20	OmpA	Outer membrane protein 3a (II*; G; d)	gi|30062494	Cell envelope
5	MopA	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein	gi|30065518	Protein fate
6	Pgm	Phosphoglucomutase	gi|30062137	Energy metabolism
7	OppA	Periplasmic oligopeptide binding protein	gi|30062764	Protein fate
8/9	AtpA	Membrane-bound ATP synthase, F1 sector, alpha-subunit	gi|30064961	Energy metabolism
10	LpdA	Lipoamide dehydrogenase (NADH)	gi|30061682	Energy metabolism
11	Gnd	Gluconate-6-phosphate dehydrogenase	gi|30063478	Energy metabolism
12/13/18	TufB	Protein chain elongation factor EF-Tu	gi|30064737	Protein synthesis
15	Tsf	Protein chain elongation factor EF-Ts	gi|30061727	Protein synthesis
19	MglB	Galactose-binding transport protein; receptor for galactose taxis	gi|30063593	Transport and binding proteins

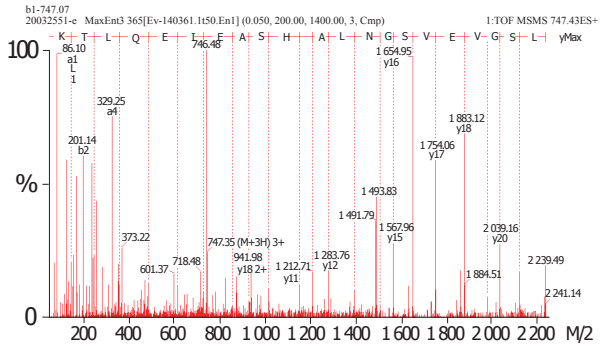

Figure 2 Mass spectra showing the determination of a partial peptide sequence of the *hypothetical* protein (spot 1).

DISCUSSION

Known antigens

Our results are in accordance with other studies[7-11].The outer membrane protein 3a (II*; G; d) is a precursor of OmpA, a major and highly conserved outer membrane protein of Gram-negative bacteria. Due to its high copies per cell[12], multiple charged isoforms[13] and its strong immunogenecity, identification of OmpA was performed several times during the immunoproteomics analysis. All these proteins were observed in our other works (unpublished).

Novel antigens

Besides the above confirmatory findings, the study detected several new immunoreactive proteins (AtpA, OppA, MglB, LpdA, ClpB, Gnd, Pgm, YaeT). AtpA, LpdA, Gnd, and Pgm are components of the energy metabolism system. ATP synthesis/hydrolysis occurs in the ATP synthase F1 sector which lies at the surface of cytoplasmic membrane. LpdA codes for an outer membrane lipoamide dehydrogenase that is highly immunogenic. It is an *in vivo*-induced antigen in *Mycobacterium tuberculosis*[14]. Since LpdA is a functional subunit of both pyruvate dehydrogenase (aceEF) and alpha-ketoglutarate dehydrogenase (sucAB), a lpdA mutant of *H. influenzae* can be significantly attenuated[15]. Gnd is an important component of pentose phosphate pathway. Phosphoglucomutase (pgm) is associated with virulence of *Brucella abortus* because the deltapgm strain is unable to assemble the O side chain in the complete LPS. Vaccination with the deltapgm strain induces effective protection[16]. The periplasmic oligopeptide binding protein OppA is part of the oligopeptide transport system. In addition to the function mentioned above, it also plays a role in mediating the adhesion or interactions of bacteria to different substrates, tissues or environments[17-19]. OppA and periplasmic galactose-binding protein MglB also display some chaperone-like functions, suggesting that they are probably involved in protein folding and protection against stress in periplasm[20]. ClpB is also a heat shock protein. The proteins described above have not been reported as antigens and may serve as candidate markers for bacterial infection though they are unlikely to be protective.

A hypothetical protein (YaeT) detected is of high homology to Oma90 of *S. flexneri* M90T (serotype 5)[21]. We also detected this protein in another study (unpublished), which is verified by ESI-MS/MS. Since it has an enhanced expression in a murine model and exhibits strong homology to genes encoding *Haemophilus influenzae* D15 and *Pasteurella multocida* Oma87, its role in Shigella infection and immunoreaction is worthy to be clarified.

REFERENCES

1 **Ahmed F,** Ansaruzzaman M, Haque E, Rao MR, Clemens JD. Epidemiology of postshigellosis persistent diarrhea in young children. *Pediatr Infect Dis J* 2001; **20:** 525-530
2 **Liao X,** Ying T, Wang H, Wang J, Shi Z, Feng E, Wei K, Wang Y, Zhang X, Huang L, Su G, Huang P. A two-dimensional proteome map of Shigella flexneri. *Electrophoresis* 2003; **24:** 2864-2882
3 **Humphery-Smith I,** Guyonnet F, Chastel C. Polypeptide cartography of Spiroplasma taiwanense. *Electrophoresis* 1994; **15:** 1212-1217
4 **Cordwell SJ.** Acquisition and archiving of information for bacterial proteomics: from sample preparation to database. *Methods Enzymol* 2002; B 207-227
5 **Wu M,** Stockley PG, Martin WJ 2nd. An improved western blotting technique effectively reduces background. *Electrophoresis* 2002; **23:** 2373-2376
6 **Liao X,** Ying TY, Wang HL, Wang J, Wei KH, Huang LY, Huang PT. A modified method of in-gel digestion of Coomassie brilliant blue-stained 2-D gels. *Shengwu Jishu Tongxun* 2003; **14:** 509-511

7 **Sanchez-Campillo M,** Bini L, Comanducci M, Raggiaschi R, Marzocchi B, Pallini V, Ratti G. Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera. *Electrophoresis* 1999; **20:** 2269-2279

8 **Bini L,** Sanchez-Campillo M, Santucci A, Magi B, Marzocchi B, Comanducci M, Christiansen G, Birkelund S, Cevenini R, Vretou E, Ratti G, Pallini V. Mapping of Chlamydia trachomatis proteins by immobiline-polyacrylamide two-dimensional electrophoresis: spot identification by N-terminal sequencing and immunoblotting. *Electrophoresis* 1996; **17:** 185-190

9 **Pardo M,** Ward M, Pitarch A, Sanchez M, Nombela C, Blackstock W, Gil C. Cross-species identification of novel Candida albicans immunogenic proteins by combination of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Electrophoresis* 2000; **21:** 2651-2659

10 **McAtee CP,** Lim MY, Fung K, Velligan M, Fry K, Chow T, Berg DE. Identification of potential diagnostic and vaccine candidates of Helicobacter pylori by two-dimensional gel electrophoresis, sequence analysis, and serum profiling. *Clin Diagn Lab Immunol* 1998; **5:** 537-542

11 **Haas G,** Karaali G, Ebermayer K, Metzger WG, Lamer S, Zimny-Arndt U, Diescher S, Goebel UB, Vogt K, Roznowski AB, Wiedenmann BJ, Meyer TF, Aebischer T, Jungblut PR. Immunoproteomics of Helicobacter pylori infection and relation to gastric disease. *Proteomics* 2002; **2:** 313-324

12 **Nikaido H.** Outer membrane. In: Neidhardt FC. Escherichia coli and Salmonella: Cellular and molecular biology. Washington DC: *ASM Press*, 1996: 29-47

13 **Molloy MP,** Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA. Proteomic analysis of the Escherichia coli outer membrane. *Eur J Biochem* 2000; **267:** 2871-2881

