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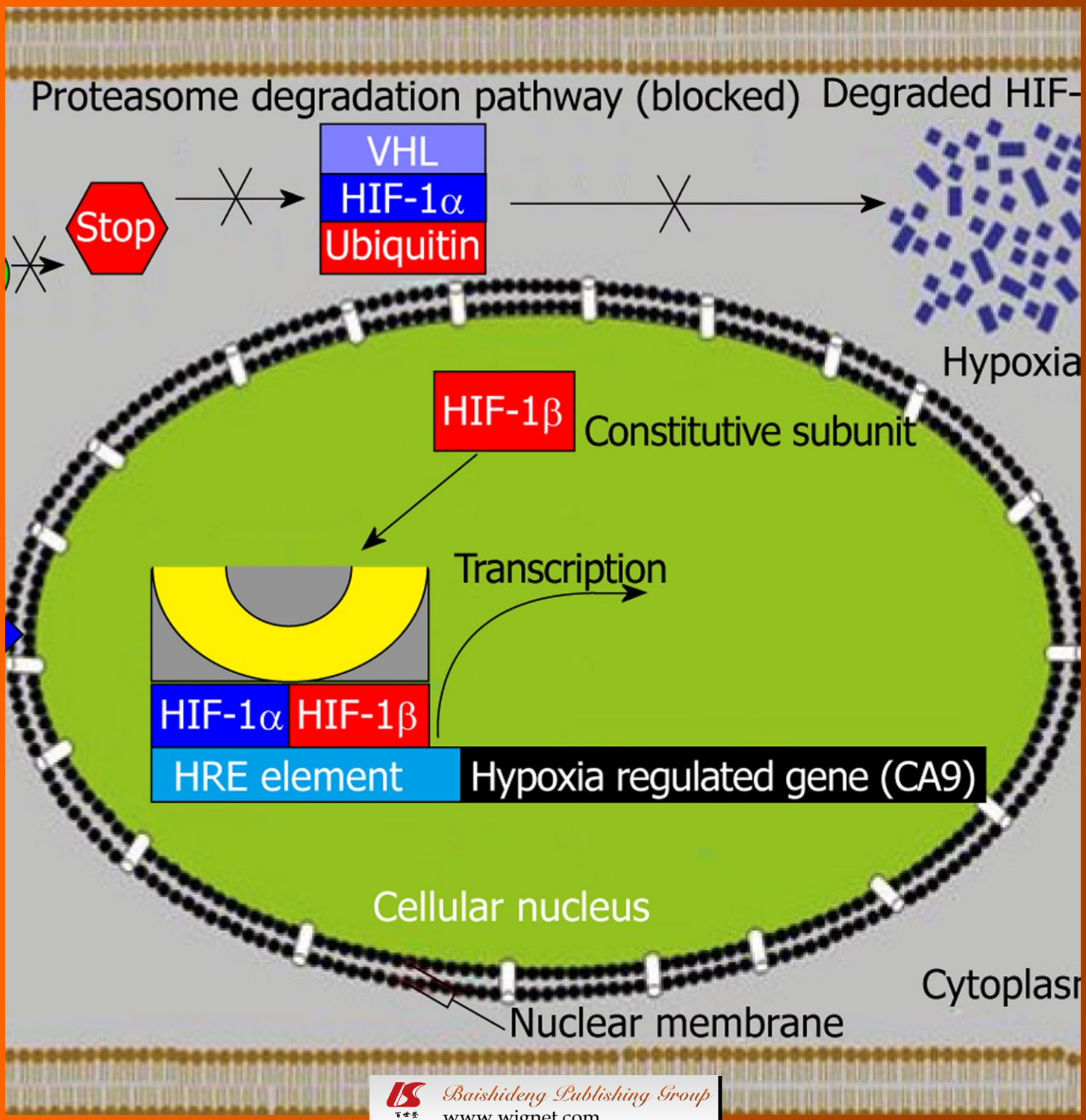

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Contents

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Contents

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ORIGINAL ARTICLE

Hypoxia and cytokines regulate carbonic anhydrase 9 expression in hepatocellular carcinoma cells *in vitro*

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Abstract

AIM: To study the expression of carbonic anhydrase (CA) 9 in human hepatocellular carcinoma (HCC) cells.

METHODS: We studied CA9 protein, CA9 mRNA and hypoxia-inducible factor-1 alpha (HIF-1α) protein levels in Hep3B cells exposed in different parallel approaches. In one of these approaches, HCC cells were exposed to extreme *in vitro* hypoxia (24 h 0.1% O_2) without or with interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) stimulation for the same hypoxic exposure time or exposed to normoxic oxygenation conditions without or with cytokine stimulation.

RESULTS: The tumour cell line analysed showed a strong hypoxic CA9 mRNA expression pattern in response to prolonged severe hypoxia with cell-line specific patterns and a marked induction of CA9 protein in response to severe hypoxia. These results were paralleled by the results for HIF-1α protein under identical oxygenation conditions with a similar expression tendency to that displayed during the CA9 protein expression experimental series. Continuous stimulation with the cytokines, IL-1, IL-6, TNF-α and TGF-β, under normoxic conditions significantly increased the carbonic anhydrase 9 expression level at both the protein and mRNA level, almost doubling the CA9 mRNA and CA9 and HIF-1α protein expression levels found under hypoxia. The findings from these experiments indicated that hypoxia is a positive regulator of CA9 expression in HCC, and the four signal transduction pathways, IL-1, IL-6, TNF-α and TGF-β, positively influence CA9 expression under both normoxic and hypoxic conditions.

CONCLUSION: These findings may potentially be considered in the design of anti- cancer therapeutic approaches involving hypoxia-induced or cytokine stimulatory effects on expression. In addition, they provide

evidence of the stimulatory role of the examined cytokine families resulting in an increase in CA9 expression under different oxygenation conditions in human cancer, especially HCC, and on the role of the *CA9* gene as a positive disease regulator in human cancer.

Key words: Angiogenesis; Carbonic anhydrase 9; Hypoxia; Hypoxia-inducible factor-1 alpha; Oxygen; Radiotherapy; Transforming growth factor-beta; Tumour microenviroment

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INTRODUCTION

Hypoxia influences the behaviour of human tumour cells *via* activation of genes involved in the adaptation to hypoxic stress which represents an important indicator of cancer prognosis and is associated with aggressive tumour growth, metastasis, poor response to treatment and malignant progression[1,2]. Hypoxia-inducible factor-1 (HIF-1) is a multi-subunit protein that regulates transcription at hypoxia response elements (HREs) and is composed of two basic helix-loop-helix proteins: a subunit, HIF-1α, and the constitutively expressed HIF-1β[3,4]. HIF-1 acts as a master regulator of numerous hypoxia inducible genes related to angiogenesis, cell proliferation/survival, and glucose/iron metabolism. Among these genes, carbonic anhydrase 9 (CA9) is one of the most strongly hypoxia-inducible genes[5] and its activity is regulated by HIF-1α under these oxygenation conditions.

CA9 is a unique transmembrane member of the *CA* gene family and is a tumour-associated protein thought to be involved in malignant cell invasion and adhesion. High levels of CA9 expression in a broad range of tumours are strongly related to its transcriptional regulation by hypoxia and high cell density, which appears to be activated by the CA9 promoter[6,7]. Induction by hypoxia occurs *via* the HIF-1 transcription factor, which accumulates in tissue under hypoxic conditions which are often present in growing tumours[8-11].

