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EDITORIAL

Adjuvant sorafenib in hepatocellular carcinoma: A cautionary comment of STORM trial

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Author contributions: Zhong JH, Du XK and Xiang BD contributed equally to this work; Zhong JH and Du XK designed the study and wrote the manuscript; Zhong JH, Xiang BD and Li LQ analyzed the data from the included studies; all authors reviewed the manuscript and approved publication.

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Abstract

Recurrence rate of hepatocellular carcinoma (HCC) is very high even after curative surgery, and no postoperative therapies have been definitively shown to prevent HCC recurrence. Sorafenib is proved to be effective for advanced HCC by two large randomized controlled trials in 2008 and 2009. Therefore it stands to reason to expect that adjuvant sorafenib may improve post-surgery outcomes of patients with HCC. However, many questions still exist about the value of sorafenib for patients with HCC after surgery or transarterial chemoembolization. In this editorial, we complehensively reviewed the safety and efficacy of adjuvant sorafenib for patients with hepatocellar carcinoma after surgery or transarterial chemoembolization. We emphasized the positive and negative role of sorafenib.

Key words: Adjuvant; Hepatocellular carcinoma; Tumor recurrence; Sorafenib

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Core tip: Sorafenib is effective for advanced hepatocellular carcinoma (HCC). However, its positive role as adjuvant therapy for HCC after surgery or transarterial chemoembolization is controversy.

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INTRODUCTION

Large randomized controlled trials have shown transarterial chemoembolization (TACE)[1,2] and sorafenib[3,4] monotherapy to extend median overall survival by approximately 3 mo over best supportive care in patients with hepatocellular carcinoma (HCC) in Barcelona Clinic Liver Cancer (BCLC) stage B or C. Though hepatic resection is the mainstay treatment for HCC, tumor recurrence is very high after surgery[5]. Therefore it stands to reason to expect that sorafenib may improve post-resection outcomes of patients with multinodular HCC or patients at high risk of HCC recurrence.

STUDY ANALYSIS

In the recent issue of the *World J Gastroenterol*, Li *et al*[6] reported a small retrospective study which enrolled 36 male patients with BCLC stage C HCC after hepatic resection. Twelve patients received resection plus sorafenib while other 24 patients received resection alone. The authors found patients in the resection plus sorafenib group had a significantly longer time-to-tumor progression (TTP) and median overall survival compared to patients in the resection alone group.

However, the phase Ⅲ placebo-controlled study STORM trial[7], which included 1602 patients from 28 countries with early-stage HCC following surgical resection or local ablation, found that adjuvant sorafenib did not significantly affect recurrence-free survival, time to recurrence or overall survival. The authors concluded that no evidence of clinical benefit exists for adjuvant sorafenib therapy in such patients.

Also, the phase Ⅱ SPACE trial comparing the efficacy and safety of TACE with or without sorafenib failed to meet its endpoint of prolonging TTP[8]. This raises important questions about the use of adjuvant sorafenib in the clinic.

The SPACE trial[8], which involved 307 Asian and non-Asian patients with multinodular HCC in BCLC stage B, showed that the combination of TACE and sorafenib did not significantly increase TTP or overall survival over TACE alone. This negative result adds to another previous study calling into question the clinical benefits of adjuvant sorafenib. A phase Ⅲ trial involving 458 Asian patients with HCC in stage B or C found that sorafenib did not significantly prolong TTP or overall survival in patients who responded to TACE[9]. In addition to non-efficacy, sorafenib add the incidence of adverse events or may worsen outcomes in certain patients[3,7,10].

REASONS OF NEGATIVE RESULTS

These negative results (Table 1) call for caution in the adjuvant use of sorafenib. Why the results would be negative when our therapeutic aim shifts from control of established tumor cells to the eradication of occult micrometastases? One reason for caution lies in the mechanism of sorafenib, which inhibits tumor angiogenesis. Preclinical studies suggest that anti-angiogenic therapy can, in principle, increase the likelihood of tumor invasion and spread[11], and that tumor angiogenesis can rapidly recover when anti-angiogenic therapy is halted[12]. Another reason for caution is that sorafenib may not be effective against recurrent or metastatic tumors, even if it is effective against primary tumors. The two types of tumors behave differently, and it is possible that recurrent or metastatic tumors are more malignant because they were not eliminated by initial therapy (TACE, resection, ablation). In fact, studies suggest that sorafenib has poor efficacy against intrahepatic metastases (derived from the primary tumor) as well as multicentric tumors arising spontaneously in the residual liver[7].

While previous works strengthens the arguments for re-assessing adjuvant use of sorafenib, some of their results should be interpreted with caution. For example, the findings of Li *et al*[6] were based on a very small retrospective study; Lencioni *et al*[8] reported that the combination of TACE and sorafenib showed greater benefit in Asian patients than in non-Asian ones, yet median TTP was nearly the same (24 mo) in Asian and non-Asian subgroups as well as the total study population[8]. This TTP is substantially longer than the 5.4 mo reported in another phase Ⅲ trial involving only Asian patients[9].

Lack of efficacy with sorafenib has been attributed to insufficient duration of therapy[8], such as because of delays in starting sorafenib after TACE, as well as to insufficient daily sorafenib doses[9]. These explanations seem less likely given that all published phase Ⅱ or Ⅲ multicenter randomized controlled trials concur that adjuvant anti-angiogenic agents, including sorafenib, are associated with negative TTP, overall survival, or recurrence-free survival for solid cancers[7-9,13]. In fact, a large dosing study involving 1943 patients with non-metastatic renal-cell carcinoma supports the notion that disease-free survival does not depend on treatment duration[13].

PERSPECTIVE

The growing evidence for lack of adjuvant sorafenib efficacy against HCC[7-9], and substantial evidence against adjuvant anti-angiogenic therapy against solid cancers in general[13-16], should lead clinicians to re-assess their treatment approaches. In this sense, some ongoing trials of adjuvant anti-angiogenic agents for solid cancers (*e.g.*, NCT00908752, NCT01009801) are already terminated.

Nowadays, more and more trials revealed the definite efficacy of postoperative antiviral treatment with nucleot(s)ide analogs for hepatitis B virus-related HCC[17-19]. Adjuvant adoptive immunotherapy may also improve recurrence-free and overall survival[20]. But more rando-

Table 1 Adjuvant sorafenib for hepatocellular carcinoma

Ref.	Recruited period	Sample size (T/C)	HCC characteristics	First therapy	Adjuvant therapy	Outcomes
Li *et al*[6], 2016	2009-2013	12/24	With portal vein thrombus	Hepatic resection	Sorafenib (200-800 mg/d)	TTP, $P = 0.041$ OS, $P = 0.01$
Bruix *et al*[7], 2015	2008-2010	556/558	Early stage HCC	Hepatic resection or ablation	Sorafenib (400 mg) twice a day	RFS, $P = 0.26$ OS, $P = 0.48$
Lencioni *et al*[8], 2016	-	154/153	Intermediate stage multinodular HCC	TACE with doxorubicin-eluting beads	Sorafenib (400 mg) twice a day	TTP, $P = 0.07$ OS, $P = 0.29$
Kudo *et al*[9], 2011	2006-2009	229/227	Unresectable HCC who responded to TACE	Conventional TACE	Sorafenib (400 mg) twice a day	TTP, $P = 0.25$ OS, $P = 0.79$

C: Control group; HCC: Hepatocellular carcinoma; OS: Overall survival; RFS: Recurrence-free survival; T: Adjuvant treated group; TACE: Transarterial chemoembolization; TTP: Time-to-tumor progression.

mized trials are warranted because of inconsistent findings from new randomized trials[21,22]. For HCC patients with high risk of recurrence, adjuvant TACE has positive effect in terms of improving overall survival[23]. However, each postoperative or adjuvant therapy has its own indication, revealing that not all patients with HCC after surgery should receive specific postoperative or adjuvant therapy. New drugs may help further define therapeutic directions for the future.

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REVIEW

Dynamics of hepatic and intestinal cholesterol and bile acid pathways: The impact of the animal model of estrogen deficiency and exercise training

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Abstract

Plasma cholesterol level is determined by a complex dynamics that involves transport lipoproteins which levels are tightly dependent on how the liver and the intestine regulate cholesterol and biliary acid metabolism. Regulation of cholesterol and biliary acids by the liver and the intestine is in turn coupled to a large array of enzymes and transporters that largely influence the inflow and the outflow of cholesterol and biliary acids through these organs. The activity of the key regulators of cholesterol and biliary acids may be influenced by several external factors such as pharmacological drugs and the nutritional status. In recent years, more information has been gathered about the impact of estrogens on regulation of cholesterol in the body. Exposure to high levels of estrogens has been reported to promote cholesterol gallstone formation and women are twice as likely as men to develop cholesterol gallstones. The impact of estrogen withdrawal, such as experienced by menopausal women, is therefore of importance and more information on how the absence of estrogens influence cholesterol regulation is started to come out, especially through the use of animal models. An interesting alternative to metabolic deterioration due to estrogen deficiency is exercise training. The present review is intended to summarize the present information that links key regulators of cholesterol and biliary acid pathways in liver and intestine to the absence of estrogens in an animal model and to discuss the potential role of exercise training as an alternative.