14 **Deb DK,** Dahiya P, Srivastava KK, Srivastava R, Srivastava BS. Selective identification of new therapeutic targets of Mycobacterium tuberculosis by IVIAT approach. *Tuberculosis* 2002; **82:** 175-182

15 **Herbert M,** Kraiss A, Hilpert AK, Schlor S, Reidl J. Aerobic growth deficient Haemophilus influenzae mutants are non-virulent: implications on metabolism. *Int J Med Microbiol* 2003; **293:** 145-152

16 **Ugalde JE,** Comerci DJ, Leguizamon MS, Ugalde RA. Evaluation of Brucella abortus phosphoglucomutase (pgm) mutant as a new live rough-phenotype vaccine. *Infect Immun* 2003; **71:** 6264-6269

17 **Sutcliffe IC,** Russell RR. Lipoproteins of gram-positive bacteria. *J Bacteriol* 1995; **177:** 1123-1128

18 **Higgins CF,** Hardie MM. Periplasmic protein associated with the oligopeptide permeases of Salmonella typhimurium and Escherichia coli. *J Bacteriol* 1983; **155:** 1434–1438

19 **Fenno JC,** Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a Treponema denticola OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun* 2000; **68:** 1884-1892

20 **Richarme G,** Caldas TD. Chaperone properties of the bacterial periplasmic substrate-binding proteins. *J Biol Chem* 1997; **272:** 15607-15612

21 **Robb CW,** Orihuela CJ, Ekkelenkamp MB, Niesel DW. Identification and characterization of an in vivo regulated D15/Oma87 homologue in Shigella flexneri using differential display polymerase chain reaction. *Gene* 2001; **262:** 169-177

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• *CASE REPORT* •

Non-parasitic splenic cysts: A report of three cases

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Abstract

Primary splenic cyst is a relatively rare disease, and the majority of cases are classified as epithelial cysts. Three cases with nonparasitic splenic cysts are presented: two epithelial and one pseudocyst. All cases had an atypical symptomatology, consisted mainly of fullness in the left upper abdomen and a palpable mass. Preoperative diagnosis was established with ultrasonography and computerized tomography. Two cases with large cysts located in the splenic hilum were treated with open complete splenectomy. The most recent case, a pseudocyst, was managed laparoscopically with partial cystectomy. All cases did not have any problems or recurrence during follow-up. Laparoscopic partial cystectomy is an acceptable procedure for the treatment of splenic cysts, because it cures the disease preserving the splenic tissue. Complete splenectomy is reserved for cases in which cyst excision cannot be done otherwise.

Key words: Spleen; Epithelial cyst; Surgery; Laparoscopy

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INTRODUCTION

Splenic cysts are unusual in everyday surgical practice. They can be parasitic (hydatid), caused by the parasite *Echinococcus granulosus*, or nonparasitic[1,2]. Non-parasitic cysts are classified as primary (true, epithelial), lined by an epithelial cover (epidermoid, dermoid, and mesothelial) or endothelial cover (hemangioma, lymphangioma), and secondary (pseudocysts, non-epithelial), which are usually of post-traumatic origin[3,4].

Primary splenic cysts comprise 30-40% of the total and are encountered more commonly in children and young adults[5,6]. Most of the cysts are asymptomatic, and they are incidental findings during abdominal ultrasonography. The number of diagnosed splenic cysts seems to rise because of the increased use of abdominal imaging techniques[7].

Laparotomy with splenectomy has been the method of choice for the treatment of primary splenic cysts[5,8]. Today, performance of more conservative surgical procedures has been advised, especially in children and young adults, in order to avoid overwhelming postsplenectomy infection[4,8]. Herein, we present three cases with non-parasitic splenic cysts, their diagnostic evaluation and surgical management.

CASE REPORTS

Case 1

A 15-year-old girl was admitted to our Department with a chief complaint of abdominal fullness. An elastic, hard mass of approximately 15 cm in diameter was palpable in the left upper abdomen. A chest X-ray showed a mild elevation of the left hemidiaphragm. Ultrasonography of the upper abdomen showed a giant cystic lesion with irregular echoic patterns. Computerized tomography confirmed the splenic localization of the cyst and demonstrated almost total displacement of the remaining splenic parenchyma.

At laparotomy, a huge splenic cyst of approximately 15 cm of maximal diameter was revealed, located in the middle of the splenic parenchyma, displacing it towards the splenic poles. First, reduction of the cyst with intraoperative drainage of 1 000 mL of serous fluid was done. However, due to the cyst location, preservation of the spleen was considered impossible, and complete splenectomy followed. The aspirated cystic fluid showed no evidence of malignancy. Histology report revealed that the cyst wall consisted of dense fibrous tissue, covered by stratified squamous or cuboid epithelium. Thus, the diagnosis of a primary epidermoid splenic cyst was established. The postoperative clinical course of the patient was satisfactory and was discharged on postoperative d 7. She received a pneumococcal vaccine and chemoprophylaxis with oral penicillin at a dose of 1 500 000 IU twice daily, for a period of 6 months. Today, the patient is in excellent condition, 8 years after surgery.

Case 2

A 27-year-old woman presented with mild dyspeptic symptoms, an atypical pain and a sensation of fullness in the epigastrium. Past medical history was negative,

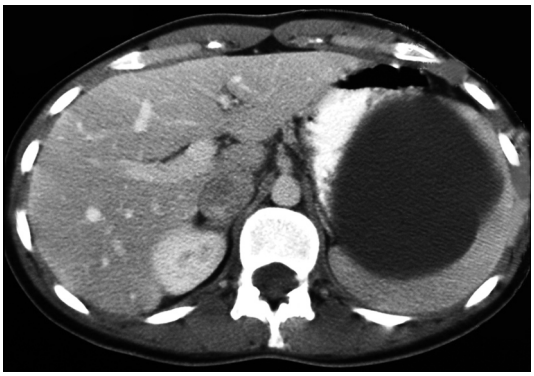

Figure 1 Plain abdominal CT scan showing the splenic localization of a large cyst displacing the remaining splenic parenchyma, the so-called "beak-sign".

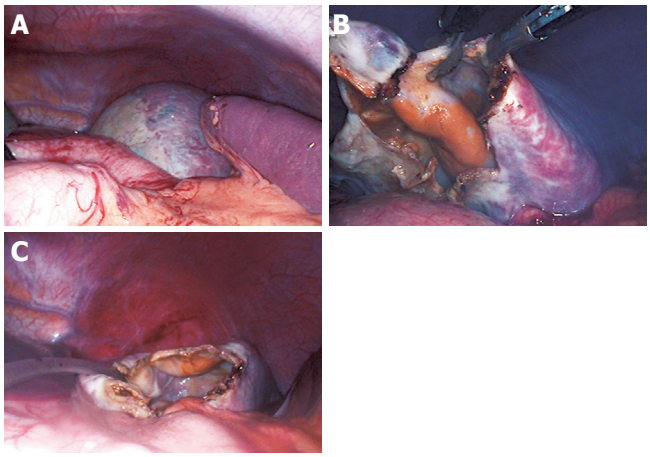

Figure 2 Laparoscopic view of the cyst. Most parts of the cyst are covered with a thin layer of splenic tissue; only a small portion in the upper pole of the spleen displays a "white roof" **(A)**. The cyst was punctured and evacuated and a 3 cm× 3 cm portion of the cyst was excised using the monopolar scissor **(B)**. A drainage tube was inserted in the remaining cavity **(C)**.

and physical examination revealed a 6-7 cm palpable mass in the left upper quadrant of her abdomen. All laboratory tests were normal, and serological tests gave no evidence of parasitic infection with *Echinococcus granulosus*. Plain abdominal films of the abdomen were negative. Ultrasound and computerized tomography of the abdomen showed a solitary cystic mass in the splenic hilum with a maximum diameter of 8 cm, displacing the splenic parenchyma (Figure 1). Endoscopic examination of the upper gastrointestinal tract did not reveal any significant findings.