In addition to hypoxia, other stimulating factors, e.g., hormones and cytokines induce HIF-1 accumulation and activity under normoxia. Moreover, transforming growth factor-beta (TGF-β) regulates the expression of its own converting enzyme, furin, *via* a recently identified HIF-1-regulated gene[12]. The expression of CA9 can only be detected in a few normal tissues, but it is abundant in several tumours, e.g., renal cell carcinoma, cervical, lung, colorectal, bladder and breast carcinomas, adenocarcinoma, hepatocellular carcinoma (HCC), lung, head and neck cancer, cervix and uteri tumours[13-16]. Although the exact mechanisms related to the functional role of CA9 underlying the contribution of TGF-β, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)[17-20] with the exception of IL-1, are not yet known, especially their role related to tumour progression, it is known that these growth factors influence the accumulation of HIF-1 under normoxic conditions by stimulatory regulation *via* different cytokine pathways. Within this context, while the functional mechanisms related to the IL-1 induced HIF-1α regulation under hypoxia is not yet known, it is known that under normoxia it may be regulated *via* the mitogen-activated protein kinase kinase kinase pathway[21-23]. On the other hand, IL-6 regulates other HIF-1α regulated genes such as vascular endothelial growth factor[24,25], while IL-6 itself is regulated *via* the signal transducers and activators of transcription-Janus kinase pathway, and TNF-α induces HIF-1α expression through 3-phosphoinositide-dependent protein kinase-1-mediated I kappa B kinase beta[26,27] and nuclear factor "kappa-light-chain-enhancer" of the activated B-cells pathway[28] and has been shown to play a positive role in the induction of the HIF-1α regulated genes in human glioma[29,30]. In addition, enhanced levels of TGF-β are a common feature in human tumour cells[31], and TGF-β is also released by infiltrating leukocytes and induces up-regulation of HIF-1α regulated genes[18,32] most probably without the involvement of the bone morphogenetic protein family[33,34] or the Smad family[35-39] pathways, despite the fact that TGF-β uses the Smad pathway which transcriptionally represses (inhibitor of differentiation 1) Ids proteins in epithelial cells. Also, CA9 causes a reduction in extracellular pH, thereby facilitating the breakdown of the extracellular matrix together with up-regulation of the genes involved in invasion and migration[40-43].

The aim of the present study was to investigate CA9 transcriptional regulation in human cancer, especially HCC, under different oxygenation conditions, namely normoxic or extreme hypoxic conditions without or with TGF-β, IL-1, IL-6, and TNF-α stimulation. The results of this experimental series provide further understanding on CA9 transcriptional regulation under different physiological conditions in HCC, and potentially in other human cancer types of identical origin. Also, the stimulatory effect of TGF-β, IL-1, IL-6, and TNF-α which underlie the contribution to HIF-1α protein accumulation under normoxia and extreme hypoxia were investigated, including its transcriptional regulation at the mRNA and protein

expression level of CA9 in Hep3B HCC cells. This also provides the fundamental information necessary for the study of cytokine regulated non-hypoxic HIF-1α regulated CA9 expression in human cancer cells, especially HCC.

MATERIALS AND METHODS

Preparation of the nuclear extracts

Nuclear extracts were prepared as previously described[44-49] with modifications. Cells/mL (5×10^7) was scratched from Petri-dishes by adding 10 mL phosphate buffered saline (PBS) to the cellular film according to previous protocols[28] with minor modifications. A cell line pellet was obtained by centrifugation (Beckman CS-6R) for 4 min at 800 r/min. After two washing steps with PBS, cells were re-suspended in 1 mL PBS, transferred into a 1.5 mL tube and centrifuged at 4 ℃ for 45 s at 14 000 r/min. The cell pellet was re-suspended in 400 μL ice-cold buffer A (10 mmol/L Hepes pH 7.9, 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L ethyleneglycoltetraacetic acid (EGTA), 1 mmol/L PMSF, 10 μL complete protease inhibitor cocktail (Roche) and 1 mmol/L DTT) and incubated on ice for 15 min. The cells were lysed by adding 25 μL of 10% NP-40 and homogenized with 10 strokes in a Dounce homogenizer at 4 ℃ followed by centrifugation for 1 min at 14 000 r/min for nuclei sedimentation. Supernatants were carefully removed and regarded as cytoplasmic fractions. Nuclear proteins were extracted by adding 50 μL of buffer C [20 mmol/L Hepes, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF and 0.1 μL protease inhibitor cocktail (Roche)] and extensively shaking the tubes for 20 min at 4 ℃ in a tube shaker followed by centrifugation at 14 000 r/min and 4 ℃ for 5 min. The supernatant was removed and stored in aliquots at -80 ℃. All steps were performed on ice.

Cell and culture conditions and hypoxia treatment

Early passage Hep3B cell lines from the American Type Culture Collection (ATCC, Rockville, MD, United States) were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, penicillin (100 IU/mL)/ streptomycin (100 μg/mL) and 2 mmol/L L-glutamine. Cells were exposed to 0.1% O_2 for 1, 6 or 24 h in a Ruskinn Invivo2 hypoxic workstation (Cincinnati, OH, United States) as previously described[44-49] for *in vitro* hypoxia. For the reoxygenation experiments, dishes were returned to the incubator following 24 h hypoxia treatment. For cytokine stimulation, cells underwent 16 h of serum starvation before 24 h stimulation under normoxic conditions or under 24 h hypoxia exposure and stimulation with 5 ng/mL TGF-β (240B), IL-1 (200LA), IL-6 (IL-6-206) or TNF-α (210TA) all from R and D Systems, Minneapolis, MN, United States.

Isolation of total RNA from tumour cell lines and tumour tissues

Total RNA was isolated from cultured tumour cells as previously reported[47-49] and described by Kaluzová *et al*[16] including the digestion of contaminating DNA with the provided DNase. Total RNA from tumour tissues was isolated with the nucleospin RNA Ⅱ kit (Promega, Germany).

Determination of CA9 mRNA expression in vitro in HCC cell lines by semi-quantitative reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed using primers designed using published information on mRNA sequences in GenBank (sequence Accession Nos., CA9: NM_001216, β-actin NM_001101 and NM_001530.2 for HIF-1α). An aliquot of 1-5 μg of total mRNA from Hep3B cell lines was transcribed at 42 ℃ for 1 h in a 20 μL reaction mixture using 200 U RevertAidTM M-MuLV RT, oligo(dT)18 primer and 40 U ribonuclease inhibitor (all from Fermentas, Ontario, Canada). The PCR primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi), based on the information indicated above in order to amplify and produce the following: a 342 bp CA9 product, forward primer was 50-ACCCTCTCTGAC ACCCTGTG-30 and reverse primer was 50-GGCTG-GCTTCTCAC ATTCTC-30, and produce a 668 bp amplification product of β-actin, the forward primer (F1) was 5'-CGTGCGTGACATTAAGGAGA-3' and the reverse primer (R1) 5'-CACCTTCACCGTTCCAGTTT-3' and produce a 233 bp amplification product of HIF-1α, the forward primer (F1) was 5'-TTACAGCAGC-CAGACGATCA-3' and the reverse primer (R1) 5' -CCCTGCAGTAGGTTTCTGCT-3'. The PCR was performed with 25-32 cycles with increments of five cycles using PCR systems and reagents acquired from Promega™ (Promega GmbH, Mannheim, Germany) and applied according to the manufacturer's instructions. The PCR products were separated on 1% agarose gels (Sigma-Aldrich, Steinheim, Germany) and visualized by ethidium bromide staining (0.07 μg/mL ethidium bromide; Bio-Rad, Munich, Germany).

Preparation of cell lysates and immunoblotting

Tumour cell lysates were prepared with 0.1 mL RIPA buffer (1X TBS, 1% Nonidet P-40 (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A (1.4 μmol/L), aprotinin (0.15 μmol/L), leupeptin (2.3 μmol/L) and 100 μmol/L PMSF (all from Sigma, St. Louis, MO, United States). To inhibit protein dephosphorylation, phosphatase inhibitor mix (Sigma) was added. Using a syringe fitted with a 21-gauge needle to shear DNA, the lysates were transferred to a prechilled microcentrifuge tube, followed by 30 min incubation on ice. The cell lysate clearance was by centrifugation at 15 000 × *g* for 12 min at 4 ℃. Whole-cell lysates (20 μg) were separated on 8% polyacrylamide SDS gel. Electrophoresis was then transferred to a 0.45 μm nitrocellulose membrane (Protran BA 85, Schleicher

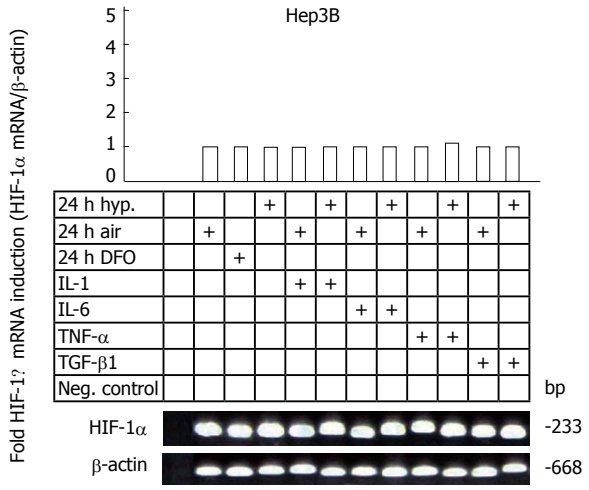


Figure 1 Hypoxia-inducible factor-1 alpha mRNA expression level determination *via* semi-quantitative reverse transcription polymerase chain reaction in the human hepatocellular carcinoma cell line Hep3B under aerobic and hypoxic conditions (0.1% O_2 for 24 h) without or with cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) under both aeration conditions examined. ?-actin was used as a loading control. Bar graphs show band intensities after densitometric evaluation. Representative experiment of three different experiments. IL: Interleukin; TNF-?: Tumor necrosis factor-alpha; TGF-?: Transforming growth factor-beta; HIF-1?: Hypoxia-inducible factor-1 alpha.