Key words: PSCK9; Low-density lipoprotein receptor; Very low-density lipoprotein; Sterol regulatory element binding proteins; Ovariectomy; High-density lipoprotein; Lipoproteins

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Core tip: The liver is considered the master piece in regulation of plasma cholesterol levels. Together with

the intestine they control the influx and the efflux of cholesterol and biliary acids in the body. Cholesterol and its conversion into biliary acids are regulated by an extended network of enzymes and transporters that largely influence plasma cholesterol levels. The key regulators of cholesterol and biliary acids in liver and intestine are in turn affected by several factors including estrogens levels and more recently exercise training. Low estrogenic levels, such as seen in post-menopausal women, are associated with higher plasma cholesterol levels. In recent years more information has been accumulated on the extent to which low estrogenic levels, such as seen in an ovariectomized animal model, influence cholesterol and biliary metabolism at the molecular level. As an alternative to a deficiency in estrogens, exercise training has been reported to exert a beneficial effect on these key regulators of cholesterol and biliary acids.

Lavoie JM. Dynamics of hepatic and intestinal cholesterol and bile acid pathways: The impact of the animal model of estrogen deficiency and exercise training. *World J Hepatol* 2016; 8(23): 961-975 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i23/961.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i23.961

INTRODUCTION

The importance of estrogens in regulating cholesterol and biliary acid metabolism in liver is enlightened by clinical studies confirming that women are twice as likely as men to develop cholesterol gallstones[1,2]. Oversaturation of biliary cholesterol is the requisite defect for the formation of gallstones[1]. This pathophysiological state is induced by either hypersecretion of biliary cholesterol or decreased secretion of bile acids. Therefore, both the cholesterol secreted into bile and the bile acids synthetized from cholesterol in liver are involved in the disease[3]. Exposure to high levels of estrogens has been reported to promote cholesterol gallstone formation[4]. Similarly, the estrogen receptor α-selective agonist propylpyrazole and tamoxifen treatment, that have estrogen-like activity, augment biliary cholesterol secretion in mice[4] and increase gallstone prevalence in women[5]. On the whole, these findings indicate that there is a close relationship between estrogens, cholesterol and biliary acid metabolism in liver. This in turn raises the question of the extent to which a deficiency in estrogens, as happens with menopause, affects cholesterol and biliary acid regulation in liver. The first element to take into consideration is the fact that estrogen withdrawal in animals decreases gene expression of HMGCoA-reductase (-r), the rate-limiting enzyme in hepatic cholesterol biosynthesis[6].

Estrogen-deficient state in Ovx animals has been repeatedly reported to result in substantial liver fat accumulation indicating that fat metabolism is perturbed by the absence of estrogens[7,8]. The information on the impact of the absence of estrogens on cholesterol metabolism, however, is scarce. An increase in plasma cholesterol levels in Ovx rats has been reported 30 years ago[9,10]. This has been confirmed in more recent studies in Ovx animals[6,11] as well as in post-menopausal women[12]. The situation of liver cholesterol levels in Ovx animals is more controversial. Liver total cholesterol level was reported not to be affected by estrogen withdrawal in some studies[11,13] while it has been found to be increased in rats ovariectomized for 5-8 wk[14,15]. Large cholesterol accumulation has also been found in liver of Ovx rats fed with a high-fat diet, that was not observed in liver of Ovx rats fed a standard diet and in Sham rats fed a high-fat diet[6]. The authors suggested a vulnerability to cholesterol accumulation in liver of Ovx animals fed a high fat diet. These findings have, at least, the merit of raising questions on the impact of the lack of estrogens on regulatory pathways involved in liver cholesterol metabolism. Cholesterol homeostasis in liver depends on cholesterol synthesis, uptake, and clearance. One of the aims of the present review is to summarize our present knowledge of the extent to which the lack of estrogens in an ovariectomized animal model affects the regulation of molecular pathways of cholesterol and bile acids in liver and intestine.

One of the best non-pharmacological strategies for the treatment of metabolic disturbances leading to coronary artery disease is exercise training[16,17]. In recent years, there has been a fair amount of studies indicating that exercise training is also beneficial in circumventing the detrimental effects of estrogen removal on metabolic pathways involved in liver fat accumulation[18]. Treadmill exercise for 12 wk has also been reported to reduce plasma low density lipoprotein (LDL)-cholesterol (-C) and total cholesterol in Ovx rats[19] while plasma LDL-C was decreased in 6-wk trained Ovx rats fed a high fat diet for 10 mo[20]. Although limited, there is recent information on the impact of exercise training on regulation of cholesterol pathways in liver and intestine in response to metabolic disturbances. For instance there are reports indicating an increased fecal cholesterol excretion in exercising animals[21]. There is also a recent report of changes with exercise training in gene expression of intestinal nuclear receptors involved in the defense system against endobiotic and xenobiotic insults suggesting that regular exercise contributes to the intestinal maintenance of cholesterol and bile acid homeostasis[22]. In the present review, a consideration will be given to the effects of exercise training on cholesterol and bile acids pathways, especially in the context of estrogens deficiency.

The present review is divided in two large sections related respectively to the pathways involved into hepatic cholesterol influx and efflux and how estrogen deficiency affects key regulators of these pathways. This will be followed by a discussion of the known effects of exercise training on these pathways.

HEPATIC CHOLESTEROL INFLUX

Western-type diets provide approximately 400 mg of cholesterol per day while our body synthesizes approximately 1 g *de novo*[23,24]. Hence, blood cholesterol levels reflect both dietary and endogenously synthesized cholesterol. The liver is a central component in regulation of cholesterol metabolism. This organ is able to acquire cholesterol through *de novo* synthesis and from all classes of circulating lipoproteins[25].

Cholesterol biosynthesis

The total body content of cholesterol is approximately 100 g, of which approximately 90% are found at the cellular levels and 10% in circulation[26]. Cholesterol is synthesised virtually in all nucleated cells[27]. For instance, the central nervous system contains approximately 25% of the unesterified cholesterol present in the body and it comes almost entirely for *in situ* synthesis[28]. It is assumed that approximately 24% of cholesterol synthesis occurs in small intestine of rats and a significant fraction of it is transported to liver where nearly 50% of total cholesterol synthesis occurs[29]. Cholesterol synthesis starts, similarly to *de novo* lipogenesis, by the transfer of acetyl CoA from mitochondria to cytosol. The further condensation of three units of acetyl CoA forms an HMG-CoA that is transported to the endoplasmic reticulum (ER) where it is reduced to melanovate by the enzyme HMGCoA-r follows by several steps leading to the formation of isoprene, squalene, lanosterol, and finally cholesterol. The action of the enzyme HMGCoA-r is the rate-limited step in endogenous cholesterol synthesis.

Regulation of cholesterol biosynthesis: The view that cholesterol is randomly distributed within cell membrane no longer holds. For instance the distribution of lipids and cholesterol in the outer leaflet is organized into domains so-called rafts and caveolae playing intricate roles to maintain cellular homeostasis[30,31]. On the other hand, membranes of the endoplasmic reticulum and the Golgi apparatus contain comparatively little cholesterol, an important factor in cholesterol homeostasis[32]. Maintenance of cholesterol homeostasis is orchestrated mainly by a feedback regulatory system that senses the level of cholesterol in cell membranes and modulates cholesterol biosynthesis and uptake from plasma lipoproteins[33]. The molecular mechanism of how hepatocytes maintain cholesterol homeostasis has become more precise with the discovery of the transcription factors sterol regulatory element binding proteins (SREBPs)[32].

Short-term regulation of the enzyme HMGCoA-r is operated by mechanisms such as phosphorylation/dephosphorylation of the catalytic domain (serine 871) by specific kinases (AMPK) and phosphatases (protein phosphatase 2A)[34,35]. HMGCoA-r is physiologically present in the cell in unphosphorylated active form (30%) and phosphorylated inactive form (70%)[36].

Long-term regulation of HMGCoA-r relies on synthesis and degradation rate of the enzyme. The cholesterol system is unique in that the regulated end-product, cholesterol, is sequestered entirely within cell membranes. Sterol regulatory elements (SREs) are nucleotidic sequences in the gene promoters, encoding proteins involved in cholesterol homeostasis such as HMGCoA-r and LDL receptor (LDL-R). These sequences are recognized by a family of transcription factors called SREBP[37]. The SREBP family members, SREBP-1 (a and c) and SREBP-2, are synthetized as membrane protein in the endoplasmic reticulum.

SREBP-2 is considered to be largely involved in the regulation of cholesterol metabolism. In ER, SREBP interacts with a cargo protein called SREBP cleavage-activated protein (SCAP), which acts as a transporter and cholesterol sensor[37,38]. The complex formation is essential for the exit of SREBPs from the ER and subsequent proteolytic activation[39]. The SREBP/SCAP containing vesicles from the ER also contain a membrane anchored serine protease of the subtilisin family called Site-1 protease (SIP-1). Sip becomes activated only during its transport to the Golgi[40]. SCAP escorts SREBP from the ER to the Golgi apparatus where the SREBPs are proteolytically processed by SIP-1 to yield active fragments that migrate to the nucleus encoding its target genes[33]. To release active SREBP, another enzyme is required, Site-2 protease. Interestingly, the nuclear action of SREBP induces new SREBP mRNA through SREs located in the promoter regions of their own genes[41]. When cholesterol builds up in the ER membrane, a conformational change in SCAP occurs through the direct cholesterol binding to the sterol domain and triggers SCAP to bind to Insig, another ER membrane protein[42]. This association hampers the transport of the SREBP/SCAP complex to the Golgi apparatus, resulting in a reduced proteolytic activation of precursor SREBP. For instance, high dietary cholesterol prevents maturation of SREBPs and cuts off cholesterol and LDL receptor synthesis.