The diagnosis of a splenic cyst was confirmed and the patient was subjected to exploratory laparotomy. On exploration, there was a medium-sized splenic cyst located in the hilum, which made splenectomy inevitable. Postoperative recovery was uneventful. Histological examination of the specimen showed a normal splenic parenchyma and an epidermoid splenic cyst with a fibrous wall lined by epithelial cells. The patient had no problems after surgery and she received pneumococcal vaccine and oral penicillin for 6 months. Today she is in good clinical condition, 4 years postoperatively.

Case 3

A 24-year-old female presented with an asymptomatic lump in the left hypochondriac region, since 2 years. The patient did not have any specific symptoms except for a mild abdominal discomfort and a sensation of fullness in her left upper abdomen. She had a negative medical history. On physical examination, a cystic mass was palpable under the left costal margin, which was moving according to the respiratory movements. The routine hematological and biochemical tests were normal. Casoni's skin test and complement fixation test for hydatid disease were negative. A clinical diagnosis of cystic splenomegaly was made and the patient was subjected to radiological investigations. Ultrasonography of the upper abdomen revealed a single unilocular spherical cystic lesion in the anterior surface of the spleen. Computerized tomography confirmed the ultrasound findings of a subcapsular cystic lesion with an almost 10 cm of maximal diameter within the spleen with attenuation value near that of water with a non-calcified wall.

With a preoperative diagnosis of a splenic cyst, the patient was subjected to laparoscopy. Before starting with the procedure, a thorough video-guided inspection of the peritoneal cavity was performed. After focusing on the left upper quadrant, the greater omentum was pulled down, and the cyst was clearly visible in the upper pole of the spleen (Figure 2A). The cyst roof was punctured and about 700 mL of yellowish (serous) fluid was aspirated and sent for culture and cytological examination. A 3 cm ×3 cm portion of the collapsed cyst wall was then excised with monopolar scissors, paying attention to remove the cyst wall segment free of splenic parenchyma (Figure 2B). Thus, there was minimal blood loss and unroofing of the cyst wall was accomplished. The specimen was extracted through a 10-mm port and sent for histological examination. The tip of an elastic drainage tube was left inside the remaining cavity (Figure 2C). The postoperative course was uneventful. The patient resumed oral diet on the first postoperative day, and was discharged on the second postoperative day after the drain was removed.

Bacteriological cultures of the fluid were negative. Cytological examination of the fluid showed a few lymphocytes and histiocytes. Histological examination of the cyst wall revealed the presence of dense cytopenic connective tissue without any epithelial lining, and confirmed the diagnosis of a splenic pseudocyst. The patient is in good clinical condition, one year after surgery, and on follow-up tomographic scan had no evidence of recurrence.

DISCUSSION

Benign true non-parasitic splenic cysts cannot be clinically distinguished from other types of splenic cysts. They have an inner lining of epithelial cells and are usually of congenital etiology[3,6]. Pseudocysts have an inner lining of connective tissue and are usually secondary to blunt trauma or hemorrhage in the splenic parenchyma, but

they may also be of infectious and degenerative origin[9,10]. Both types of splenic cysts do not produce any specific symptoms, until they reach a significant size. Large cysts may cause atypical pain and heaviness in the left hypochondriac region, due to distension of the capsule or space-occupying mechanisms within the abdominal cavity, or they may present as a palpable mass[3,5,9]. Indeed, in our patients, symptomatology was atypical with a sensation of fullness and a palpable mass in the left upper abdominal quadrant, as well as mild dyspeptic symptoms. Symptoms secondary to pressure on surrounding organs, such as nausea, vomiting, flatulence, and diarrhea may gradually appear. Also, pressure in the cardiorespiratory system may cause pleuritic pain or dyspnea, and irritation of the left diaphragm may cause persistent cough[10]. Occasionally splenic cysts may present with complications, such as infection, rupture and hemorrhage[9,11].

When a lump is detected in the left upper quadrant of the abdomen, it is necessary to exclude any disease associated with splenomegaly, mononucleosis, fever of unknown origin, hemolytic anemia, chronic leukemias, collagen vascular disease, and liver diseases[12]. Serological studies are useful in excluding most of the above-mentioned diagnosis. In our cases hematological, biochemical, and serological investigations were negative. Angiography is useful in differentiating a splenic cyst, which is usually avascular, from solid malignant tumors (lymphoma, sarcoma), which usually have neoplastic vasculature in a disorganized pattern[10,13]. Ultrasonography is able to see that the cysts are either anechoic or hypoechoic and they have a smooth thin wall[14], whereas solid tumors are either isoechoic or hypoechoic. In addition, computerized tomography and magnetic resonance imaging may give most of the necessary information, regarding the morphology of the cyst, the composition of the cystic fluid, the location in the spleen, the position of the cyst and its relationship with the surrounding tissues[5,7,10]. Calcifications of both the primary and secondary cysts are frequently found, which are useful in diagnosing cysts from other causes of splenomegaly[5]. In our cases, ultrasonography and computerized tomography had preoperatively set the diagnosis of solitary unilocular noncalcified splenic cysts.

Due to the increased risk of complications in splenic cysts with a diameter larger than 4-5 cm should be managed surgically[9,11,15], because conservative options, such as percutaneous aspiration or sclerosis, do not result in long-term control[5,8,16]. There are different types of surgical treatment according to the patient's age and the size, location and nature of the cyst. The classical approach to splenic cysts has been open complete splenectomy[5,8,17]. However, there was a trend towards more conservative surgery after the 1970s, because of the appearance of overwhelming life-threatening septicemia, especially in children who underwent splenectomy[5,10,18]. Indeed, the spleen plays an important role in hematopoiesis, immune function, and protection against infections and malignancies[5,19]. Today the optimal treatment options are partial splenectomy, total cystectomy, marsupialization, or cyst decapsulation (unroofing), accessed either by open laparotomy or laparoscopy[5,11,19,20,21].

Partial splenectomy preserves more than 25% of splenic parenchyma, which is the minimal splenic tissue to preserve immunologic protection without increasing the risk of recurrence[5,20]. Partial splenectomy can be performed safely with the laparoscopic approach[4,11,20,21]. This procedure is recommended, if the cyst is located in the poles of the spleen, or if the cyst cavity is deep, due to the higher risk of recurrence[5,8]. Incision of the splenic capsule and hemostasis is performed with the ultrasonic or the monopolar scissors[5,11]. A more conservative option could be a partial cystectomy (unroofing) of the cyst. However, it has yet to be determined how much of the cyst wall should be resected, and whether unroofing should be partial or radical. It is supported that as much of the cyst wall as possible should be resected to prevent reclosure of the cyst[11,15].

Marsupialization of the cyst is another conservative option recommended for superficial splenic cysts, and can be performed safely with the laparoscopic method. This approach reduces the duration of the operation and carries no risk of recurrence[5,8]. In general, the laparoscopic management of splenic cysts offers the benefits of minimally invasive surgery: minimal postoperative pain, faster recovery, shorter hospital stay, and reduced morbidity and recovery[21].

However, any type of conservative procedure is difficult to perform, if the cyst is very large, is located in the splenic hilum, or is covered completely by the splenic parenchyma (intrasplenic cyst), or if there are multiple cysts (polycystic cases): in these cases, a complete splenectomy should be performed either using the open or the laparoscopic appro ach[1,21,22,23].