and Schuell, Dassel, Germany). Non-specific binding was blocked by 5% non-fat milk powder in Tris-buffered saline (TBS) overnight at 4 ℃ followed by incubation with the NDRG1 primary antibody (ab8448, Abcam, Cambridge, United Kingdom), diluted 1:1000 in 2.5% non-fat milk powder in TBS for 1 h at room temperature. Blots were washed twice in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and then three times in TBS for 5-10 min, each. The secondary antibody, goat anti-rabbit-HRP (stock solution: 400 ?g/mL, DakoCytomation, Denmark), was incubated at a dilution of 1:2000 for one additional hour at room temperature followed by five washing steps as described above. Detection of the bound antibodies was accomplished by membrane development with electroluminescence (ECL) plus a Western blotting detection system (Amersham Biosciences, Cambridge, United Kingdom) for 5 min with subsequent development of the Hyperfilm ECL (Amersham) used for detection purposes.

Determination of CA9 expression via fluorescence activated cell sorter analysis

CA9 expression was determined by flow cytofluorometry employing the fluorescence activated cell sorter (FACS) Calibur™ flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany, low-power argon laser excitation at 488 nm) and CellQuest Pro™ software as cell-associated fluorescence. For each analysis, about 10.000 gated events were collected. The experimental basis was previously described[46] and was successfully applied after modification. Hep3B cells were kept under aerobic as well as hypoxic (0.1% O_2) conditions for 24 h.

Densitometric evaluation of Western blotting and the statistical analysis of measurements

Protein expression signal strengths on Western blotting were determined with 1D Kodak Image analysis software. The signals were measured in Kodak light units (KLU) and divided by the corresponding signals of the loading controls ?-tubulin and ?-actin, as previously described[44-49]. The relative changes in protein expression resulting from hypoxic conditions, or hypoxic conditions with subsequent reoxygenation were analysed in relation to the 24 h normoxic value. Three to four individual experiments were always performed. The Mann-Whitney *U* test for independent samples was used to analyse these data. The Student's *t* test for unpaired samples was used to analyse overall cell numbers. In the two tests $P \leqslant 0.05$ was considered to be statistically significant. All tests were carried out using the statistical package SPSS, release 12.0.1 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Analysis of HIF-1? regulation by IL-1, IL-6, TNF-? , TGF-? and hypoxia by Western blotting and flow cytometry

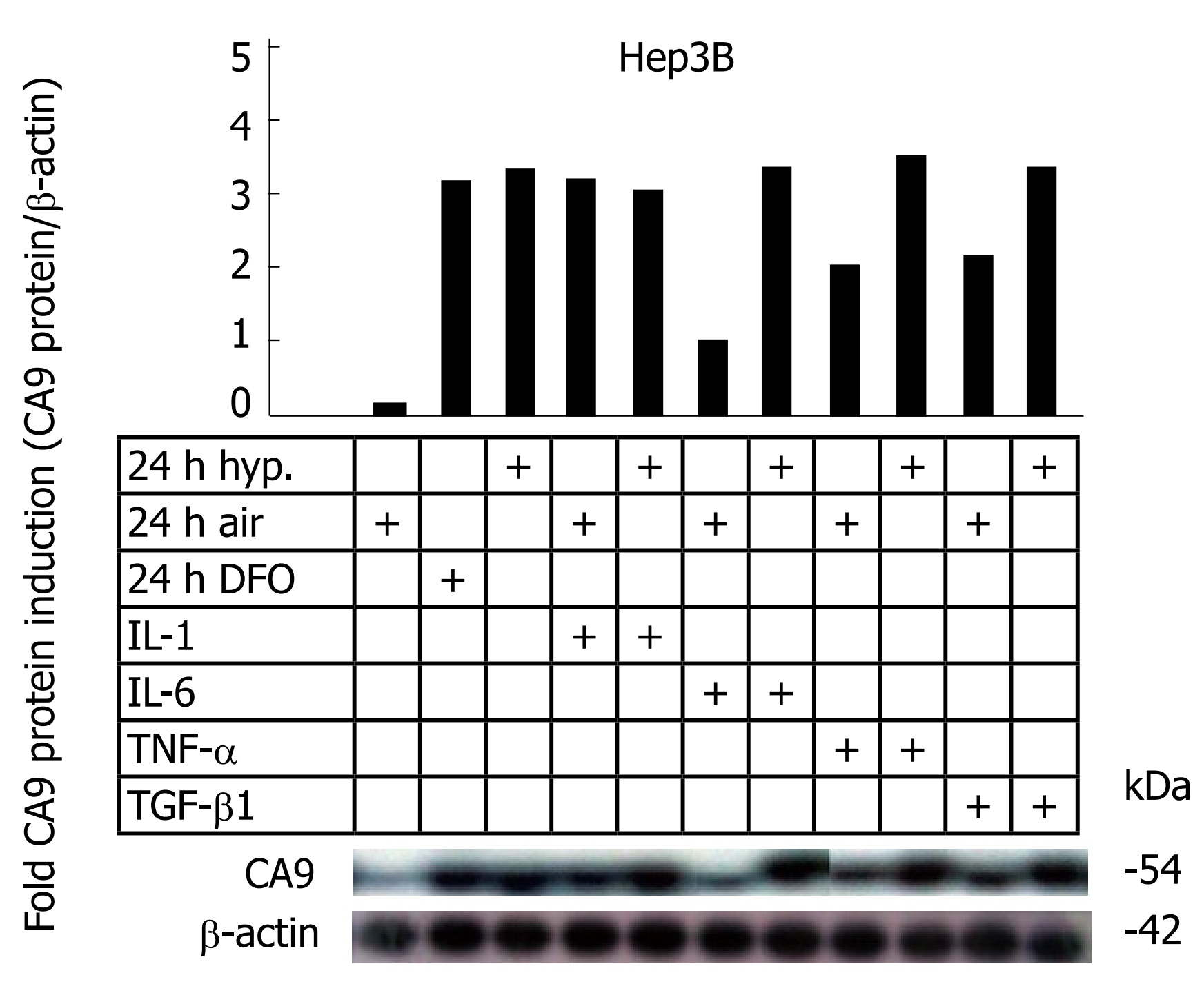
Results of the semiquantitative RT-PCR series of experiments showed that HIF-1? mRNA was evenly expressed in the cells examined (Figure 1) which were under normoxic aeration or conditions with or without IL-1, IL-6, TNF-? or TGF-? stimulation for 24 h, and showed no HIF-1? mRNA up-regulation which is a common characteristic of tumour cells under hypoxic aeration conditions.

In contrast, and in parallel sets of experiments, HIF-1? nuclear protein expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without IL-1, IL-6, TNF-? or TGF-? stimulation for 24 h, confirming oxygen-dependent HIF-1? expression regulation. This severe hypoxia-induced expression rate was almost doubled with IL-1, IL-6, TNF-? or TGF-? stimulation for 24 h under severe hypoxic conditions (0.1% O_2) (Figure 2).

Analysis of CA9 regulation by IL-1, IL-6, TNF-? , TGF-? and hypoxia by Western blotting and FacsScan analysis

Intracellular CA9 protein levels expressed in response to *in vitro* hypoxia in addition to CA9 protein levels from whole-cell lysates maintained under normoxic conditions were detectable in a cell type-specific fashion (Figure 3). Here, CA9 protein expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without stimulation with IL-1, IL-6, TNF-? or TGF-? for 24 h respectively, confirming the oxygen-dependent CA9 protein regulation. This severe hypoxia induced CA9 expression rate was almost doubled with IL-1, IL-6, TNF-? or TGF-? stimulation for 24 h under severe hypoxic conditions (0.1% O_2) (Figure 3).