Estrogen deficiency and HMGCoA-r regulation: Since plasma cholesterol level is increased in Ovx animals[6,11] one might expect an increase in cholesterol synthesis. However, HMGCoA-r mRNA secondary to Ovx was found to be decreased in several studies in rats[6,11,43,44] and in mice fed a high-fat high-cholesterol diet[13]. Along with HMGCoA-r, gene expression of SREBP-2, the transcription factor involved in the regulation of HMGCoA-r, was also decreased in Ovx animals[14,43]. On the opposite, an increase in HMGCoA-r protein content has been reported in frog and rat after 5 d of estrogen administration[45,46]. On the whole these results strongly suggest that an increased cholesterol biosynthesis is not responsible for the increased higher plasma cholesterol found with estrogen deficiency in animals and in post-menopausal women. They also suggest an accumulation of cholesterol in the ER membrane.

Receptors involved in hepatic uptake of cholesterol from lipoproteins

Lipoprotein remnant receptors: Upon completion of hydrolysis (approximately 50% of TG removal) chylomicrons and VLDL lose affinity for lipoprotein lipase (LPL) and dissociate[47]. The apoproteins A1 and C are then transferred to high-density lipoprotein (HDL) in exchange for apo E upon what they are then called chylomicrons and VLDL remnants[48,49]. The acquisition of apo E is crucial since it will serve eventually as ligands for receptor mediated clearance. Intermediate density lipoproteins (IDL) which are VLDLs that interact for prolonged period with LPL are also remnants particles. The remnant lipoproteins are then small enough to enter the space of Disse. Once into the space of Disse, remnant lipoproteins small enough to fit between the endothelial cells are sequestrated by high-molecular-weight heparin proteoglycan (HSPG) molecules. Within the space of Disse the particles are remodeled by hepatic lipase. Final uptake by the hepatocytes is receptor mediated that include LDL-R, LDL related protein (LRP), a complex LRP-HSPG or HSPG alone[25,50]. These mechanisms are efficient so that half-life of remnants in plasma is 30 min. The apoB-48 containing chylomicron remnants are completely cleared from the plasma. However the presence of apoB-100 in VLDL alters their metabolism so that only 50% of VLDL remnants are cleared by lipoproteins remnant receptors.

Receptors involved in hepatic uptake of LDL-cholesterol: VLDL remnants that are not taken up by the remnant receptors are metabolized to a greater extent by LPL, become increasing smaller, relatively deficient in TG and enriched in cholesterol esters. These particles are called IDL. Because IDL contains apoE a fraction of these particles may be taken up by the liver through the remnant receptors[51]. However, the remainder will be changed to LDL following further hydrolysis of the TG by the hepatic lipase. The apoE and apoC-Ⅱ molecules will then transfer to HDL and leave apoB as their only apolipoprotein[52]. The LDL-R is the only receptor able to clear up LDL from the circulation. Because of the lack of apoE, the LDL particle is a relatively weak ligand for the LDL receptor[53]. As a result, the half-life of the LDL particle is relatively long (two to four days) thus accounting for 65%-75% of total plasma cholesterol. Interaction of apoB with the LDL-R facilitates the internalisation and the further degradation of LDL[53]. Inside the cell, the LDL particle is hydrolysed to release unesterified cholesterol. The LDL-R is expressed on the cell surface of several tissues including liver, macrophages, lymphocytes, adrenal cortex, gonads, and smooth muscle[25].

Metabolism of the LDL-R: The LDL-R is a cell surface receptor that mediates specific uptake and catabolism of plasma lipoproteins containing apoB or apoE[53]. The primary function of this receptor is the removal of highly atherogenic LDL particles from circulation[53]. Since the liver contains approximately 70% of total LDL-R found in the body[54], hepatic LDL-R activity is an important contributor to regulation of plasma cholesterol LDL levels. The LDL-R activity is downregulated post-transcriptionnally by a protease, proprotein convertase subtilisin kexin type 9 (PCSK9)[55]. PCSK9 is highly expressed in liver and intestine[56]. However, circulating PCSK9 originates exclusively from hepatocytes[57]. The gene expressions of LDL-R and PCSK9 as well as HMGCoA-r are regulated by a transcription factor, SREBP-2[58]. Within the endoplasmic reticulum, PCSK9 undergoes an auto catalytic cleavage[56] that results in a tightly bound secretable heterodimeric complex[59]. PCSK9 is, therefore, readily measured in plasma. PCSK9 binds to the LDL-R at the surface of the hepatocytes and/or within the cell[60]. LDL-R is then directed from the cell surface recycling toward degradation in the endosome/lysosome pathway[61]. Mutations leading to a loss of function or genetic invalidation of PCSK9 largely reduce circulating LDL-C levels and reduce cardiovascular events (88%) in humans (for a review see[60]).

The co-regulation of PCSK9 and HMGCoA-r by the same transcription factor has consequences. As discussed by Poirier *et al*[60], statins that lower LDL-C by inhibiting HMGCoA-r also increase the expression of PCSK9[62] which decreases their capacity at increasing LDL-R. This may explain why LDL-C levels do not reach therapeutic goals in many patients with statins therapy. Hepatocyte nuclear factor 1 alpha, a key mediator of the effects of bile acids on gene expression, also regulates PCSK9[63].

Estrogen deficiency and LDL-R: In line with the reduction in HMGCoA-r, gene expression of hepatic LDL-R has been repeatedly reported to be reduced in Ovx animals[11,13,14,43,64]. Along with LDL-R, PCSK9 transcripts in liver and PCSK9 plasma levels have also been shown to be reduced in Ovx rats[14]. These results concord with the reports that estrogens administration upregulates *LDL-R* gene expression in rat liver[46,65]. In a recent study, Roubtsova *et al*[66] showed, using PCSK9 KO mice, that the interaction between PCSK9 and LDL-R was sex-specific, thus depending on estrogens. The similar decrease in PCSK9 and LDL-R in Ovx animals is, however, puzzling considering that a decrease in PCSK9 should lead to an increase in LDL-R. It has been proposed that the rate of cycling of hepatic LDL-R on cell surface might be an explanation. When hepatic cholesterol increases, as it is observed in Ovx animals[11,14], the transcriptional regulation of PCSK9 and LDL-R both mediated by SREBP-2 would be inhibited, and the rate of cycling of the hepatic LDL-R slowed down leading to higher levels in circulating LDL-cholesterol. The transcriptional regulation of the LDL-R is, however, paradoxical since SREBP-2 also regulates the transcription of PCSK9, thus leading to two opposing effects initiated by the same signal. In a recent publication, Starr *et al*[67] proposed a more dynamic role for PCSK9, suggesting that phosphorylated PCSK9 promotes degradation of LDL-R, whereas nonphosphorylated PCSK9 is in an LDL-R-protective state. Taken together, these results emphasize

the need to a better understanding of the sex specific interaction between LDL-R and PCSK9, especially in view of a new class of cholesterol lowering drugs, the PCSK9 inhibitors[68].

Metabolism of the LRP1 receptor: LRP1 is a member of the *LDL-R* gene family which also includes receptors such as LRP2 (megalin), LRP8 (apoE receptor 2), and the VLDL receptor (VLDLR)[69]. LRP1 is expressed in several types of cells including hepatocytes, fibroblasts, smooth muscle cells, and neurons[70]. This transmembrane protein displays both scavenging and signaling functions. LRP1 mediates removal of at least 30 different ligands, including VLDL remnants or IDL and chylomicron remnants from the circulation[71], but also several molecules unrelated to lipid homeostasis including proteases, protease inhibitor complexes, extracellular matrix proteins, growth factors, toxins, and viral proteins[72]. LRP1 also acts as an endocytic receptor for several intracellular proteins released by necrotic cells, which failure to be efficiency cleared may be associated with the onset of autoimmune disease[73,74]. Interestingly, LRP1, by regulating cell signaling through several mechanisms, may change the activity of other receptors by controlling the abundance of these receptors in the plasma membrane[75]. For instance, disruption of the LRP gene in adult normal mice resulted in a compensatory upregulation of the LDL-R in the liver[76].

The gene expression of LRP1 is complex and appears to be regulated by hormones and growth factors[77]. LRP1, as well as other members of the LDL-R family, are bound by a molecule called receptor-associated protein (RAP) that blocks the bindings of ligands to these receptors[78]. RAP functions as a molecular chaperone that assists in the trafficking of the LRP1 to the cell surface[79]. In different tissues, LRP1 gene expression has been reported to be affected by factors such as hypercholesterolemia, lipopolysaccharides, growth factors, and hypoxia (for a review see[80]). Hepatic LRP1 expression has been reported to be negatively associated with intracellular cholesterol level and positively associated with expression of SREBP-2[81]. On the whole, LRP1 may be seen as a complex biosensor allowing the cells to answer to micro-environmental variations[80].