In all our cases, the cysts were of significant size and had produced clinical manifestations. Therefore, surgical treatment was absolutely indicated. Our first and second cases have been treated earlier, when laparoscopic splenectomy was not performed routinely in our center. In both the cases, we had to treat large cysts located in the splenic hilum, whereas the splenic parenchyma consisted of a rim of tissue pushed to the periphery. Therefore, both indications were met, location and dimension, and a successful open complete splenectomy was accomplished. In both cases the cysts were proved to be true epithelial cysts.

In the third case, there was a large subcapsular cyst located in the lower pole of the spleen, and thus a laparoscopic cystectomy looked as a feasible option. A partial cystectomy was done using the monopolar scissors followed by placement of a drainage tube in the remaining cavity. In this case, histology revealed a cyst without an epithelial lining (pseudocyst), and thus recurrence is not expectable[15,24]. Since there was no history of trauma, this cyst was probably of degenerative origin. We conclude that laparoscopic partial cystectomy is considered as an adequate procedure in cysts devoid of epithelial lining[24], as was our third case. No neoplastic growth has been found in any of our cases.

In conclusion, splenic cysts larger than 5 cm or symptomatic ones should be treated surgically, trying to preserve as much of splenic parenchyma as possible. If the cyst is very large and almost completely covered by splenic parenchyma, or if it is located in the splenic hilum, complete splenectomy is recommended, because of the risk of intractable bleeding from the spleen. Partial cystectomy (unroofing) could be an acceptable procedure in the majority of other cases. The laparoscopic approach seems to be a safe procedure, having all the benefits of minimally invasive surgery.

REFERENCES

1 **Avital S**, Kashtan H. A large epithelial splenic cyst. *N Engl J Med* 2003; **349**: 2173-2174
2 **Safioleas M**, Misiakos E, Manti C. Surgical treatment for splenic hydatidosis. *World J Surg* 1997; **21**: 374-378 discussion
3 **Reddi VR**, Reddy MK, Srinivas B, Sekhar CC, Ramesh O. Mesothelial splenic cyst-a case report. *Ann Acad Med Singapore* 1998; **27**: 880-882
4 **Heidenreich A**, Canero A, di Pasquo A. Laparoscopic approach for treatment of a primary splenic cyst. *Surg Laparosc Endosc* 1996; **6**: 243-246
5 **Hansen MB**, Moller AC. Splenic cysts. *Surg Laparosc Endosc percutan Tech* 2004; **14**: 316-322
6 **Ough YD**, Nash HR, Wood DA. Mesothelial cysts of the spleen with squamous metaplasia. *Am J Clin Pathol* 1981; **76**: 666-669
7 **Robertson F**, Leander P, Ekberg O. Radiology of the spleen. *Eur Radiol* 2001; **11**: 80-95
8 **Smith ST**, Scott DJ, Burdick JS, Rege RV, Jones DB. Laparoscopic marsupialization and hemisplenectomy for splenic cysts. *J Laparoendosc Adv Surg Tech A* 2001; **11**: 243-249
9 **Trompetas V**, Panagopoulos E, Priovolou-Papaevangelou M, Ramantanis G. Giant benign true cyst of the spleen with high serum level of CA 19-9. *Eur J Gastroenterol Hepatol* 2002; **14**: 85-88
10 **Labruzzo C**, Haritopoulos KN, EL Tayar AR, Hakim NS. Posttraumatic cyst of the spleen: a case report and review of the literature. *Int Surg* 2002; **87**: 152-156
11 **Till H,** Schaarschmidt K. Partial laparoscopic decapsulation of congenital splenic cysts. *Surg Endosc* 2004; **18;** 626-628
12 **Knudson P,** Coon W, Schnitzer B, Liepman M. Splenomegaly without an apparent cause. Surg Gynecol Obstetr 1982; **155:** 705-708
13 **Nakashima A,** Nakashima K, Seto H, Kamei T, Kakishita M, Kitagawa M. Primary splenic lymphoma presenting as a large cyst. *Radiat Med* 1994; **12:** 42-45
14 **Siniluoto TM,** Paivansalo MJ, Lahde ST, Alavaikko MJ, Lohela PK, Typpo AB, Suramo IJ. Nonparasitic splenic cysts. Ultrasonographic features and follow-up. *Acta Radiol* 1994; **35:** 447-451
15 **Morgenstern L.** Nonparasitic splenic cysts: pathogenesis, classification and treatment. *J Am Coll Surg* 2002; **194:** 306-314
16 **Cowles RA,** Yahanda AM. Epidermoid cyst of the spleen. *Am J Surg* 2000; **180:** 227
17 **Desai MB,** Kamdar MS, Bapat R, Modhe JM, Medhekar ST, Kokal KC, Abraham P. Splenic cysts: (report of 2 cases and review of the literature). *J Postgrad Med* 1981; **27:** 251-252
18 **Grinblat J,** Gilboa Y. Overwhelming pneumococcal sepsis 25 years after splenectomy. *Am J Med Sci* 1975; **270:** 523-524
19 **Sakamoto Y**, Yunotani S, Edakuni G, Mori M, Iyama A, Miyazaki K. Laparoscopic splenectomy for a giant splenic epidermoid cyst: report of a case. *Surg Today* 1999; **29:** 1268-1272
20 **Touloukian RJ**, Maharaj A, Ghoussoub R, Reyes M. Partial decapsulation of splenic epithelial cysts: studies on etiology and outcome. *J Pediatr Surg* 1997; **32**: 272-274
21 **Tagaya N,** Oda N, Furihata M, Nemoto T, Suzuki N, Kubota K. Experience with laparoscopic management of solitary symptomatic splenic cysts. *Surg Laparosc Endosc* Percutan Tech 2002; **12:** 279-282
22 **Birmole BJ**, Kulkarni BK, Vaidya MM, Borwankar SS. Splenic cyst. *J Postgrad Med* 1993; **39**: 40-41
23 **Yagi S,** Isaji S, Iida T, Mizuno S, Tabata M, Yamagiwa K, Yokoi H, Imai H, Uemoto S. Laparoscopic splenectomy for a huge splenic cyst without preoperative drainage: report of a case. *Surg Laparosc Endosc Percutan Tech* **2003; 13:** 397-400
24 **Losanoff JE,** Richman BW, Jones JW. Laparoscopic management of splenic cysts. *Surg Laparosc Endosc Percutan Tech* 2003; **13:** 63-**64; author reply 64**

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• *CASE REPORT* •

Secondary pouchitis in a post-operative patient with ulcerative colitis, successfully treated by salvage surgery

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Abstract

We report a case of secondary pouchitis, defined as a mucosal inflammatory lesion in the ileal reservoir provoked by pouch-related complication following total colectomy and pouch anal anastomosis, which was successfully treated by salvage surgery. A 20-year-old woman with ulcerative colitis developed acute severe bloody diarrhea following proctocolectomy, ileal pouch-anal anastomosis and diverting ileostomy. She was diagnosed as having a secondary pouchitis mainly caused by a peripouch abscess and partly concerned with the abnormal pouch formation. The remnant rectum and ileal pouch were excised and ileal pouch-anal anastomosis and diverting ileostomy were constructed. The postoperative course was uneventful with no sign of pouchitis. Salvage surgery may be indicated to treat secondary pouchitis when caused by surgery-related complications.