Furthermore, CA9 expression was determined by flow cytofluorometry employing the FACSCalibur™ flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany, low-power argon laser excitation at 488 nm)


Hep3B
Fold CA9 protein induction (CA9 protein/β-actin)
5
4
3
2
1
0
24 h hyp.
24 h air
24 h DFO
IL-1
IL-6
TNF-α
TGF-β1
kDa
CA9
-54
β-actin
-42

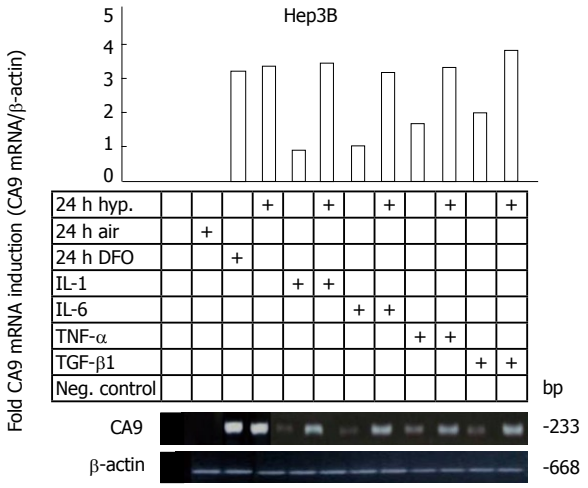


Figure 5 Regulation of hypoxia-inducible carbonic anhydrase 9 mRNA level *via* semiquantitative reverse transcription polymerase chain reaction *in vitro* in the hepatocellular carcinoma cell line Hep3B under aerobic and hypoxic conditions (0.1% O_2 for 24 h) with or without cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) under both aeration conditions and β-actin as loading control. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta; CA9: Carbonic anhydrase 9.

regulated by HIF-1α, has been shown to be induced by hypoxia in various malignant cells *in vitro*.

CA9 is under the control of a vHL tumour suppressor gene[52], while the loss of function of this gene may result in the up-regulation of HIF-1α and CA9 in affected cells. The observed relative (Figures 3-5, 6 respectively), differences in expression between CA9 protein and the corresponding CA9 mRNA may be explained by different post-transcriptional processing or post-transcriptional regulation of CA9 mRNA by CA9 protein levels[53,54]. High basal levels of HIF-1α and CA9 under normoxic conditions may be an adaptive response in cells with increased metabolic demands or altered signal transduction pathways which are unrelated to hypoxia, but govern HIF-1 cellular activity[55]. Using flow cytometry we were able to accurately discriminate CA9-negative and -positive cells *in vitro* (Figure 5). The measured percentages of CA9 positive cells closely reflected the hypoxic status of the cells. Cytokines belonging to the examined families regulated the HIF-1α-dependent CA9 expression under normoxic conditions and enhanced the development of this expression pattern due to the shift in oxygenation conditions towards the extreme hypoxic conditions[17-19,25,30,54-64].

Within this context, TGF-β-influenced functional CA9 regulation was indirect and complex. TGF-β activates hCA9 gene transcription, thereby causing an increase in mRNA and protein levels of hCA9. This up-regulation may occur in an indirect manner since there are no Smad binding elements available in the hCA9 promoter region. TGF has a clear role in HIF-1α stabilization under normoxia[42]; in addition, the hCA9 promoter is transcriptionally regulated by the HRE present within the promoter region[44], therefore, TGF-β1 transcriptionally up-regulates the hCA9 promoter. This might suggest that the TGF-β1-mediated HIF-1α regulated up-regulation of *hCA9* gene expression is *via* the TGF-β pathway which as a consequence is responsible of this functionally important regulation leading to the high level of *hCA9* expression in Hep3B cells.

The cytokines belonging to the examined cytokine families positively regulated the HIF-1α dependent CA9 expression under normoxic conditions, and enhanced the CA9 mRNA and protein expression level pattern due to the oxygenation conditions under extreme hypoxic conditions. Within this context, cancer cell type expression pattern or tendency specificity resulted from stimulation with cytokines and may be true for all cytokines examined, with the exception of TNF-α which might display other expression patterns or tendencies (Figure 6), as in the case of HT-29 human colon adenocarcinoma cells[65].

Until now, the detailed functional *CA9* gene regulation in human tumours, especially HCC, was unknown both under hypoxia and normoxic oxygenation conditions, and combined with the stimulatory effect of the different signal transduction pathways, especially IL-1, IL-6, TNF-α and TGF-β1, it is now partially clear in which cancer tissue they play a regulatory role[66,67]. IL-1, IL-6, TNF-α and TGF-β1 induced an increase in the CA9 protein level in HCC cells, which may suggest potential targets for new and more specific approaches to cancer treatment and prevention, since CA9 overexpression in the tumour is mainly responsible for tumour resistance to both radiotherapy and/or chemotherapy-based treatment approaches (it was indicated that IL-1 was responsible for radio-resistance in murine tissues[68], and that IL-1 regulated stimulation is responsible alone or in combination with the other cytokines due to the tumour starvation effect)[55]. The down-regulation of CA9 expression (with respect to the role of the post-translational regulatory effect on expression[69] by interfering with IL-1, IL-6, TNF-α and TGF-β1 signalling leading to basal expression level without affecting the fundamental cellular functions of the targeted HCC cells) should be respected when designing new approaches or modifying existing approaches with respect to the consequences associated with the knockout of these genes during human cancer progression. We also demonstrated that IL-1, IL-6, TNF-α and TGF-β transcription up-regulated *hCA9* gene expression in HCC which has an important role in hypoxia and consequently metastasis of tumours. This regulatory cycle is of potential importance in the induction, as well as, in the activation of numerous factors implicated in the pathogenesis of cancer. Therefore, further studies to explore the mechanism(s) involved or related to this regulative process are required. There are two possible explanations for the regulatory behaviour of the regulated HIF-1α-dependent CA9 expression under normoxic or hypoxic conditions, either it enhanced this expression or it was due to the stimulatory effect of IL-1, IL-6, TNF-α and TGF-β1, or there was dynamic interplay between hypoxia and the stimulatory