Estrogen deficiency and LRP1receptor: A reduction in gene expression of LRP1 in Ovx rats was first reported by Ngo Sock *et al*[14] and confirmed in recent studies at the protein levels[15]. This decrease in LRP1 in Ovx animals may be associated with the decrease in the SREBP-2 transcription factor[81]. Interestingly, it has been recently reported that LRP1 is also a target for PCSK9 in HepG2 cells[82]. These authors postulated that LDL-R can effectively compete with LRP1 for PCSK9 activity. A reduction in *LRP1* gene expression could contribute to the increase in plasma cholesterol in Ovx rats by reducing the uptake of circulating lipoprotein remnants. Finally, inducible degrader of the low-density lipoprotein receptor an ubiquitin ligase that also mediates the degradation of the LDL-R was found not to be affected by an ovariectomy[66].

VLDLR

In addition to LRP, the LDL-R gene family includes a further member that functions as receptor for VLDL[83]. The VLDLR is expressed in several tissues including heart, muscle, adipose tissue, and macrophages but barely detectable in liver under normal conditions[83,84]. This receptor has been suggested to be important for the metabolism of apoE-containing triacylglycerol-rich lipoproteins, such as VLDL and IDL.

Interestingly, circulating PCSK9 originating from liver can regulate VLDLR in adipose tissue, which tissue does not express PCSK9[57]. In that manner, the absence of circulating PCSK9 resulted in an increase in the level of surface of VLDLR in the perigonadal tissue[57]. Interestingly, the increase was 10 times higher in female than in male mice[57]. This response was in line with the typical female pattern in mice that implies a high surface VLDLR levels in perigonadal fat and low surface LDLR levels in hepatocytes[66].

Hepatic cholesterol uptake from HDL

HDL is a class of lipoproteins that is able to remove excess cholesterol from cells and transport it through plasma to the liver. The apoA1 is the major structural determinant of HDL. It is involved in the formation as well as in the interaction with its receptor, scavenger receptor class B, type 1 (SR-B1)[85]. HDL formation occurs mainly in the liver and to a lesser extent in the intestine[85]. The events start when lipid-poor apoA1 is secreted by the liver or the intestine[86] or dissociates from lipoprotein particles in the plasma[87]. ApoA1 interacts with the membrane-embedded ATP binding cassette A1 (ABCA1) and incorporates small amount of phospholipids and unesterified cholesterol into the apoA1 molecule[88]. Maturation of these preβHDL in the plasma occurs due two enzymes, lecithin: Cholesterol acyl transferase (LCAT) that esterifies cholesterol and phospholipid transfer protein (PLTP) that transfers phospholipids from remnant particles to HDL.

HDLs have the ability of removing excess cholesterol from cells. The first mechanism involved the action of preβHDL interacting with ABCA1 that in addition of forming a new HDL by the liver is used to remove excess cholesterol from macrophages[89]. Spherical mature HDL may remove cholesterol from cells using several mechanisms. The particle may interact with SR-B1 on the plasma membrane. Macrophages also express ABCG1 transporters that mediate transfer of excess cholesterol to HDL. Finally excess cholesterol from cells may also efflux in absence of binding to transport protein, travels short distance through plasma and be taken up by HDL[25]. The activity of LCAT and PLTP prevents the HDL from being saturated with cholesterol. The enzyme cholesterol ester transfer protein (CETP) that transfer cholesteryl ester molecules from HDL to remnant particles in exchange for TG also increases the capacity of HDL to accept

unesterified cholesterol from cells.

HDLs circulating to the liver interact with SR-B1 the main HDL receptor[90]. SR-B1 in the liver facilitates the uptake of cholesterol and cholesterol esters from the HDL particle without the apoA1[86]. ApoA1 may then be recycled to form a new preβHDL. The action of SR-B1 is facilitated by the hydrolysis of TG by the hepatic lipase. The adrenal gland and gonads also highly express SR-B1 most likely due to their requirement in cholesterol[86].

HDLs are considered limiting for the reverse cholesterol transport because it is assumed that they deliver peripheral cholesterol to the liver for biliary secretion and eventually fecal excretion[91,92]. As discussed by Temel and Brown[93], however, there is evidence that HDL-driven cholesterol efflux does not correlate with how much is lost in bile or in the feces. Mice genetically lacking ApoA1 or ABCA1 and, therefore having very low circulating levels of HDL, or showing different steady-state concentrations of HDL-C have normal biliary and fecal cholesterol loss[94,95]. Some authors argue that apoB-containing lipoproteins and particularly the activity of CETP play a substantial role in reverse cholesterol transport[96].

Estrogen deficiency and hepatic HDL receptor: SR-B1 mRNA in liver that allows the return of cholesterol to liver *via* HDL was reported to be higher in Ovx compared to Sham rats[14]. Interestingly, *ABCA1* gene expression, involved in biosynthesis of nascent HDL was also found to be increased in Ovx rats[14]. An increase in gene expression of ABCA1 was also found in jejunum of Ovx rats[14]. Although limited, these findings point to the direction as if the hepatic contribution to HDL metabolism was increased with estrogen withdrawal.

HEPATIC CHOLESTEROL EFFLUX

There are essentially two ways by which liver can excrete cholesterol: (1) secretion of unmodified cholesterol or after its transformation in bile salts into bile caniculi; and (2) through VLDL secretion.

Hepatic cholesterol-bile acid metabolism

The liver is the only organ that has ability to eliminate cholesterol through its secretion into bile or its transformation into bile salts. Bile acids synthesis from cholesterol is stimulated by the nuclear factor liver X receptor (LXR) through its target gene cytochrome P450, family 7, subfamily a, polypeptide 1 (CYP7A1), the main enzyme in the conversion of cholesterol into bile acids[97]. The synthesis of a full complement of bile acids requires 17 enzymes[98]. The bile acid pool size is reduced by 75% in mice deficient in CYP7A1[99]. An alternative biosynthetic pathway is initiated by the enzyme cholesterol 27α-hydroxylase (Cyp27α1[99]). Bile salts are highly soluble in water. They form aggregate with phospholipids derived from hepatocyte membranes and solubilize cholesterol in bile for transport from liver to intestine[100]. Nuclear factor farnesoid X receptor (FXR) activated by bile acids, stimulates bile and cholesterol efflux from liver. Opposite to LXR, FXR suppresses bile acids synthesis by inhibiting Cyp7A1. At the canalicular membrane of the hepatocytes, bile salts are pump into bile by a membrane transporter, ABCB11, also referred to as bile salt export pump (BSEP) and to a lesser extent by the multidrug resistance-associated protein 2 (MDR2; ABCC2[101]), which activates two other transporters, ABCB4 involved in the transport of phospholipids and ABCG5/G8 a heterodimer involved in the secretion of cholesterol[102-104]. Alternative mechanisms to ABCG5/G8 cholesterol secretion involve ATP8B1 and diffusion[105]. Altogether bile salts and phospholipids form micelles which are stored in the gall bladder during fasting. In addition, bile salts may be exported to the blood at the sinusoidal membrane mediated by MRP3 (ABCC3) and MRP4 (ABCC4), as well as the organic solute transporter OST α/β[106]. Conversion of cholesterol to bile salts accounts for about 50% of daily cholesterol excretion[107].

Estrogen deficiency and hepatic cholesterol-bile acid metabolism: Cyp7A1and Cyp8b1 transcripts have been reported to be decreased in Ovx rats and mice[6,11,13,43] suggesting a reduction in cholesterol elimination *via* bile acid formation. This decrease has been found in Ovx rats fed a standard diet and even more so when Ovx rats were fed a high-fat (42%) diet[6]. On the opposite, estrogen treatment has been reported to result in an increase in biliary cholesterol hypersecretion in mice[4].

Estrogen deficiency was associated with lower transcript levels of BSEP and MDR2 suggesting that, in addition to synthesis, excretion of bile acids from hepatocytes to caniculi was decreased in Ovx rats[15,43]. Furthermore, the gene expression of nuclear receptors FXR and LXR was found to be lower in Ovx compared to Sham animals[43]. The decrease in gene expression of FXR suggests that bile acids did not accumulate in liver of Ovx rats. FXR mRNA levels are controlled by bile acids[108]. The specific role of hepatic FXR is to prevent bile acid hepato-toxicity by initiating the expression of a gene network involved in the synthesis and excretion of bile acids. Accordingly, FXR-null mice show massive accumulation of cholesterol in hepatocytes[109]. The indication that bile acid metabolism is disrupted in Ovx rats may in turn favours cholesterol accumulation in liver since bile acid secretion exerts a driven force for biliary cholesterol excretion[110]. Supporting the hypothesis that biliary metabolic pathways are indeed disrupted in Ovx animals is the finding of a decrease in total bile production in Ovx rats[111].

Gene expression of ABCG5/G8 transporters involved in exportation of cholesterol from the liver to the bile ducts was unchanged in Ovx compared to Sham rats[6,15,43] and in aromatase knockout mice[112] suggesting that these transporters are not regulated by estrogens.

Hepatic excretion of cholesterol through VLDL

VLDL assembly in liver is initiated by the entry of apoB100 in the lumen of the endoplasmic reticulum[113].