Key words: Ulcerative colitis; Ileal pouch-anal anastomosis; Secondary pouchitis; Salvage operation

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INTRODUCTION

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the current treatment of surgical choice for ulcerative colitis and for selected familial adenomatous polyposis[1,2]. One of the main long-term complications after IPAA is inflammation of the pouch (pouchitis). Symptomatic inflammation of the ileal pouch develops in 7-40% of patients who undergo IPAA[3-6]. The etiology of pouchitis is probably a multifactorial event involving genetic, immune, microbial, and toxic mediators, but is poorly understood. Heusten first identified "secondary pouchitis" caused by surgical complication specific to the ileal reservoir and pouch anal anastomosis[7]. Most cases of pouchitis are primary with an acute course and show a good response to medical therapy, but patients with secondary pouchitis underlying surgical complications need surgical management because medical treatment alone is ineffective. In this report, we described a case of secondary pouchitis due to a peripouch abscess, blind loop formation, which had no response to medical treatment, and so we selected a surgical procedure suitable to the disease state.

CASE REPORT

This case was about a 20-year-old woman who had been diagnosed as having an ulcerative colitis when she was 12 years old. She had been controlled in remission with medical therapy (prednisone and 5-aminosalicylic acid), but on July 2001, she relapsed to severe colitis and was admitted in another hospital. She was treated there with intravenous fluids, broad spectrum parenteral antibiotics, systemic steroids (prednisone 60 mg/d), and granulocytapheresis (GCAP). Despite this conservative therapy, her condition deteriorated and she had underwent laparoscopic total colectomy, ileal pouch-anal anastomosis and diverting ileostomy.

About 3 mo after the primary operation, she had episodes 10 times a day of continuous severe bloody diarrhea. Colonoscopy showed inflammatory mucosa spontaneously bleeding from the anal verge to the ileal J pouch; and was diagnosed as severe pouchitis. She received metronidazole in the form of enemas, but did not respond.

On admission to our hospital, she was not pale, afebrile with a pulse rate of 90 beats/min, and had an anal bloody mucus discharge of 5-10 times per day. Laboratory studies showed that all parameters were almost normal. Culture analysis of the bloody mucus discharge showed multiplication of streptococcus agalactiae and Gram-negative rods. Pouchoscopy showed easy bleeding, inflammatory mucosa with ulcer formation, apical bride formation in the ileal J pouch and edematous colonic mucosa with inflammation from the anal verge to the anastomosis (Figure 1). A pouchogram showed an about

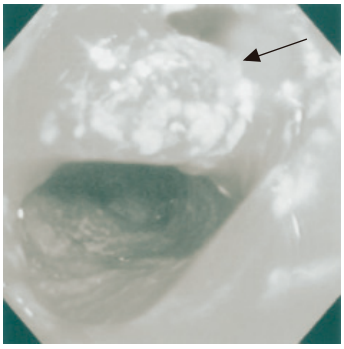

Figure 1 Pouchoscopy shows inflamed hemorrhagic mucosa. An apical bridge is seen (arrow head).

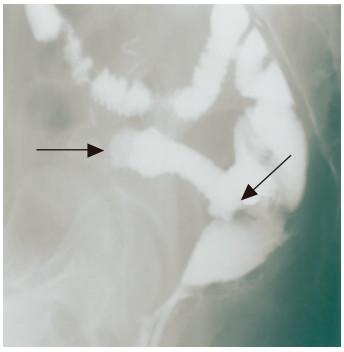

Figure 2 Pouchography shows a blind loop 10 cm in length (arrow head) and apical bridge formation (arrow).

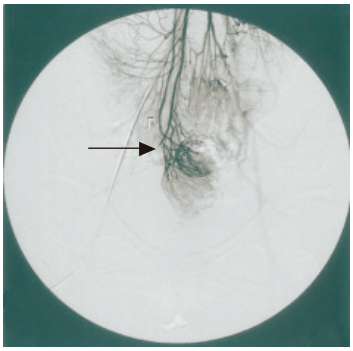

Figure 3 Superior mesenteric arteriography shows a marginal disconnection of the arcade (superior mesenteric artery–ileocolic artery) (arrow).

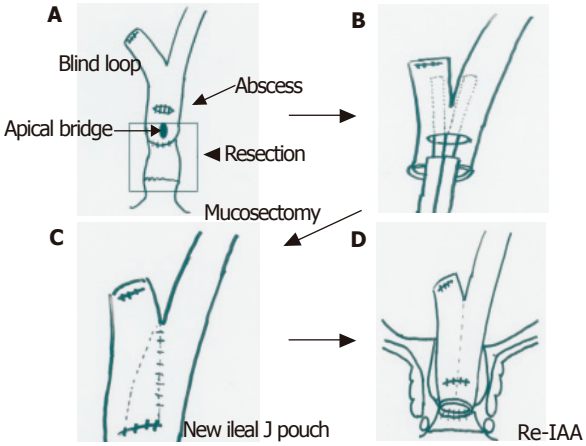

Figure 4 A: Mucosectomy and ileal pouch excision; **B:** side to side anastomosis of blind loop by linear stapler; **C:** reconstruction of a new ileal pouch; **D:** re-ileal pouch anal anastomosis.

10-cm blind loop formation of the ileal J pouch and apical bridge formation (Figure 2). Since we assumed that the operative procedures had not causally affected the outcomes, conservative therapy using metronidasole enemas, steroid enemas, and leukocytapheresis was undertaken for 1 mo.

However, no improvement of clinical symptoms or endoscopic findings was obtained. We attempted to re-evaluate using more extensive examinations. Abdominal CT showed that bilateral ovarian cysts but no remarkable intrapelvic abscess was recognized. Superior mesenteric arterial angiography showed that the ileal J pouch was supplied from the superior mesenteric artery (SMA) and the ileocolic artery (ICA), but that the arcades of these arteries were divided (Figure 3). These findings suggested that the pathogenesis of the disease was secondary pouchitis, due to abnormal formation of the ileal J pouch constructed apical bridge and blind loop or/and ischemic change of the pouch.

On 27 February 2002, she underwent laparotomy. At laparotomy, adhesion in the intra-abdominal space was recognized, but no ascites was seen. From the dentate line, rectal mucosectomy and ileal J pouch excision was performed at first. A peripouch abscess was recognized on the posterior wall of the pouch, and was tightly adhesive to the sacrum. Pouch mucosa on the site of the abscess had erosion and was partly deficient. We diagnosed secondary pouchitis due to a late peripouch abscess. We undertook abscess drainage, excision of the remnant rectum and ileal J pouch including the apical bridge. Side to side anastomosis of the blind loop was performed using a linear stapler into the site of the wall deficiency. After re-ileoanal anastomosis was performed, a covering ileostomy was constructed (Figures 4A-D).

There was a sustained clinical improvement in the patient after this re-operation without any complications. She was discharged on March 25, 2002. Three months later, ileostomy closure was performed. She remains well, with no sign of pouchitis, and has complete continence with bowel movements, 5 times per day.

DISCUSSION

Pouchitis is a term coined by Kock[8] that described the reservoir ileitis that developed in a number of patients with ulcerative colitis who underwent continent ileostomy operations. In 1980, proctocolectomy with ileal reservoir and anal anastomosis was described by Parks[9] and this operation, when performed for ulcerative colitis,

also produced pouchitis in a number of patients. The causes of the pouchitis have been poorly understood. Possible causes advanced include fecal stases resulting in bacterial overgrowth and infection[10], ischemia[11], oxygen free radical injury[12] and deprivation of short chain fatty acids[13].