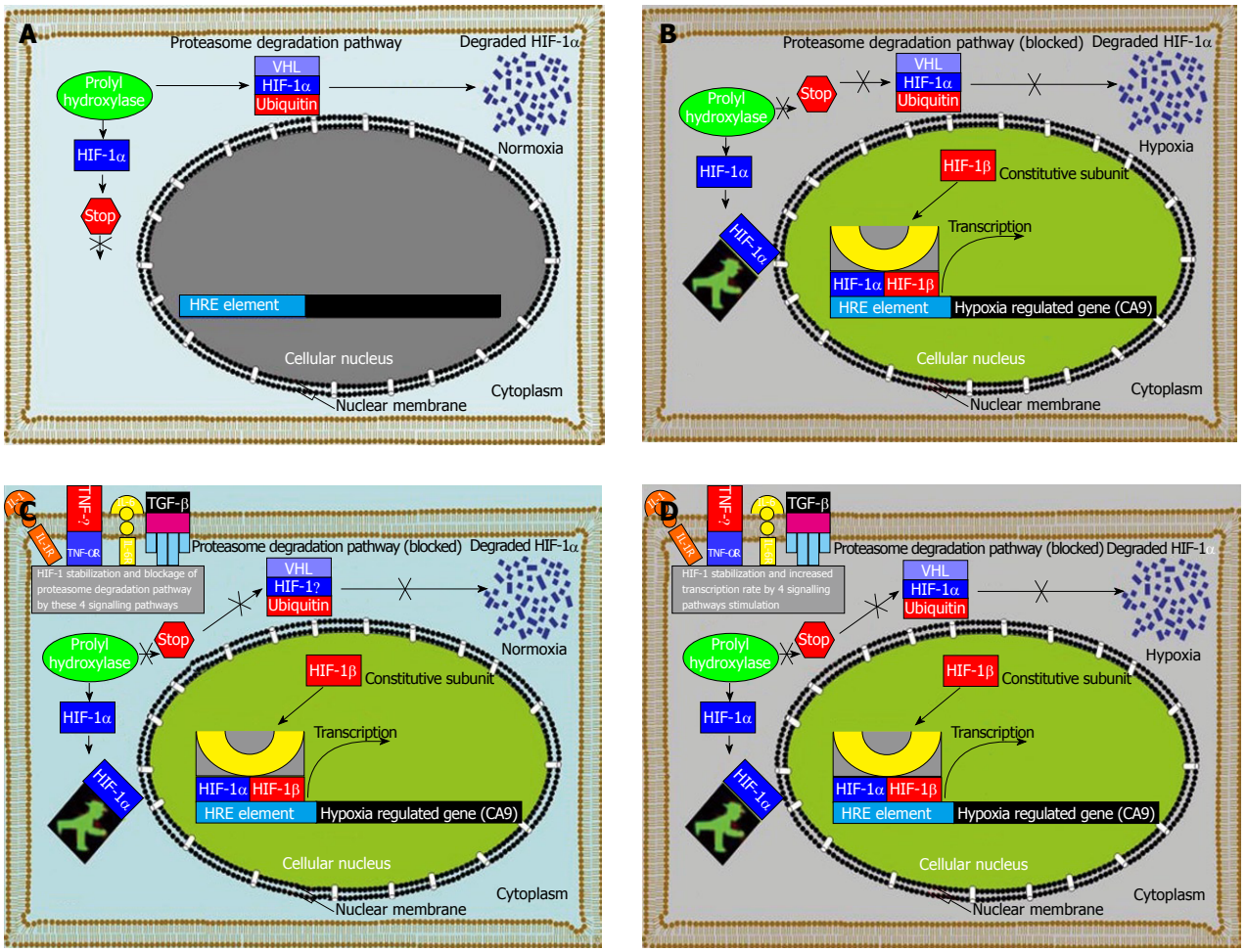


Figure 6 Hypoxia-inducible factor-1 alpha induced regulation of hypoxia-induced carbonic anhydrase 9 expression in human tumor cells without or with stimulation. A: Under normoxic conditions in the tumour cell microenvironment, hypoxia-inducible factor-1 alpha (HIF-1?) is rapidly degraded *via* the von Hippel-Lindau tumour suppressor gene product (pVHL) - mediated proteasome pathway; B: Following a shift in tumour environment aeration conditions from normoxic to hypoxic aeration conditions, HIF-1? subunit becomes stable and translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery consists if e.g. p300/CBP to modulate the transcriptional activity of numerous hypoxia inducible genes, such as carbonic anhydrase 9 (CA9) in our case, and about 61 other hypoxia induced genes[51]; C: When the cells are stimulated under normoxia with either interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-?) or transforming growth factor-beta (TGF-?), the transcription factor HIF-1? subunit becomes stable despite the oxygenation status of the tumour environment and translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery consists of e.g. p300/CBP to modulate the transcriptional activity of CA9 with a similar expression to that under hypoxia; D: Experimental stimulation with either IL-1, IL-6, TNF-? or TGF-?1 under hypoxia increases the CA9 level to almost double the expression rate under hypoxic conditions with the stimulation of these cytokines due to increased HIF-1α translocation into the nucleus and increased binding rate to the hypoxia response element element within the CA9 promoter region.

effect of IL-1, IL-6, TNF-? and TGF-?1.

In conclusion, we showed in HCC Hep3B cell lines that CA9 protein and CA9 mRNA are generally up-regulated due to stimulation with IL-1, IL-6, TNF-? and TGF-?1, and exposure to prolonged severe (0.1% O_2) hypoxic or normoxic conditions. CA9 pathway up-regulation might be accomplished by the effect of cytokine stimulation on hypoxia induced and regulating genes might represent an interesting aspect of gene conditions under these environmental conditions. Thus, in cancer development, it is undoubtedly a promising target for anti-cancer treatment. The results of this series of experiments are useful for the potential optimization of applied tumour therapeutic approaches against HCC. Since tumour tissue oxygenation conditions are dynamic and CA9 expression in such tissues is chronic this feature is involved in the radio-resistance of this type of cancer tissue.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most prominent type of liver cancer, while alcoholism is the most common cause of hepatic cirrhosis. This is a result of hepatic fibrogenesis which is characterized by increased and altered deposition of newly generated extracellular matrix in response to injury. Transdifferentia-

tion of hepatic stellate cells (HSCs) is driven by an array of cytokines of which transforming growth factor beta (TGF-β) has proven to be the fibrogenic master cytokine stimulating HSCs by autocrine and paracrine mechanisms. As a result of treatment *via* hepato-surgery and because only 10%-20% of HCCs can be removed completely, patients have a short life expectancy of 3 mo to 6 mo.

Research frontiers

Carbonic anhydrase 9 (CA9), is a hypoxia induced gene and acts as a tumour hypoxia marker as well as an indicator and a potential therapeutic target in different human cancers. Transcriptional regulation of transmembrane protein CA9 is complex and the transcriptional activation of CA9 by TGF-β and other cytokine families is consistent with hCA9 mRNA levels revealed by reverse transcription polymerase chain reaction and human CA9 protein expression levels by flow cytometry in Hep3B cells in a cell-specific manner. The findings from this series of experiments showed that besides hypoxia as a positive regulator of CA9 expression in HCC, the four signal transduction pathways, interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) and TGF-β, positively influence CA9 expression in HCC both under normoxic and hypoxic conditions. These findings may potentially be considered in the design of anti-cancer therapeutic approaches involving the CA9 gene as a positive disease regulator in human cancers.

Innovations and breakthroughs

Due to the regulatory effect of CA9 on the physiological condition of the human tumor tissue microenviroment, therapeutic approaches used against these tumors are not normally successful due to resistance to treatment modalities such as radiotherapeutic or chemotherapeutic modalities. The results of this experimental series highlight the functional regulatory role of IL-1, IL-6, TGF-β1 and TNF-α which led to an increase in the expression of CA9 in tumor tissue cells. As a consequence, these important data provide functional points of interaction for the inhibition or down-regulation of CA9 expression *via* tailored gene therapeutic modalities such as siRNA, adenoviral or retroviral vectors carrying genes acting as functional down-regulators. This is a pre-requisite step for the application of therapeutic approaches aiming to optimize the outcome of these therapies and provide a better quality of life for cancer patients undergoing treatment.

Applications

The results of this study show the regulatory events of the hypoxia induced gene CA9 both *via* oxygenation deprivation in tumor cells and *via* the stimulatory effect of different cytokine families with related expression patterns.

Terminology

Hypoxia is a pathological condition in a certain region of the body, namely the tissues of organs are deprived of adequate oxygen due to the failure to deliver oxygen to target tissues. The difference between normal oxygen supply and demand at the cellular level may result in hypoxic conditions. The oxygenation state where oxygen is absent is called anoxia. Normoxia, normal oxygen concentration as a result of a normal or adequate oxygen supply at the cellular level is typically 20%-21% in the atmosphere or 2%-3% in the physiological context. HCC accounts for most liver cancers and differs from metastatic liver cancer, which starts in another body organ such as breast or lung and disseminates towards the liver. The cause of liver cancer is usually the unidirectional development of liver fibrosis into liver cirrhosis. Different factors, besides genetic predisposition, favour this development and include; alcohol abuse, autoimmune diseases of the liver, hepatitis B or C virus infection, chronic liver inflammation, and hemochromatosis; cytokines are small cell-signalling non-hormonal protein molecules functioning in the intercellular communication. Cytokines can be classified as proteins, peptides, or glycoproteins and include a large family of regulators produced by cells of different embryonic origin; tumour therapy refers to the approaches applied for various cancers in humans. These include radiation therapy, surgical removal of cancer tissue, drugs or other substances that block cancer growth and spread by interfering with specific molecules involved in tumour growth and progression including agents which interfere with cell growth signalling or tumour blood vessel development, cancer cells specific death promotion, stimulating the immune system to destroy specific cancer cells, and toxic chemical agents delivered into cancer cells as well as gene therapeutic modalities; Tumour microenvironment: The extracellular environment present in a very small region of a solid tumour. Cells in different areas of solid tumours will have markedly different microenvironments; Angiogenesis: The formation of new blood vessels.

Peer review

Due to its clear regulatory behaviour under hypoxic condition in human tumor cells, NDRG1 represents an additional diagnostic marker for brain tumor detection, due to the role of hypoxia in regulating this gene, and it can represent a potential target for tumor treatment in human glioblastoma.

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CASE REPORT

Primary duodenal NK/T-cell lymphoma with massive bleeding: A case report

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Abstract

Primary natural killer/T-cell (NK/T-cell) lymphoma of the gastrointestinal tract is a very rare disease with a poor prognosis, and the duodenum is quite extraordinary as a primary lesion site. Here, we describe a unique case of a primary duodenal NK/T-cell lymphoma in a 26-year-old man who presented with abdominal pain and weight loss. Abdominal computed tomography scan demonstrated a hypodense tumor in the duodenum. Because of massive upper gastrointestinal tract bleeding during hospitalization, the patient was examined by emergency upper gastrointestinal endoscopy. Under endoscopy, an irregular ulcer with mucosal edema, destruction, necrosis, a hyperplastic nodule and active bleeding was observed on the duodenal posterior wall. Following endoscopic hemostasis, a biopsy was obtained for pathological evaluation. The lesion was subsequently confirmed to be a duodenal NK/T-cell lymphoma. The presenting symptoms of primary duodenal NK-/T-cell lymphoma in this patient were abdominal pain and gastrointestinal bleeding, and endoscopy was important for diagnosis. Despite aggressive treatments, the prognosis was very poor.