The apoB protein is lipidated by the action of microsomal transfer protein (MTP) accumulating TG as well as cholesterol esters molecules. Besides MTP and apoB100, other molecular markers of VLDL assembly include diacylglycerol acyltransferase 2 (DGAT2), involved in the reesterification of TG[114], and acyl-CoA: Cholesterol acyltransferase 2 (ACAT2) that converts free cholesterol into cholesterol esters[115]. Further lipidation of the VLDL particles after they exit the endoplasmic reticulum compartment is carried on by a lipid droplet-associated protein, cell death-inducing DNA fragmentation factor alpha-like-effector B (Cideb)[116]. The importance of Cideb has been enlightened by the finding of a reduction in plasma LDL levels in Cideb-null mice[117]. However, hepatic cholesterol storage was increased in liver of these animals due to its increased LDL-R and ACAT expression. Finally, small GTP binding protein (Sar1a), an intracellular vesicular trafficking protein, facilitates the movements of VLDL particles between the endoplasmic reticulum and the Golgi apparatus where they are secreted in the plasma.

Estrogen deficiency and hepatic VLDL metabolism: The observation that plasma cholesterol level is increased in Ovx animals[6,11] might suggest an increased cholesterol excretion through VLDL. On the opposite, a decrease in VLDL-TG production has been reported in estrogen-deficient animals[118,119]. Supporting such a decrease in VLDL production at the molecular level is the repeatedly reported decrease in gene expression of MTP, the rate-limiting molecule for VLDL assembly and secretion, in Ovx animals[15,43,118]. Transcripts of other genes involved in VLDL synthesis, including apoB, DGAT2, ACAT2, Cideb, and Sar1a have also been reported to be decreased in Ovx rats fed a standard diet[15,43] and even more so for some genes (MTP and apoB100) in Ovx rats fed an enriched-cholesterol diet[15]. The additive effect of estrogen withdrawal and high-cholesterol diet on reducing markers of VLDL production was corroborated by an accumulation of total cholesterol and TG in liver and lower levels of these two forms of lipids in plasma[15]. In search of an explanation for the postulated reduced VLDL production in Ovx rats fed the cholesterol diet, it has been suggested that cholesterol may induce ER stress through cholesterol accumulation[120] and that ER stress limits VLDL assembly and secretion through apoB degradation[121]. Collectively, these results points toward the interpretation that VLDL assembly is disrupted upon ovariectomy leading to reduced excretion of TG and cholesterol from the liver, thus contributing to exacerbate liver fat and cholesterol accumulation[14,15].

Molecular mechanisms by which estrogens regulate transcription of target genes involved in VLDL pathway are not well known. The classical genomic mechanism of estrogen action involves activation of its nuclear receptor (ERα and β) and subsequent binding to estrogen response elements located in the promoters of target genes[122,123]. Estrogens have also been shown to have non-genomic actions mediated through a subpopulation of ERα and β located at the plasma membrane[124]. It is thus possible that estrogens affect expression of target genes involved in different metabolic pathways through interaction in the nucleus and/or activation of signal transduction pathways at the plasma membrane.

Intestinal excretion of biliary cholesterol

As mentioned above, hepatic cholesterol is secreted into bile unmodified or after its conversion into bile salts. These bile salts participate in cholesterol transport and eventually in fat digestion in the intestine. However, rather than being lost in the feces, most of the bile salts are recycled when they are taken up by transport proteins in the distal ileum. FXR controls the absorption of bile acids in the intestine through the regulation of bile acid transporters from the intestine to the portal system[125]. These include apical sodium-dependent bile acid transporter, the ileal bile acid binding protein, and at the basolateral membrane of enterocytes the heterodimeric organite solute transporters α and β (OSTα, OSTβ)[126,127]. Bile salts picked up by these transporters enter the portal circulation and are transported back to the liver where they are eventually re-secreted into bile. This process of recycling back the bile salts between the intestine and the liver is called the enterohepatic circulation[128]. The Na^+-taurocholate cotransporting polypeptide (NTCP) is the major uptake system to transport bile salts from the blood into parenchymal cells[129]. Together with several organic anions transporting polypeptide, it controls bile salt uptake at the sinusoidal membrane[130]. Bile salt accumulation down-regulates NTCP at the transcriptional level mediated by FXR and the short heterodimer partner 1[131].

Less than 10% of transported bile salts are lost in the feces (0.4 g/d)[132]. Therefore, dietary cholesterol (0.4 g/d) constitutes only 25% compared to endogenous cholesterol (1.2 g/d) that passes through intestine in one day[133]. Coordination between intestinal bile acids levels and hepatic bile acids biosynthesis is assured through the intestinal secretion of fibroblast growth factor 15/19 that inhibits Cyp7α1 in liver under FXR activation[134].

Excretion of intestinal absorbed cholesterol: The cellular mechanisms by which chylomicrons in the intestine and VLDL in the liver are assembly are very similar. Their assembly depends of the availability of apoB, triglycerides, and the TG transfer protein MTP. However, opposite to liver, enterocytes express a protein called apoB editing complex-1[135]. As a result of the action of this enzyme, translation of apoB comes to a premature stop making intestinal apoB in the intestine 48% as long as the protein expressed in the liver (apoB100). Cholesteryl esters added to the core molecule of chylomicrons come from biliary acids (75%) and from dietary sources. During digestion, cholesteryl esters in food are hydrolyzed to form unesterified cholesterol[136]. Dietary and biliary cholesterol from micelles enter the enterocytes mainly (80%) *via* a protein channel, Neimann-Pick C-1 like 1 protein (NPC1L1)[137]. Some of

this cholesterol is immediately pumped back into the lumen by the heterodimer transporter ABCG5/G8[138]. A portion of cholesterol is also transferred to apoA1 by the ABCA1 transporter to form a nascent HDL. The fraction of cholesterol remaining is esterified to a long-chain fatty acid by ACAT2[139].

Estrogen deficiency and intestinal bile acid-cholesterol metabolism: The information is rather limited in regard to biliary cholesterol metabolism in the intestine. A greater faecal excretion of bile acids has been reported in Ovx rats[11]. The authors explain this response by suggesting a decreased reabsorption of bile acids from the ileum through a decrease in bile acid transporters. Gene expression of NTCP, the major uptake system to transport bile salts from the blood into parenchymal cells, was found to be unchanged in Ovx compared to Sham rats[15]. On the other hand, gene expression of ABCA1 was reported to be increased in jejunum of Ovx rats, suggesting an increased efflux of intestinal cholesterol through HDL synthesis in Ovx animals[14].

Transintestinal cholesterol excretion

The hepatobiliary pathway also referred to as the reverse cholesterol transport pathway is considered the major elimination cholesterol route. Nevertheless, fecal cholesterol excretion was observed in several states of disturbances in cholesterol biliary excretion supporting the existence of a new route for cholesterol excretion[140-142]. In other words, a large part of the cholesterol found in the feces originates from a source other than bile and diet. The non-biliary alternative called the transintestinal cholesterol excretion pathway implies the direct secretion of plasma lipoprotein-derived cholesterol by the small intestine[94,143,144]. Among the numerous studies on trans-intestinal cholesterol excretion (TICE), there is some agreement that under normal conditions TICE contributes to less than 30% of cholesterol found in the feces (for a review see[93]). However, the TICE pathway may be stimulated under pathophysiological or pharmacological conditions. For instance, intestinal cholesterol excretion is inducible by a high-fat diet[145] or pharmacologically by ligands of LXR[146]. The importance of the role of TICE has been recently highlighted by the demonstration that TICE is essential to macrophage reverse cholesterol transport in mice[142].

It seems that the liver initiates the activation of the TICE[93]. Findings in mice with impaired hepatobiliary cholesterol excretion indicate that cholesterol is first transported to the liver before being delivered to the intestine[93]. Temel and Brown[93] summarized evidence that indicate that it is the subsequent steps within the liver that determine the amount of cholesterol eliminated through the biliary and non-biliary excretory mechanism. The excess cholesterol is most likely repacked into apoB rich lipoproteins secreted by the liver. These liver-derived apoB-containing lipoproteins are recognized by the proximal small intestine through LDL-R and probably other mechanisms[147]. Le May *et al*[147] provided data suggesting that PCSK9 is a repressor of TICE dependent on the LDL-R. They also demonstrated that both LDL and HDL (possibly through SR-B1 transporter) provided cholesterol to TICE. Once the free cholesterol is liberated from the TICE lipoproteins, it may efflux from the apical side of the enterocyte through the ABCG5/G8 transporters or the multidrug transporter ABCG1a/b[93].

EFFECTS OF EXERCISE TRAINING ON LIVER AND INTESTINAL CHOLESTEROL METABOLISM

The main finding supporting the contention that exercise training improves lipid and cholesterol metabolism is the reported increase in plasma HDL levels and the concomitant decrease in LDL-cholesterol and triglycerides in human studies[148,149]. In animals, positive effects of exercise training on the outcome of disturbances in lipid and cholesterol metabolism has been demonstrated by Ramachandran *et al*[150] who reported a 50% reduction in pre-existing atherosclerotic lesions in LDL-R KO mice. Similarly, Matsumoto *et al*[151] reported that exercise training in LDL-R KO mice prevented aortic valve sclerosis. These authors specified that exercise exerted several numerous favourable effects that include preservation of valvular endothelial integrity, reduced recruitment of inflammatory cells, and oxidative stress. A decrease in aortic lesion size was also reported by Meissner *et al*[21] after 12 wk of voluntary running wheel in LDL-R deficient mice.