Data on incidence and prevalence of pouchitis in the literature shows great disparities[14,15]. One reason for this disparity is related to the varying durations and modes of follow-up, the lack of a commonly accepted definition of pouchitis, and the lack of valid scoring for estimating the severity of inflammation[16-20]. Another reason is that pouchitis caused by surgical complication is not widely recognized. Heuschen[7] coined the term "secondary pouchitis" caused by surgical complication specific to the ileal reservoir and pouch-anal anastomosis. These surgical complications can cause clinical symptoms, such as an elevated frequency of defecation, hematochesia, fever, and malaise, which are very similar to those of idiopathic pouchitis. Nonspecific medical treatment of these symptoms as with primary pouchitis appears to be totally inadequate.

In our case, the patient had inflammatory changes in ileal J pouch mucosa with a defunctioned pouch. Pouch formation was abnormal with an apical pouch bridge[21] and an about 10-cm blind loop of the ileal pouch. Furthermore, medical treatment such as with metronidazole, topical steroids in the form of enemas, and GCAP were not effective. SMA angiography could suspect ischemic changes of the pouch mucosa because of the dividing of the arcades of SMA and ICA. However, a resected specimen (remnant rectum and ileal pouch) near the abscess formation showed extensive erosion and diffuse thickening. So we diagnosed that this secondary pouchitis was mainly caused by a peripouch abscess and was partly concerned with the abnormal pouch formation.

Moreover, crucial elements in our salvaged surgery were the initial resection of the peripouch abscess and the damaged portion of the pouch, and then the reconstruction of the pouch and re-IAA; thus improving hopes of curability.

In conclusion, salvage surgery might be indicated as a first choice treatment for secondary pouchitis caused by surgery-related complications. A surgical procedure suitable for the state of the disease should be selected after instituting a comprehensive series of examinations[22].

REFERENCES

1 **Williams NS.** Restorative proctocolectomy is the first choice elective surgical treatment for ulcerative colitis. *Br J Surg* 1989; **76**: 1109-1110

2 **Ambroze WL** Jr, Dozois RR, Pemberton JH, Beart RW Jr, Ilstrup DM. Familial adenomatous polyposis: results following ileal pouch-anal anastomosis and ileorectostomy. *Dis Colon Rectum* 1992; **35**: 12-15

3 **Nicholls RJ,** Belliveau P, Neill M, Wilks M, Tabaqchali S. Restorative proctocolectomy with ileal reservoir: a pathophysiological assessment. *Gut* 1981; **22**: 462-468

4 **Keighley MR.** Review article: the management of pouchitis. *Aliment Pharmacol Ther* 1996; **10**: 449-457

5 **Zuccaro G Jr**, Fazio VW, Church JM, Lavery IC, Ruderman WB, Farmer RG. Pouch ileitis. *Dig Dis Sci* 1989; **34**: 1505-1510

6 **Tytgat GN**, van Deventer SJ. Pouchitis. *Int J Colorectal Dis* 1988; **3**: 226-228

7 **Heuschen UA**, Autschbach F, Allemeyer EH, Zollinger AM, Heuschen G, Uehlein T, Herfarth C, Stern J. Long-term follow-up after ileoanal pouch procedure: algorithm for diagnosis, classification, and management of pouchitis. *Dis Colon Rectum* 2001;**44**:487-499

8 **Kock NG**, Darle N, Hulten L, Kewenter J, Myrvold H, Philipson B. ileostomy. *Curr Probl Surg* 1977; **14**: 1-52

9 **Parks AG**, Nicholls RJ, Belliveau P. Proctocolectomy with ileal reservoir and anal anastomosis. *Br J Surg* 1980; **67**: 553-558

10 **Fonkalsrud EW**, Phillips JD. Reconstruction of malfunctioning ileoanal pouch procedures as an alternative to permanent ileostomy. *Am J Surg* 1990; **227**: 245-251

11 **Chaussade S**, Denizot Y, Valleur P, Nicoli J, Raibaud P, Guerre J, Hautefeuille P, Couturier D, Benveniste J. Presence of PAF-acether in stool of patients with pouch ileoanal anastomosis and pouchitis. *Gastroenterology* 1991; **100**: 1509-1514

12 **Levin KE**, Pemberton JH, Phillips SF, Zinsmeister AR, Pezim ME. Role of oxygen free radicals in the etiology of pouchitis. *Dis Colon Rectum* 1992; **35**: 452-456

13 **Clausen MR**, Tvede M, Mortensen PB. Short-chain fatty acids in pouch contents from patients with and without pouchitis after ileal pouch-anal anastomosis. *Gastroenterology* 1992; **103**: 1144-1153

14 **Sandborn WJ.** Pouchitis following ileal pouch-anal anastomosis: definition, pathogenesis, and treatment. *Gastroenterology* 1994; **107**: 1856-1860

15 **Kuhbacher T**, Schreiber S, Runkel N. Pouchitis: pathophysiology and treatment. *Int J Colorectal Dis* 1998; **13**: 196-207

16 **Rauh SM**, Schoetz DJ Jr, Roberts PL, Murray JJ, Coller JA, Veidenheimer MC. Pouchitis--is it a wastebasket diagnosis? *Dis Colon Rectum* 1991; **34**: 685-689

17 **Dozois RR**, Kelly KA, Welling DR, Gordon H, Beart RW Jr, Wolff BG, Pemberton JH, Ilstrup DM. Ileal pouch-anal anastomosis: comparison of results in familial adenomatous polyposis and chronic ulcerative colitis. *Ann Surg* 1989; **210:** 268-71; discussion 272-273

18 **Luukkonen P**, Jarvinen H, Tanskanen M, Kahri A. Pouchitis--recurrence of the inflammatory bowel disease? *Gut* 1994; **35**: 243-246

19 **Keranen U**, Luukkonen P, Jarvinen H. Functional results after restorative proctocolectomy complicated by pouchitis. *Dis Colon Rectum* 1997; **40**: 764-769

20 **Svaninger G**, Nordgren S, Oresland T, Hulten L. Incidence and characteristics of pouchitis in the Kock continent ileostomy and the pelvic pouch. *Scand J Gastroenterol* 1993; **28**: 695-700

21 **Sakanoue Y**, Shoji Y, Kusunoki M, Utsunomiya J. Transanal division of an apical pouch bridge after restorative proctocolectomy with a J shaped reservoir. *Br J Surg* 1993; **80**: 248

22 **Thoeni RF**, Fell SC, Engelstad B, Schrock TB. Ileoanal pouches: comparison of CT, scintigraphy, and contrast enemas for diagnosing postsurgical complications. *Am J Roentgenol* 1990; **154**: 73-78

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• *CASE REPORT* •

Crohn's disease and recurrent appendicitis: A case report

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Abstract

The clinical diagnosis of classic Crohn's disease (CD) of the small bowel is based on a typical history, tender right lower quadrant fullness or mass, and characteristic radiographic findings of the terminal ileum. Appendicitis may as well present with chronic or recurrent symptoms and this presentation may be confused with CD. We herein describe the case of a young teenage girl with a presumptive diagnosis of CD, who was ultimately diagnosed as having chronic nongranulomatous appendicitis. The literature on the subject is reviewed.