Key words: Bleeding; Duodenum; Natural killer/T-cell lymphoma

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INTRODUCTION

Lymphoma is a heterogeneous disease as there is a great

variety in the biology of normal lymphocytes and clinical behavior is different. The World Health Organization classification divides lymphoma biologically into B cell and T cell/natural killer (NK) cell lineages which are commonly called non-Hodgkin's lymphoma (NHL). The incidence of the various subtypes varies widely as some lesions appear to be peculiar to particular sites and ethnic groups[1]. Primary NHL of the duodenum is an uncommon primary tumor of the gastrointestinal tract which accounts for less than 12% of all NHL[2,3]. The majority of these lymphomas arise in the stomach, with less than 30% arising in the small intestine[3]. The incidence of lymphoma decreases from the ileum to the jejunum to the duodenum[1]. The NK cell type can be classified into 3 subgroups: NK/T-cell lymphoma nasal/nasal type, NK cell leukemia and chronic lymphoproliferative disorders of NK cells[4,5]. Of the NHL subtypes, NK/T-cell lymphoma nasal type is very rare, predominantly found in East Asia, and there it makes up 2%-10% of NHL[6]. It is primarily located in the nasal/nasopharyngeal region (75%), the skin (4%), the gastrointestinal tract (6%), the bone marrow and the spleen[7]. NK/T-cell lymphomas in the gastrointestinal tract, especially those occurring in the duodenum, are rare. Herein, we report a rare case of primary duodenal NK/T-cell lymphoma with massive bleeding.

CASE REPORT

A 26-year-old man was admitted to our hospital due to a 2 mo history of abdominal pain with weight loss. He had a medical history of hyperlipidemia and hypertension, but denied history of alcohol consumption or drug abuse. Both his grandfather and uncle had a history of pulmonary tuberculosis (TB). Two months prior to admission, he developed symptoms of early satiety, poor appetite, and abdominal fullness. Thereafter, he developed a dry cough, malaise, and dyspnea on exertion. Low fever, night sweats, and a 14 kg weight loss were also noted during this period. On physical examination, his chest showed bilateral decreased breath sounds at the lung bases. His abdomen was hard and tender without organomegaly. Shifting dullness and rebound tenderness were also noted. Enlargement of the cervical or axillary lymph nodes were identified. Laboratory tests revealed the presence of occult blood in his stools, microcytic anemia (Hb: 11.1 g/dL, normal 13.1-17.2 g/dL), and hypoalbuminemia (albumin: 2.3 g/dL, normal 3.6-5.1 g/dL). The white blood cell count, platelet count, results of coagulation studies, liver and renal function tests, and levels of electrolytes were normal. The fecal occult blood test using an enzyme immunoassay was 214.6 ng/mL (normal, < 12 ng/mL).

A chest radiograph and thoracic ultrasonography revealed bilateral moderate pleural effusions. Abdominal ultrasonography demonstrated thickened peritoneum with a moderate amount of ascites without liver cirrhosis. Diagnostic paracentesis and thoracocentesis both yielded exudative fluid that predominantly contained mononuclear cells. The serum level of CA-125 was 727.2 U/mL (normal, < 35 U/mL), while the serum carcinoembryonic antigen and CA-199 levels were within the normal range. Serum anti-immunodeficiency virus was negative. The level of CA-125 in the ascitic fluid was markedly elevated at 2008.5 U/mL. Although the results of acidfast staining tests were negative in the ascites and pleural effusions, TB peritonitis was initially suspected on the basis of the patient's symptoms, and elevated CA-125 level in the serum and ascites. However, the adenosine deaminase level in the ascitic fluid was 30 U/mL. In addition, TB-PCR and TB culture of ascitic fluid yielded negative findings. Analysis of ascites did not reveal growth of common microorganisms or the presence of malignant cells.

Abdominal computed tomography scan revealed multiple enlarged retroperitoneal lymph nodes, surrounding the abdominal blood vessels and organs (Figure 1A-C). Abdominal ultrasonography examination indicated a cavitary mass between the distal jejunum and proximal ileum (Figure 1D). A barium study of the small intestine also showed a cavitary mass lesion with mucosal destruction between the distal jejunum and proximal ileum, and focal segmental jejunal wall rigidity with loss of mucosal folds (data not shown). Bone marrow laboratory testing showed tumor infiltration.

After two weeks of hospitalization, abdominal pain progressed markedly within a few days, with the development of hematemesis and melena. Thus, emergency upper gastrointestinal endoscopy was carried out in our endoscopy unit. The upper gastrointestinal endoscopy showed a large amount of fresh blood in the stomach without a bleeding site or mucosal lesion. Vascular deformity and erosive or active ulcers were not observed in the duodenal bulb. When the endoscope was placed in the descendent duodenum and horizontal junction, an irregular ulcer with mucosal edema, destruction, necrosis, a hyperplastic nodule and active bleeding were observed in the duodenal posterior wall (Figure 2). After endoscopic hemostasis, a biopsy was obtained for pathological evaluation. Colonoscopy showed a smooth mucosa without erosion, ulcer or neoplasm (data not shown). Microscopically, the biopsy specimens revealed a monotonous population of atypical lymphoid cells with scanty cytoplasm, mucosal glands widely spaced or lost, mucosal necrosis and hemorrhage (Figure 3). Pathologic features did not support Crohn's disease. The immunohistochemical study was positive for CD3, CD56 and T cell intracytoplasmic antigen 1 (TIA1), but negative for CD20 and CD23 (Figure 4A-E). The *in situ* hybridization of Epstein-Barr virus encoded small nuclear RNAs was positive (Figure 4F). On the basis of the pathological characteristics, a diagnosis of NK/T-cell lymphoma was made. A whole body fluorodeoxyglucose (FDG) positron emission tomography scan showed intensely increased FDG uptake involving most of the intra-abdominal region (omentum/mesentary/peritoneum) and in the lumbar pre/para-vertebral

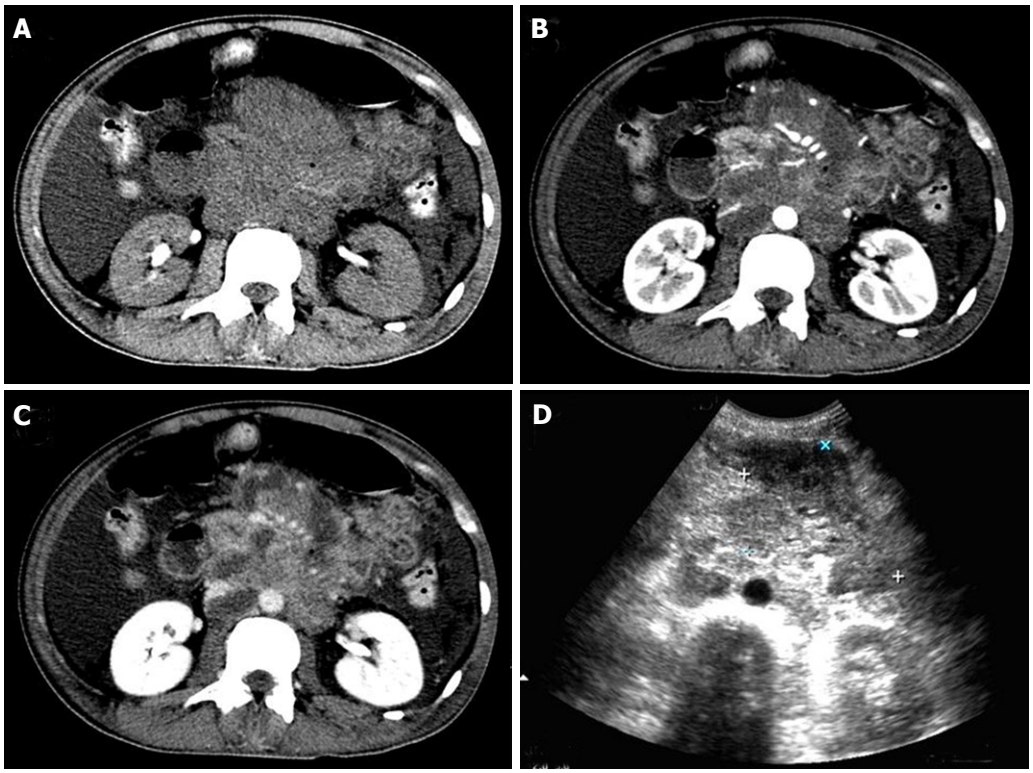

Figure 1 Abdominal computed tomography scan and ultrasonography examination revealed a cavitary mass between the distal jejunum and proximal ileum with multiple enlarged retroperitoneal lymph nodes. A: Plain computed tomography (CT) scan image; B: CT image in the arterial phase of contrast enhancement; C: CT image in the parenchymal phase of contrast enhancement; D: Ultrasonography image.