However, as mentioned by Meissner *et al*[152], the molecular pathways behind such exercise-induced improvements in plasma lipids are not well defined. In addition, the analysis of the effects of exercise training on the molecular components of cholesterol metabolism in liver is complicated by the variety of animal models used.

HMGCoA-r and exercise training

There is a paucity of information on the effects of exercise training on cholesterol biosynthesis. Ngo Sock *et al*[14] reported that training (8 wk) did not appear to have any effect on HMGCoA-r as well as on SREBP-2 transcripts whether in Sham or in Ovx rats. Previously, Meissner *et al*[152] reported an increase in lanosterol/cholesterol ratio in mice submitted to two weeks of voluntary exercise suggesting an increase in cholesterol biosynthesis. However, the same group of authors reported a decrease in HMGCoA-r after 12 wk of voluntary wheel running in LDL-R deficient mice[21]. On the whole, there is no clear indication that hepatic cholesterol biosynthesis is changed with exercise training.

LDL-R and exercise training

Using CETP transgenic mice, an animal model that simulates reverse cholesterol transport (RCT) in human, Rocco *et al*[153] found an increase in hepatic LDL-R protein

levels following 6 wk of treadmill exercise. Using this animal model they also found that exercise training improved macrophage RCT. An increase in *LDL-R* gene expression in liver of normal mice exercised for two weeks had been previously found[154]. At the same time, Wilund *et al*[155] reported an increase in *LDL-R* gene expression and a reduction in gallstone development in gallstone-sensitive mice fed a lithogenic diet after 12 wk of exercise training.

In a recent study, Wen *et al*[156] found that treadmill exercise for 8 wk resulted in an increase in PCSK9, LDL-R, and SREBP-2 mRNA in high-fat fed mice. On the other hand, they found a reduction in plasma PCSK9 levels and no difference in LDL-R protein abundance. They attributed these latter responses to the lower levels of circulating LDL-C in trained animals.

In other respects, exercise training (8 wk) did not alter *LDL-R*, *PCSK9*, and *LRP1* gene expression in Sham rats as well as being ineffective in correcting reductions in these molecular markers in Ovx rats[14]. On the opposite, Pinto *et al*[157] recently reported an increase in LDL-R protein levels in male mice trained for 6 wk. Taken together, there is indication that exercise training may favour liver cholesterol uptake from circulation through LDL-R thus, supporting the general finding of a reduction in circulating LDL-C in human[149].

HDL metabolism and exercise training

Exercise training (8 wk) did not influence SR-B1 and ABCA1 responses in Sham as well as in Ovx rats[14]. On the other hand, an increase in ABCA1 mRNA had previously been reported following 6 wk of treadmill exercise in rats accompanied by an increase in plasma HDL-C concentration[158].

Two weeks of exercise training resulted in an increase in SR-B1 in livers of exercised mice[154]. Wilund *et al*[155] also reported an increase in *SR-B1* gene expression and a reduction in gallstone development in gallstone-sensitive mice fed a lithogenic diet after 12 wk of exercise training. An increase in SR-B1 protein level in liver has also been reported in male mice trained for 6 wk along with the demonstration of an increased macrophage cholesterol flux to the liver[157].

In CETP transgenic mice, Rocco *et al*[153] found an increase in hepatic ABCA1 protein levels following 6 wk of treadmill exercise but no effects on SR-B1. On the whole, it appears that exercise training stimulates positive adaptations of molecular markers of HDL metabolism that would tend to support the finding of an increase circulating HDL levels with exercise training in human[149].

Bile acids and exercise training

Wilund *et al*[155] reported an increase in gene expression of Cyp27A1 in mice fed a lithogenic diet after 12 wk of exercise training. On the opposite, Meissner *et al*[21,152] did not observe any effects of exercise on key genes expression involved in bile acid synthesis (*CYP7A1*, *CYP8B1*, and *CYP27A1*) in mice despite an increased fecal bile acid and cholesterol excretion, leading the authors to assume a posttranscriptional regulation of these genes. The authors hypothesized that physical activity might increase bile acid synthesis to increase the capacity for micelle formation, thus increasing fatty acid absorption[21]. More recently, Pinto *et al*[157] reported an increase in *CYP7A1* gene expression in male mice trained for 6 wk. On the whole the existing molecular data would tend to support the physiological finding of an increase in fecal bile acid and cholesterol excretion in exercise trained animals.

VLDL and exercise training

There is a report that VLDL-TG secretion rate is reduced in human following exercise training[159]. A decrease in VLDL-TG accumulation and apoB mRNA after exercise training has also been reported in male Wistar rats[160]. Accordingly, liver MTP protein content has been found to be decreased with exercise training in mice[21] and in standard and high-fat fed female Sprague-Dawley strain rats[161]. Since liver fat accumulation is reduced with exercise training[162], the latter authors argue that the reduced liver VLDL production induced by regular exercise is a consequence of an increased lipid disposal through oxidation[163]. It is also possible that an increased hepatic insulin sensitivity following exercise training may have resulted in a decrease in VLDL-TG synthesis and secretion. It is well documented that insulin suppresses the secretion of VLDL particles by the liver[164] and *MTP* gene expression has been reported to be reduced by insulin in culture liver cells[165].

Plasma VLDL-TG levels have also been reported to be reduced following exercise training in Ovx rats for which VLDL-TG levels were already reduced[118]. This suggests that the effects of exercise training and estrogen withdrawal on VLDL-TG synthesis and/or secretion are additive and most likely take place through different pathways. On the other hand, the reduction in VLDL-TG production with exercise training in Ovx rats did not result in an accumulation of liver TG[118]. This was explained by the fact that exercise training increases the use of lipids, therefore, reducing fat delivery to the liver.

Intestinal markers and exercise training

Gene expression of ABCA1 was reported to be increased in jejunum of Ovx rats but unchanged by exercise training (8 wk)[14]. On the other hand, the same group of authors found an increase in ABCA1 in ileum of 8-wk trained rats[22]. An increase in ABCA1 mRNA in the upper part of the small intestine in Wistar rats trained for 6 wk had been previously reported[166]. Although limited, these findings concord with what has been found in liver and suggest that HDL synthesis from the intestine is increased following exercise training.

Wilund *et al*[155] found a decrease in *NPC1L1* and *ABCG5/G8* gene expression in duodenum of mice after 12 wk of exercise training. The authors explain that the reduction in ABCG5/G8 might have been the consequence of the reduction in NPC1L1 and less cholesterol transported into the enterocytes. A decrease in NPC1L1 and ABCG5/G8 was also recently reported in the ileum of

8-wk trained rats[22].

On the other hand, Meissner *et al*[152] reported an increase in fecal bile and cholesterol loss and a decrease in jejunal NPC1L1, suggesting a decrease intestinal cholesterol absorption, in male mice submitted to voluntary exercise for two weeks. Running mice also displayed lower ileal OSTα, OSTβ, and NTCP transporters, all involved in the enterohepatic circulation of bile acids. However, running did not affect mRNA levels of cholesterol efflux ABCG5/G8 in jejunum. On the whole these authors[152] reached the conclusion of an increase cholesterol turnover with regular exercise. In a subsequent study, Meissner *et al*[21] found a massive fecal bile acid loss in hypercholesterolemic LDL-R deficient mice trained for 12 wk. Decreases in ileal OSTα and OSTβ mRNA have also been reported in 8-wk trained rats along with a decrease in FXR transcription factor indicating that the need to protect the intestine against bile acid overload is reduced in trained animals[22]. Finally, Ngo Sock *et al*[22] found a decrease in pregnane X receptor (PXR) mRNA in ileum of trained rats. Since PXR receptors protect organisms from exogenous chemical insults, and several endobiotics such as lipids, steroids, and bile acids[167], the authors advocate that exercise training contributes to the maintenance of cholesterol and bile acid homeostasis[22].

On the whole it appears that, at the molecular level, exercise training would contribute to the maintenance of normal circulating cholesterol levels by increasing hepatic LDL-R and HDL metabolism and by favouring adaptations to bile acid metabolism that stimulate fecal bile and cholesterol excretion. When discussing the effects of exercise training on cholesterol metabolism one has to consider that on contrary of fatty acids and glucose or glycogen, cholesterol is not metabolized during exercise. Therefore, it might be an interesting avenue to look at the impact of exercise training on cholesterol metabolism through its link with lipid and glucose metabolism such as intestinal lipid absorption or hepatic *de novo* lipogenesis.

IN SUMMARY (ESTROGEN DEFICIENCY EFFECTS)

HMGCoA-r gene expression in liver along with its transcription factor SREBP-2 is decreased in Ovx animals suggesting a decrease in cholesterol synthesis. There are also indications that bile acid synthesis (*i.e.,* CYP7A1) and transporters of bile acid excretion into caniculi (*i.e.,* BSEP) are also decreased with estrogen deficiency. The reduction in hepatic bile acid metabolism would support the finding that total bile production is reduced in Ovx rats[111].