Key words: Chronic appendicitis; Crohn's disease; Diagnosis; Terminal ileitis; Adolescent

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INTRODUCTION

Crohn's disease (CD) is a chronic transmural inflammation, which may involve any part of the alimentary tract from mouth to the anus. CD typically affects the ileum, colon, or perianal region[1]. The clinical diagnosis of classic CD of the small bowel is based on a typical history, tender right lower quadrant fullness or mass, characteristic radiographic findings of the terminal ileum and endoscopic findings. Although granulomas are regarded as the characteristic feature of CD, their absence does not rule out the diagnosis[1]. Only half of the documented cases of CD reveal epithelioid granulomas in surgical specimens[1]. CD can involve the appendix by extension from the terminal ileum or the cecum and present as an acute or subacute appendicitis. About 25% of patients with ileal CD and 50% of those with colonic Crohn's disease have appendiceal involvement[2]. A clinical picture similar to acute appendicitis is not an uncommon presentation of Crohn's disease.

Among the inflammatory diseases of the right lower quadrant that may mimic CD, acute appendicitis is the most common and potentially the most dangerous one. The principal distinguishing features of acute appendicitis are its onset without any pre-existing history of chronic bowel symptoms and the change in the location of pain and tenderness from epigastrium to the right lower quadrant (RLQ)[1]. Appendicitis, however, may present with chronic or recurrent symptoms and this presentation may be confused with CD. Although an entity of recurrent attacks of RLQ pain, apparently self-limiting, is not infrequently encountered, the entity of chronic or recurrent appendicitis is controversial[3-7]. Nevertheless, it is probably an authentic clinical and pathologic entity[3-15]. Intermittent bouts of obstruction of the appendiceal lumen with spontaneous remission may be the cause[4]. True chronic inflammation of the appendix is difficult to define as a pathologic entity, although occasionally granulation tissue and fibrosis associated with acute and chronic inflammation of the appendix are demonstrated, suggesting an organizing acute appendicitis[16]. In the pediatric population, children with cystic fibrosis can develop chronic appendicitis due to mucous engorgement of the lumen. The occurrence of chronic appendicitis in otherwise healthy children is nevertheless debatable[6].

Herein, we have described the case of a young teenage girl with a presumptive diagnosis of CD, who was ultimately diagnosed as having chronic non-granulomatous appendicitis. The literature on the subject has been reviewed.

CASE REPORT

A 17-year-old girl presented to our pediatric emergency room because of persistent RLQ abdominal pain. She had been previously healthy. Three weeks prior to admission, she was admitted to another hospital for severe RLQ pain. A computerized tomography (CT) scan of the abdomen with barium contrast showed a bowel conglomerate in the cecal area and a stricture of the distal ileum. A presumptive diagnosis of CD was made and the patient was discharged

Table 1 Laboratory values

	1st admission	2nd admission	3rd admission	4th admission	Normal values
HB	12	12.4	13.2	13.8	11.5–16.5 g/dL
WBC	8.0	17.7	5.7	9.1	4.0–11.0×109/L
PLT	322	197	371	309	150–400×109/L
ESR	NA	20	53	6	0–20 mm/h
CRP	2.4	27	0	0.28	0–1 mg/L
ALB	3.7	3.6	4.4	4.8	3.2–5 g/dL
AST	27	15	65	17	10–40 U/L
ALT	18	46	61	15	10–40 U/L
GGT	10	16	105	47	7–33 U/L

HB: hemoglobin; WBC: white blood cells; PLT: platelets; ESR: erythrocyte sedimentation rate; CRP: C reactive protein; ALB: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase.

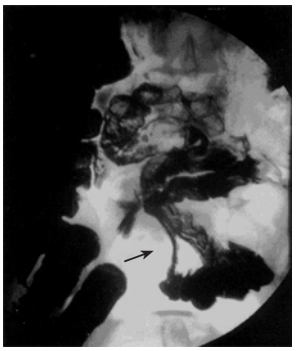

Figure 1 Upper gastrointestinal and barium follow-through study showing a stricture of an ileal loop without involvement of the terminal ileum (arrow).

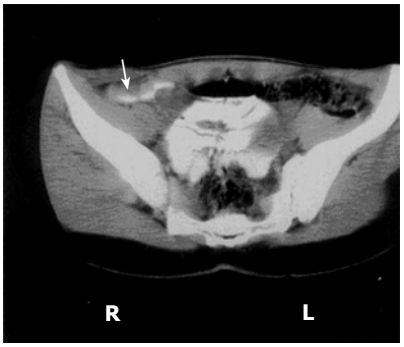

Figure 2 Abdominal CT with contrast media showing a bowel conglomerate in the cecal area and a stricture of the distal ileum (arrow).

on 5-ASA, antibiotics and steroids. Colonoscopy, performed a week later, showed no gross pathology and the treatment was discontinued. Biopsy samples from the colon and terminal ileum were normal.

She was admitted to our Pediatric Department because of the continuation of RLQ abdominal pain, recurrent non-bilious vomiting and a 6-kg weight loss over 2 wk.

Physical examination revealed a well-looking young girl with 45 kg body weight and 167 cm height. Diffuse tenderness of the abdomen and right flank was noted. Rectal examination revealed no masses, normal stools and no localized tenderness.

Laboratory results are shown in Table 1 (1st admission). Abdominal ultrasound (US) was normal; a gynecological US showed a left ovarian cyst and a tubular structure in the RLQ. An upper gastrointestinal and barium follow-through study showed a stricture of an ileal loop without involvement of the terminal ileum. This finding was considered to be compatible with CD (Figure 1). Gastroscopy showed gastritis with hemorrhagic areas in the body and antrum. A quick urease test for *H pylori* was negative. Biopsies from the antral mucosa showed remodeling of the foveolar layer, regeneration, atypia, and mild chronic inflammation. Biopsies from the small bowel, gastric corpus and esophagus were normal.

Despite the absence of characteristic histological findings of CD, the clinical presentation and the radiological findings were thought to be compatible with CD and therefore oral steroids and omeprazole were prescribed with resolution of symptoms.

Four weeks later, she was readmitted because of a 24-h history of abdominal pain mainly over the RLQ, fever of 39 °C and non-bilious vomiting.

Physical examination revealed a sick-looking girl having 37.8 °C temperature, 47.5 kg body weight and severe diffuse tenderness over the right lower abdomen. Laboratory results are shown in Table 1 (2nd admission). Abdominal X-ray was normal. Abdominal US showed a thickened bowel loop located over the RLQ. A repeat CT of the abdomen with contrast media showed a bowel conglomerate in the cecal area and a stricture of the distal ileum similar to the findings previously demonstrated. No signs of appendicitis or periappendicular abscess were noted (Figure 2). After 5 d of intravenous treatment with steroids, ciprofloxacin, and metronidazole, the patient's symptoms and clinical signs improved and she was discharged on oral steroids and azathioprine.

For the following 2 mo, the patient was asymptomatic. She was gradually weaned from steroids and maintained on azathioprine. She was then readmitted because of abdominal pain and non-bilious vomiting for 10 d. She had no fever or diarrhea, her appetite was poor and had a 1 kg weight loss. Her physical examination revealed severe diffuse tenderness over the right abdomen. Laboratory results are shown in Table 1 (3rd admission). Budesonide was started and the azathioprine dose was decreased due to abnormal liver enzymes. She was discharged after a clinical improvement but readmitted a week later due to vomiting and epigastric and RLQ pain for 4 d. Laboratory results are shown in Table 1 (4th admission). Gastroscopy showed a mild inflammation with normal antral and duodenal biopsies. A diagnostic laparoscopy showed a phlegmonous appendicitis and an appendectomy was performed. No other abnormality was noted and the ileum appeared to be normal. Histological examination of the appendix and terminal ileum showed periappendicular inflammation and normal small bowel. No granulomata were found. A diagnosis of chronic appendicitis mimicking CD was made. Follow-up of 4 years showed a healthy asymptomatic girl.