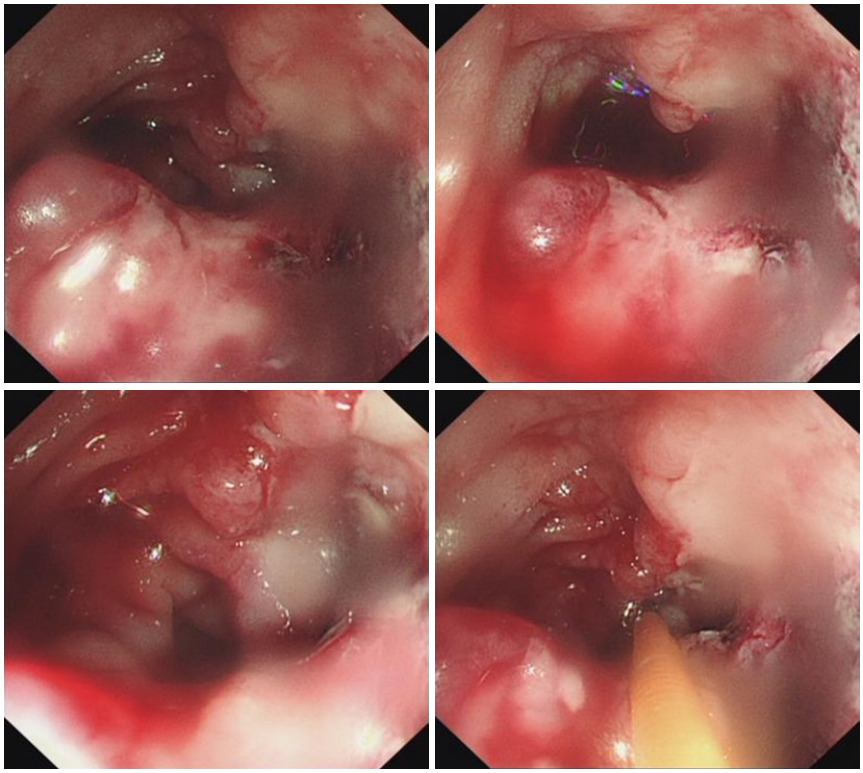

Figure 2 Images of the emergency endoscopy. The endoscopy showed an irregular ulcer with mucosal edema, destruction, necrosis, a hyperplastic nodule and active bleeding in the duodenal posterior wall.

region (retroperitoneal lymph nodes) (data not shown). Ann Arbor staging in this patient was stage Ⅳ. Hemor-

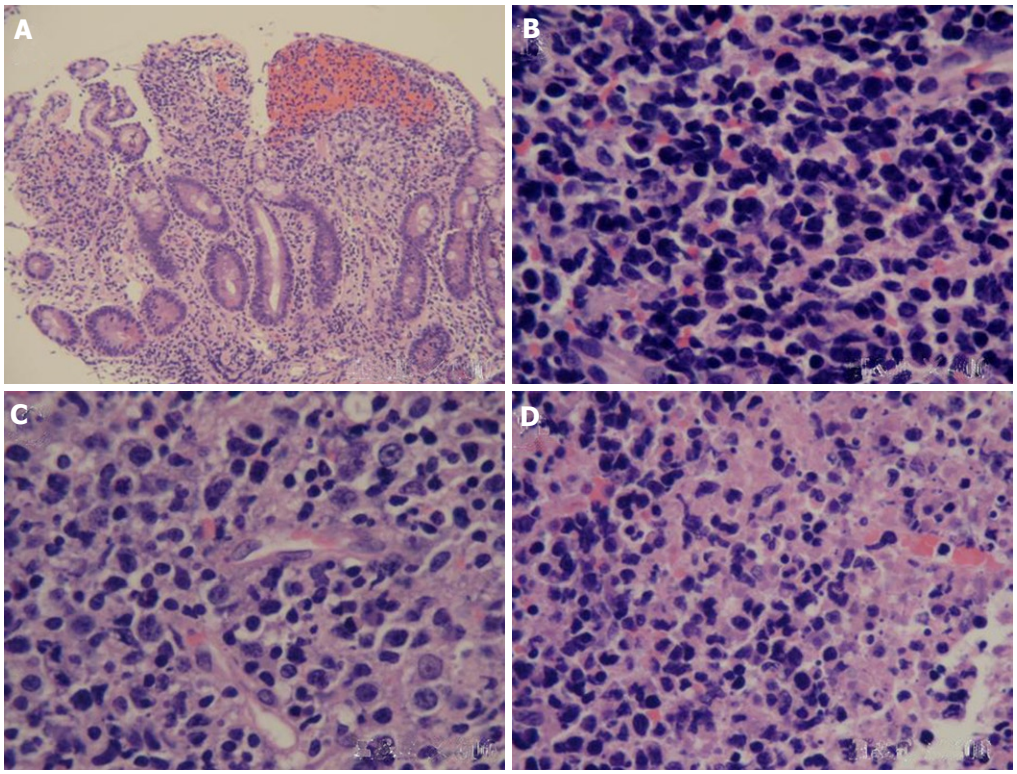

Figure 3 Photomicrograph of biopsy specimens. A: A large numbers of atypical cells infiltrated the mucosa and mucosal glands became widely spaced or lost [hematoxylin and eosin (HE) stain , × 100]; B: The tumor was composed of medium-sized cells (HE stain, × 400); C: The large cells admixed with small cells (HE stain, × 400); D: Coagulative necrosis and admixed apoptotic bodies were observed in the specimens (HE stain, × 400).

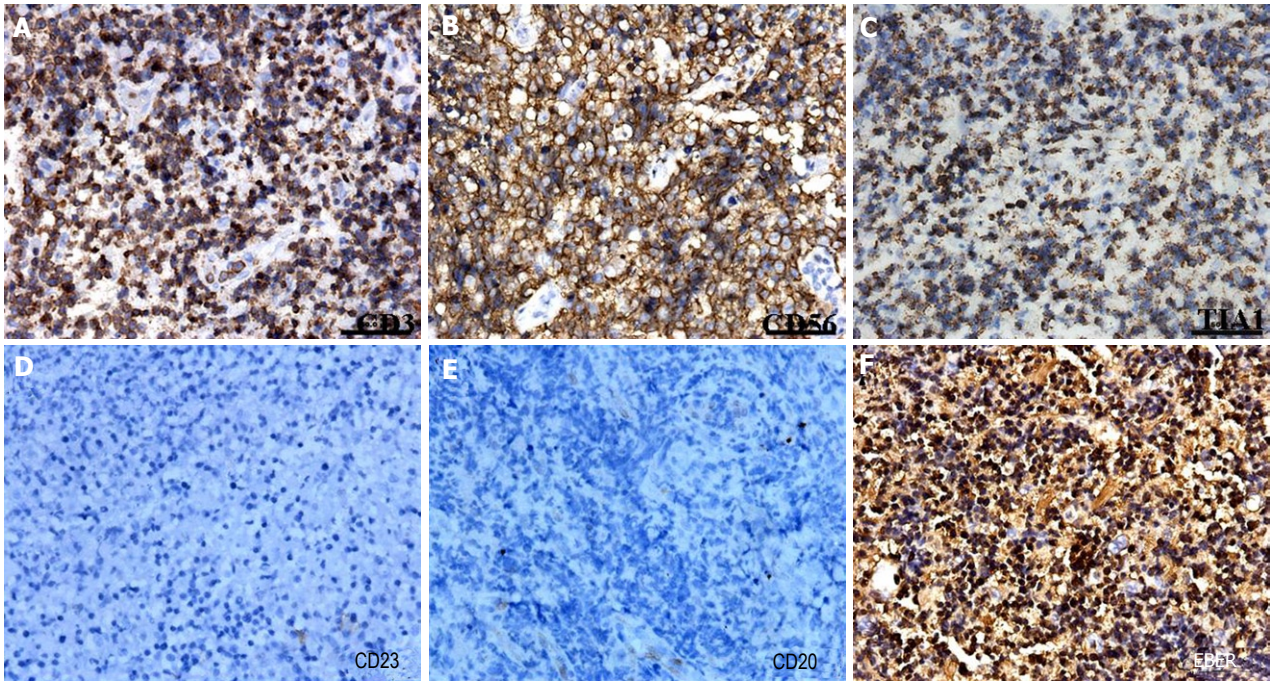

Figure 4 Immunohistochemical characteristics of tumor cells. A: Neoplastic cells showed strong staining for cytoplasmic CD3; B: Positive membranous staining for CD56; C: Strong granular staining for TIA1; D: Negative staining for CD23; E: Negative staining for CD20; F: *In situ* hybridization showed marked nuclear labeling of EBER. EBER: Epstein-Barr virus encoded small nuclear RNA.

rhage was controlled following esophagogastroscopy. He received several courses of EPCOH formula chemotherapy, but died of disease progression and consequential severe infections 2 mo later.