Although it has been shown that hepatic PCSK9 as well as SREBP-2 and LDL-R mRNA levels are reduced in estrogen deficient animals, there is on the whole data supporting the contention that LDL-R protein levels are increased in Ovx animals most likely associated with a reduction in *PCSK9* gene expression. Although it is difficult at the present time to reconcile clearly the impact of the absence of estrogens on the dynamics of hepatic PCSK9 and LDL-R and its consequence on plasma LDL-cholesterol, it is evident that estrogen levels play a critical role. The sex specific interaction between LDL-R and PCSK9 would be particularly relevant to post-menopausal women, especially in view of a new class of cholesterol lowering drugs, the PCSK9 inhibitors[68].

There are also data supporting the finding that VLDL and HDL metabolism are changed with the absence of estrogens. VLDL production and its main regulatory factor (MTP) have been repeatedly reported to be decreased in Ovx animals. On the other hand, increases in SR-B1 and ABCA1 mRNA in liver of Ovx animals support the contention that HDL metabolism is increased in these animals. An increase in ABCA1 in intestine suggesting an increase in HDL biosynthesis has also been reported[14].

Although it is obvious that more work has to be done to clearly understand the changes in cholesterol and bile acid metabolism in liver and intestine with the absence of estrogens, the data actually available in Ovx models tend to indicate an increase in cholesterol influx into the liver and a decrease in cholesterol efflux.

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ORIGINAL ARTICLE

Basic Study

Interplay between microRNA-17-5p, insulin-like growth factor-Ⅱ through binding protein-3 in hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of microRNA on insulin-like growth factor binding protein-3 (IGFBP-3) and hence on insulin-like growth factor-Ⅱ (IGF-Ⅱ) bioavailability in hepatocellular carcinoma (HCC).

METHODS: Bioinformatic analysis was performed using microrna.org, DIANA lab and Segal lab softwares. Total RNA was extracted from 23 HCC and 10 healthy liver tissues using mirVana miRNA Isolation Kit. microRNA-17-5p (miR-17-5p) expression was mimicked and antagonized in HuH-7 cell lines using HiPerFect

Transfection Reagent, then total RNA was extracted using Biozol reagent then reverse transcribed into cDNA followed by quantification of miR-17-5p and IGFBP-3 expression using TaqMan real-time quantitative PCR. Luciferase reporter assay was performed to validate the binding of miR-17-5p to the 3'UTR of IGFBP-3. Free IGF-Ⅱ protein was measured in transfected HuH-7 cells using IGF-Ⅱ ELISA kit.

RESULTS: Bioinformatic analysis revealed IGFBP-3 as a potential target for miR-17-5p. Screening of miR-17-5p and IGFBP-3 revealed a moderate negative correlation in HCC patients, where miR-17-5p was extensively underexpressed in HCC tissues (P = 0.0012), while IGFBP-3 showed significant upregulation in the same set of patients (P = 0.0041) compared to healthy donors. Forcing miR-17-5p expression in HuH-7 cell lines showed a significant downregulation of IGFBP-3 mRNA expression (P = 0.0267) and a significant increase in free IGF-Ⅱ protein (P = 0.0339) compared to mock untransfected cells using unpaired t-test. Luciferase assay validated IGFBP-3 as a direct target of miR-17-5p; luciferase activity was inhibited by 27.5% in cells co-transfected with miR-17-5p mimics and the construct harboring the wild-type binding region 2 of IGFBP-3 compared to cells transfected with this construct alone (P = 0.0474).

CONCLUSION: These data suggest that regulating IGF-Ⅱ bioavailability and hence HCC progression can be achieved through targeting IGFBP-3 *via* manipulating the expression of miRNAs.

Key words: Insulin-like growth factor binding protein-3; Insulin-like growth factor signaling pathway; MicroRNA; Insulin-like growth factor-Ⅱ; Hepatocellular carcinoma

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Core tip: microRNA-17-5p (miR-17-5p) was extensively underexpressed in hepatocellular carcinoma tissues, while insulin-like growth factor binding protein-3 (IGFBP-3) mRNA showed significant upregulation in the same set of patients. In HuH-7 cell line, miR-17-5p directly targets and downregulates IGFBP-3, consequently elevating the level of free insulin-like growth factor-Ⅱ (IGF-Ⅱ). Thus, manipulation of microRNAs can potentially control the activation of the oncogenic IGF axis.

Habashy DA, El Tayebi HM, Fawzy IO, Hosny KA, Esmat G, Abdelaziz AI. Interplay between microRNA-17-5p, insulin-like growth factor-Ⅱ through binding protein-3 in hepatocellular carcinoma. *World J Hepatol* 2016; 8(23): 976-984 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i23/976.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i23.976

INTRODUCTION

The insulin-like growth factor (IGF) signaling pathway is composed of IGF ligands, IGF receptors and insulin-like growth factor binding proteins (IGFBPs) which work in unison to regulate cell growth, differentiation, proliferation, and apoptosis. This axis is activated when the IGFs, IGF-Ⅰ and IGF-Ⅱ, bind to the insulin-like growth factor-1 receptor (IGF-1R) and activate a series of downstream signaling pathways controlling the cell cycle[1,2]. IGFBPs are transport proteins which bind to IGF-Ⅱ with high affinity thereby prolonging their half-life and circulation turnover, and negatively regulate the activity of IGFs by controlling their binding to IGF receptors[3]. The levels of IGFBPs are modulated by various IGFBP proteases, such as matrix metalloproteinases (MMPs), which regulate the bioavailability and activity of IGFBPs, by mediating their proteolytic cleavage[4].

Multiple IGF axis members were found to play an important role in hepatocellular carcinoma (HCC) pathogenesis. IGF-Ⅱ was found to be overexpressed in HCC and to promote tumor cell migration, proliferation and extra-hepatic metastasis[5-8]. Moreover, our research group has shown IGF-Ⅱ to be overexpressed in peripheral blood monocytes of HCC patients, and this aberrant expression was directly correlated with elevated serum levels of alfa-fetoprotein and poor prognosis[9]. IGF-1R was reported to be upregulated in 59% of HCC tissues in which it was associated with poor prognosis and tumors exceeding the Milan criteria[10]. The tumorigenic effect of IGF-1R was reversed through its efficient blockage by combination of two IGF-1R antibodies which dramatically reduced liver tumor growth[11]. On the other hand, IGFBP-3 expression was found to be inversely correlated to HCC metastasis and proliferation[12,13].

The potential regulation of IGF axis members by microRNAs is an appealing subject of investigation. We have previously shown that miR-615-5p downregulates IGF-Ⅱ expression and forcing its expression reduces tumorigenesis in HCC[14]. miR-122 was found to suppress IGF-1R expression thus inhibiting HCC progression[15,16]. Conversely, we have demonstrated that forcing the expression of the oncomiR miR-96 leads to the up-regulation of IGF-1R and IGFBP-3 expression, while forcing the expression of the oncomiR-182 leads to the downregulation of IGF-1R and the upregulation of IGFBP-3 expression[17]. On the other hand, our research group reported that miR-155 induces the expression of IGF-Ⅱ and IGF-1R and downregulates IGFBP-3 expression[18]. Nevertheless, the regulation of IGF-axis members by microRNAs still needs further investigation, particularly for the IGFBP-3. *In silico* analysis revealed IGFBP-3 as a potential downstream target for several microRNAs, one of which is microRNA-17-5p (miR-17-5p). This microRNA is an oncomiR that belongs to miR-17-92 cluster[19]. We have previously shown miR-17-5p to be significantly downregulated in non-metastatic HCC tissues compared to healthy tissues, where forcing its expression in HuH-7 cells resulted in enhancement of tumor cell growth, proliferation, migration, and colony-formation[20]. Therefore, this study aimed at identifying the impact of this important microRNA on IGFBP-3 expression, and

Table 1 Characteristic features of non-metastatic hepatocellular carcinoma patients and healthy controls

	Average ± SD
HCC and cirrhotic patient parameters	
Mean age	49 ± 13.5
Sex: Male/female	22/1
Ethanol abuse	None
AST (U/L)	100.5 ± 65.8
ALT (U/L)	85.6 ± 95.6
Alkaline phosphatase (U/L)	110.2 ± 60.7
Serum albumin (g/dL)	4.6 ± 1.5
Serum AFP (ng/mL)	155.7 ± 22.3
HCV Ab	100% (23/23 HCC patients)
HBV Ab	17.3% (4/23 HCC patients)
Healthy control (liver donor) parameters	
Mean age	31 ± 10.5
Sex: Male/female	7/3
Ethanol abuse	None
HCV Ab	None
HBV Ab	None

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; AFP: Alpha fetal protein; HBV: Hepatitis B virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Standard deviation.