DISCUSSION

Controversy and skepticism surround the diagnoses of chronic and recurrent appendicitis[3-6]. Nevertheless, a sufficient histological and radiological evidence to support that this entity exists[3-16].

Chronic appendicitis is defined as lower abdominal pain lasting for 3 wk with radiological and pathological findings of appendicitis. Recurrent appendicitis is defined as recurring episodes of right lower abdominal pain with radiological or pathological finding of appendicitis[17].

Five to ten percent of patients suffering from acute appendicitis describe prior episodes of RLQ pain resolving spontaneously. USG studies have demonstrated a spontaneous resolution of acute appendicitis, in as many as 1 in 13 patients suffering from symptoms of acute appendicitis. The recurrence rate, however, was 38%, mostly in the year following the initial episode[9]. The CT diagnosis of acute appendicitis includes distended or thickened appendix with or without appendicolith, greater than approximately 5-7 mm in size. The wall of the inflamed appendix is circumferentially thickened and may appear as a "halo" or "target." CT findings of periappendiceal inflammation would also support the diagnosis of appendicitis[4,18]. Moreover, CT findings of appendices in patients suffering from prolonged (more than 3 wk) abdominal pain as well as recurring episodes of abdominal pain are identical to those of patients suffering from acute appendicitis[17]. None of these findings were present in the CT done in our patient. It is important to mention that advances in CT with high-resolution techniques have yielded a sensitivity as high as 100% and specificity as high as 98% for the diagnosis of acute appendicitis[18]. The yield of CT in the diagnosis of CD is not good.

Mattei *et al*.[14] reviewed a series of seven patients matching the criteria of chronic or recurrent appendicitis and concluded that recurrent and chronic appendicitis should be considered in the differential diagnosis of recurrent or non-resolving lower abdominal pain. Barber *et al*.[3], in their retrospective study encompassing 1 084 patients who had inflamed appendices removed, found that 6.5% of them had attended the emergency ward prior to their operation for symptoms compatible with recurrent appendicitis. Our patient had clinical and radiological findings compatible with Crohn's ileitis, without evidence of acute or chronic appendicitis on US and CT. It has been suggested that these modalities can aid diagnosis in questionable situations[1]. Although there was no histopathological evidence of CD at biopsy, the clinical manifestations, the radiological findings and the response to treatment supported the diagnosis.

Consideration was also given to the diagnosis of isolated CD of the appendice, an uncommon chronic appendicitis with histological findings (granulomas) resembling a CD[2]. This rare presentation of CD is confined to the appendice and progression to systemic disease is rarely noted. The reluctance to intervene surgically in a patient with a suspected CD is another barrier to diagnosis.

Considering the data presented, chronic or recurrent appendicitis seemed to be the probable diagnosis. It must be considered in every case of unexplained long-standing or recurrent abdominal pain over RLQ. The great improvements in laparoscopic techniques should be valuable in those cases where endoscopical, histological and radiological findings do not provide a definitive answer.

REFERENCES

1 **Kornbluth A**, Sachar DB, Salomon P. Crohn's disease. In: Feldman M, Scharschmidt BF, Sleisenger MH, Klein S, editors. Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 6th ed. W. B. Saunders Company, 1998: 1708-1734

2 Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 10-1996. A 36-year-old man with right-lower-quadrant pain of two years' duration. *N Engl J Med* 1996; **334:** 849-854

3 **Barber MD**, McLaren J, Rainey JB. Recurrent appendicitis. *Br J Surg* 1997; **84**: 110-112

4 **Lally KP**, Cox CS, Andrassy RJ. Appendicitis. In: Townsend CM, Beauchamp RD, Evers BM, Mattox KL, editors. Sabiston Textbook of Surgery. 16th ed. W. B. Saunders Company, 2001: 918-926

5 **Leardi S**, Delmonaco S, Ventura T, **Chiominto A, De Rubeis G,** Simi M. Recurrent abdominal pain and "chronic appendicitis". *Minerva Chir* 2000; **55**: 39-44

6 **Schrock TR**. Appendicitis. In: Feldman M, Scharschmidt BF, Sleisenger MH, Klein S, editors. Sleisenger & Fordtran' s Gastrointestinal and Liver Disease. 6th ed. W. B. Saunders Company, 1998: 1778-1787

7 **Seidman JD,** Andersen DK, Ulrich S, Hoy GR, Chun B. Recurrent abdominal pain due to chronic appendiceal disease. *South Med J* 1991; **84:** 913-916

8 **Babb RR,** Trollope ML. Recurrent appendicitis. Uncommon, but it does occur. *Postgrad Med* 1999; **106:** 135-138

9 **Cobben LP**, de Van Otterloo AM, Puylaert JB. Spontaneously resolving appendicitis: frequency and natural history in 60 patients. *Radiology* 2000; **215**: 349-352

10 **Crabbe MM,** Norwood SH, Robertson HD, Silva JS. Recurrent and chronic appendicitis. *Surg Gynecol Obstet* 1986; **163:** 11-13

11 **Falk S**, Schutze U, Guth H, Stutte HJ. Chronic recurrent appendicitis. A clinicopathologic study of 47 cases. *Eur J Pediatr Surg* 1991; **1**: 277-281

12 **Fayez JA**, Toy NJ, Flanagan TM. The appendix as the cause of chronic lower abdominal pain. *Am J Obstet Gynecol* 1995; **172**: 122-123

13 **Graham D**. Chronic appendicitis: it does exist. *Tenn Med* 2001; **94**: 23-24

14 **Mattei P**, Sola JE, Yeo CJ. Chronic and recurrent appendicitis are uncommon entities often misdiagnosed. *J Am Coll Surg* 1994; **178**: 385-389

15 **Van Winter JT**, Wilkinson JM, Goerss MW, Davis PM. Chronic appendicitis: does it exist? *J Fam Pract* 1998; **46**: 507-509

16 **Crawford JM**. The Gastrointestinal tract. In: Cotran RS, Kumar V, Collins T, editors. Robbins Pathologic Basis of Disease. 6th ed. W.B. Saunders Company, 1999: 775-844

17 **Rao PM**, Rhea JT, Novelline RA, McCabe CJ. The computed tomography appearance of recurrent and chronic appendicitis. *Am J Emerg Med* 1998; **16**: 26-33

18 **Pena BM**, Taylor GA, Fishman SJ, Mandl KD. Costs and effectiveness of ultrasonography and limited computed tomography for diagnosing appendicitis in children. *Pediatrics* 2000; **106**: 672-676

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2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]

3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287 [CMFAID:1082371101835979]

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4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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5 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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6 **Morse SS.** Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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Present as mean±SD and mean±SE.

Statistical expression

Express *t* test as *t*(in italics), *F* test as *F*(in italics), chi square test as χ^2(in Greek), related coefficient as *r*(in italics), degree of freedom as γ (in Greek), sample number as *n*(in italics), and probability as *P*(in italics).

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Use SI units. For example: body mass, m(B) = 78 kg; blood pressure, p(B)=16.2/12.3 kPa; incubation time, t(incubation)=96 h, blood glucose concentration, c(glucose) 6.4±2.1 mmol/L; blood CEA mass concentration, p(CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2 not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23,243,641 should be read 23 243 641.

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Italicization

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc*.

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.

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Number	Nonstandard	Standard	Notice
1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku,18 500u or M_r18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μmol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD_{260}	A_{260}	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance in statistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If there is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^{\#}F$=67.4	^1F=9.87,^2F=25.9, ^3F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	10×10×10cm^3	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$\bar{x}\pm s$	mean±SD	In figures, tables or text narration
68	– Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	