DISCUSSION

Primary extranodal lymphomas are a heterogeneous group of diseases which have diverse etiology, pathogenesis, patterns of presentation and outcomes. The commonest site of extranodal NHL is the gastrointestinal tract, but virtually every extranodal location has been reported[8]. Of these 50%-60% occur in the stomach and 20%-30% in the small intestine. The most common site in the small intestine is the ileocecal region, and the least common site is the duodenum. The incidence of lymphoma in the gastrointestinal tract depends on the amount of lymphoid tissue present in a particular segment[9]. The most common pathology types are diffuse large B-cell lymphoma, marginal zone B cell lymphoma-mucosa associated tissue and mantle cell lymphoma[10]. As only a few gastrointestinal NK/T-cell lymphomas have been reported to date, duodenal NK/T-cell lymphoma is extremely rare.

NK/T-cell lymphoma is an aggressive malignancy with vascular destruction and tissue necrosis, and is an unusual type of NHL which is categorized as nasal and extranodal NK/T-cell lymphoma by the primary tumor lesion. The nasal cavity is the most common location, while the others show an extranodal presentation such as the skin, soft tissue, testis, lung, gastrointestinal tract, and central nervous system[5]. Lymphomas in the gastrointestinal tract are difficult to diagnose early due to their nonspecific symptoms[11]. Gastric and colonic lymphomas can be diagnosed *via* conventional endoscopy[12]. However, NK/T-cell lymphoma derived from the small intestine, which has a low incidence, is mostly diagnosed incidentally or when the patient presents with abdominal pain. Vigilance against "hints" of small bowel tumors and proper diagnostic tools are the key to preventing complications including perforation, massive bleeding, ileus, and to treat the disease when its spread is limited and it is easy to cure.

Most patients with gastrointestinal lymphoma present with vomiting, abdominal pain, weight loss, and fever. Other features such as early satiety, symptoms of peptic ulceration, bleeding in the form of hematemesis, melena, anemia or non-specific symptoms like loss of appetite may also be present. Bleeding duodenal ulcer is rarely a presentation of duodenal lymphoma. Cases of primary gastrointestinal lymphoma, particularly primary duodenal lymphoma can also present as jaundice. Most gastrointestinal tract lymphomas are initially asymptomatic until the tumor causes abdominal pain, abdominal mass, bleeding and obstruction. Usually, patients do not pay much attention to mild gastrointestinal tract discomfort or changes in bowel habits. The lack of specific clinical symptoms and signs probably accounts for the delay in diagnosis. In our case, as the tumor grew in the duodenum the patient experienced abdominal pain initially and the tumor was identified by endoscopy when upper gastrointestinal tract bleeding appeared. Although the diagnosis was very clear then, the disease was incurable.

In patients with primary gastrointestinal lymphoma, the overall 5-year survival rate is 47%, 5-year disease-free survival is 40%, and 79% of patients die within the first year of diagnosis. Important prognostic factors include stage of the disease, extent of surgical resection, response to treatment, serosal involvement, multimodality treatment, and performance status of the patient. Therefore, the best outcome for the patient depends on early diagnosis and treatment. The most effective standard treatment has not been established for this tumor[13-15]. A better response might be achieved in some cases with accurate diagnosis in the early stage (stage Ⅰ/Ⅱ), after intensive systemic chemotherapy-assisted radiotherapy. In summary, the presenting symptoms of primary duodenal NK-/T-cell lymphoma in our patient were abdominal pain and gastrointestinal bleeding. The duodenal NK-/T-cell lymphoma was endoscopically characterized by mucosal superficial erosion, irregular ulcer, edema, destruction, necrosis, a hyperplastic nodule and active bleeding.

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January 16-17, 2012
Biomarkers Summit Egypt
London, United Kingdom

January 25-26, 2012
Multi-Disciplinary Appoaches to Cancer Therapy
Dubai, United Arab Emirates

January 26-27, 2012
3rd National Conference: Renal and Bladder Cancer 2012
London, United Kingdom

January 30-31, 2012
2nd Annual Clinical Trials in Oncology
Rome, Italy

February 2-3, 2012
Stem Cells 2012 Conference and Exhibition
San Diego, CA, United States

February 6-8, 2012
Mahidol International Conference on Infections and Cancers 2012
Bangkok, Thailand

February 12-17, 2012
Keystone Symposia: Cancer and Metabolism
Alberta, Canada

February 22-25, 2012
Excellence in Oncology
Istanbul, Turkey

March 8-10, 2012
10th International Congress on Targeted Anticancer Therapies
Amsterdam, Netherlands

March 9-10, 2012
13th European Congress: Perspectives in Lung Cancer
Amsterdam, Netherlands

March 14-16, 2012
BTOC-11 Biological Therapy of Cancer
Munich, Germany

March 15-17, 2012
3rd Conference on Therapeutic Resistance in Cancer
Quebec, Canada

March 29-30, 2012
Modern methods of diagnosis and treatment of malignant tumors
Kiev, Ukraine

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 20-21, 2012
Diagnosis and treatment of advanced forms of prostate cancer, bladder cancer and kidney cancer
Kiev, Ukraine

April 20-22, 2012
The 9th Meeting of Asian Society for Neuro-Oncology
Taipei, Taiwan

April 26-28, 2012
3rd International Video Workshop on Radical Surgery in Gynaecological Oncology
Prague, Czech Republic

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 5-6, 2012
Radiation Research Methods as A Diagnostic and Therapeutic Support in Oncology
Kiev, Ukraine

May 17-18, 2012
Eurasian forum on the management of patients with tumors of the gastrointestinal tract
Uman, Ukraine

June 16-17, 2012
Issues of Neurosurgery, vascular neurosurgery, neurooncology, spinal surgery and spinal cord
Kiev, Ukraine

July 7-10, 2012
22nd Biennial Congress of the European Association for Cancer Research
Barcelona, Spain

July 21-28, 2012
Cancer In Women
Hawaii, HI, United States

July 25-27, 2012
5th Latin American Conference on Lung Cancer
Rio de Janeiro, Brazil

August 27-30, 2012
UICC World Cancer Congress 2012
Québec, Canada

September 6-8, 2012
The 8th International Jordanian Oncology Society Conference
Amman, Jordan

September 27-28, 2012
Current issues of diagnosis and treatment of oncogynecology diseases
Ivano Frankivsk, Ukraine

September 27-29, 2012
European Conference of Oncology Pharmacy
Budapest, Hungary

October 5-8, 2012
44th Congress of the International Society of Paediatric Oncology
London, United Kingdom

October 13-16, 2012
14th Biennial Meeting of the International Gynecologic Cancer Society
Vancouver, Canada

October 19, 2012
Modern aspects of diagnosis and treatment of breast cancer
Kiev, Ukraine

October 23-26, 2012
Sydney International Breast Cancer Congress 2012
Sydney, Australia

October 27-28, 2012
Optimization methods for radiation diagnosis in oncology
Odessa, Ukraine

November 6-9, 2012
24th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
Dublin, Ireland

November 16-17, 2012
17th Annual Perspectives in Thoracic Oncology
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GENERAL INFORMATION

World Journal of Clinical Oncology (*World J Clin Oncol*, *WJCO*, online ISSN 2218-4333, DOI: 10.5306) is a monthly peer-reviewed, online, open-access (OA), journal supported by an editorial board consisting of 316 experts in oncology from 33 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJCO* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article *via* online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJCO* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJCO* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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