Table 2 Number/sizes of focal lesions according to Milan criteria

Patients	No. of focal lesions	Size of focal lesions (cm)
Patient 1	3 focal lesions	1.5, 1 and 1
Patient 2	Unifocal	2.5
Patient 3	3 focal lesions	2, 2.5 and 3
Patient 4	3 focal lesions	2, 2 and 3.5
Patient 5	Unifocal	1.5-2
Patient 6	3 focal lesions	3-4, 1 and 1
Patient 7	Unifocal	4
Patient 8	3 focal lesions	4, 1 and 1
Patient 9	3 focal lesions	1, 1 and 1.5
Patient 10	Unifocal	2.5
Patient 11	2 focal lesions	1 and 1.7
Patient 12	3 focal lesions	1, 1 and 1
Patient 13	Unifocal	3
Patient 14	3 focal lesions	3, 1.5 and 2
Patient 15	3 focal lesions	1, 1 and 4
Patient 16	2 focal lesions	3 and 1.5
Patient 17	2 focal lesions	1.5 and 3
Patient 18	3 focal lesions	2.5, 2.5 and 1.5
Patient 19	3 focal lesions	1.5, 1 and 1
Patient 20	Unifocal	2
Patient 21	Unifocal	1.5
Patient 22	3 focal lesion	3, 2.5 and 1
Patient 23	Unifocal	3

consequently on the IGF-Ⅱ bioavailability, and hence on HCC tumorigenesis.

MATERIALS AND METHODS

Bioinformatics

Bioinformatics algorithms microrna.org, DIANA Lab, and Segal lab were used to predict microRNAs that may target IGFBP-3.

Study subjects

This study included 23 HCC patients who underwent liver transplantation surgery in the Kasr Al Aini Hospital, Cairo University, Egypt. Ten healthy liver tissues were obtained from the healthy liver donors. Healthy donors were non-diabetic, non-hypertensive and negative for hepatitis B and C viruses (Table 1). The study was approved by the ethical review committees of the German University in Cairo and Cairo University, and is in accordance with the standards set by the Declaration of Helsinki. All participants gave their written informed consent. All patients were non-metastatic with no extrahepatic manifestations and no vascular invasion. Most of the patients (65.5%) had more than one focal lesion as indicated in the pathology report and were subjected to clinical assessment as shown in (Table 2).

Cell cultures and transfection of microRNA oligonucleotides

HuH-7 cells were maintained in Dulbecco's modified Eagle's medium (Lonza, Switzerland) supplemented with 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% fetal bovine serum and Mycozap (1:500, Lonza, Switzerland) at 37 ℃ in 5% CO_2 atmosphere. HuH-7 cells were transfected with mimics and inhibitors of miR-17-5p (Qiagen, Germany) (Qiagen ID: MSY0000070 and MIN0000070, respectively). All transfection experiments were carried out in triplicates using HiPerFect Transfection Reagent (Qiagen, Germany), according to the manufacturer's protocol; the experiments were repeated three times. Cells that were only exposed to transfection reagent are designated as mock. Cells transfected with miR-17-5p mimics are designated as miR-17-5p, whereas cells transfected with miR-17-5p inhibitor are designated as anti-miR-17-5p.

mRNA and microRNA isolation from liver tissues and HCC cell lines

mRNAs and microRNAs were extracted from liver tissues and HCC cell lines. Fresh liver samples (HCC and healthy tissues) were collected during surgery and were immediately snapfrozen in liquid nitrogen. The specimens were manually pulverized in liquid nitrogen, and about 100 mg of tissues powder were used for large and small RNA extraction using mirVana miRNA Isolation Kit (Ambion, United States), according to the manufacturer's protocol. HCC cell lines were harvested 48 h after transfection according to HiPerFect Transfection Reagent protocol and total RNA was extracted using Biozol Reagent (Bioer Technology, China).

miRNA and mRNA quantification

The extracted microRNAs were reverse transcribed into single stranded complementary DNA (cDNA) using TaqMan MicroRNA Reverse Transcription Kit (ABI, United States) and specific primers for has-miR-17-5p and RNU6B. mRNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (ABI, United States)

Table 3 The forward and reverse primer sequences used in the wild type 1 and 2, and the mutant type 1 and 2 insulin-like growth factor binding protein-3 3'UTR constructs

Primer name	Primer sequence
WT1 forward	5′-CAATGGTAAACTTGAGCATCTTTTCACTTTCCAGTAGT-3′
WT1 reverse	5′-CTAGACTACTGGAAAGTGAAAAGATGCTCAAGTTTACCATTGAGCT-3′
WT2 forward	5′-CGTCGAAGCGGCCGACCACTGACTTTGTGACTTT-3′
WT2 reverse	5′-CTAGAAAGTCACAAAGTCAGTGGTCGGCCGCTTCGACGAGCT-3′
MUT1 forward	5′-CAATGGTAAACTTGAGCATCTTTTCATCCAGTAGT3′
MUT1 reverse	5′-CTAGACTACTGGATGAAAAGATGCTCAAGTTTACCATTGAGCT-3′
MUT2 forward	5′-CGTCGAAGCGGCCGACCACTGACGTGACTTT-3′
MUT2 reverse	5′-CTAGAAAGTCACGTCAGTGGTCGGCCGCTTCGACGAGCT-3′

WT: Wild type; MUT: Mutant type.

according to the manufacturer's instructions. Relative expression of miR-17-5p and RNU6B (for normalization) as well as IGFBP-3 and beta-2 microglobulin (B2M; as housekeeping gene for normalization) was quantified using TaqMan Real-Time quantitative PCR (ABI Assay IDs: 002308, 001093, Hs00365742_g1 and Hs00984230_m1, respectively) using StepOne™ Systems (ABI, United States). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. All PCR reactions including controls were run in duplicate reactions.

IGFBP-3 3'UTR construct and luciferase assay

The two predicted target sites for miR-17-5p on IGFBP-3 3'UTR were each designed as sticky ended oligonucleotides flanked by *Sac* Ⅰ and *Xba* Ⅰ restriction sites, and ligated into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Germany) to form the two wild-type (WT) constructs. Also, two mutant constructs (MUT) were designed where 3 nucleotides from the binding region had been deleted from each site. The first target site is denoted as WT1 and its mutant form as MUT1; the second target site is WT2 and its mutant form is MUT2. The forward and reverse primer sequences for each construct are as shown in (Table 3). HuH-7 cells were seeded in 24-well plates and either WT or MUT constructs were transfected by lipofection technique using SuperFect Transfection Reagent (Qiagen, Germany). The following day, the cells were co-transfected with miR-17-5p mimics using HiPerFect according to the protocol (Qiagen). After 48 h, luciferase assay was performed using Steady-GLO Luciferase Reporter System (Promega, Germany) according to the manufacturer's protocol. After 5 min, luminescence was measured at 545 nm. Luciferase experiments were done in triplicates.

Quantitative detection of free IGF-II protein

Free IGF-Ⅱ protein was measured in the cell culture supernatant from miR-17-5p mimicked, miR-17-5p antagonized, and mock untransfected HuH-7 cells, using the human IGF-Ⅱ ELISA kit (CUSABIO, China), according to the manufacturer's instructions. Absorbance was measured at 450 nm in a microplate reader.

Statistical analysis

miRNA and gene expression data analysis was performed according to the $2^{-\Delta\Delta CT}$ method. An assessment of the normality of data was done as a prerequisite for all the statistical tests to identify the correct statistical methods to analyze our data with. We used Shapiro Wilks test since the size of the sample is less than 50. The normality test for miR-17-5p and IGFBP-3 screening experiments of "Healthy controls" and "HCC patients" showed that the dependent variable, "RQ", isn't normally distributed since the significant value of the Shapiro Wilks test is less than 0.05, so the data significantly deviate from a normal distribution, with an exception in the data obtained from IGFBP-3 expression in the healthy controls were found to be normally distributed. In view of this fact the statistical significance of the data was analyzed by performing the non-parametric Mann-Whitney test. The degree of the relationship between linear related variables was measured by the Pearson *r* correlation test. The normality test for the transfection and binding confirmation experiments showed that the data are normally distributed; therefore the parametric unpaired *t*-test was used. The specific types of tests, when applicable, are indicated in the figure legends. All data are presented as mean ± standard error of the mean (SEM). All tests were 2-tailed and a two-tailed *P* value < 0.05 was required for statistical significance. All the data were statistically analyzed using GraphPad Prism 5 software.

The statistical methods of this study were reviewed by Dr. Nihal Aly Etman, Department of Statistics, Mathematics and Insurance, Faculty of Commerce, Ain Shams University.

RESULTS

Bioinformatics

miR-17-5p accession number and mature sequence were retrieved using miRBase database (http://www.mirbase.org/). *In silico* predictions were carried out using three different softwares, and results showed IGFBP-3 to be a potential downstream target to miR-17-5p, where the microRNA was predicted to bind to the 3'UTR of IGFBP-3 at two different regions. The interactions between miR-17-5p seed sequence and its target sequence on the 3'UTR of IGFBP-3 are as shown in (Table 4). Where, the seed sequence of miR-17-5p

Table 4 Predicted target region-seed sequence binding for miR-17-5p on the 3'UTR of insulin-like growth factor binding protein-3

Target region	hsa-miR-17-5p (seed sequence) binding to IGFBP-3 (target sequence)	Target sequence position on 3'UTR of IGFBP-3	6mer/7mer/8mer
Region 1	miR-17-5p 3'gaUGGACGUG-ACAUUCG*UGAAA*c 5' IGFBP-3 5'aaACUUGAGCAUCUUUU<u>CACUUU</u>c 3'	196-204	6mer
Region 2	miR-17-5p 3'GAUGGAC- GUGACAUUCG*UGAAAC* 5' IGFBP-3 5' CGGCCGACCACUG----------<u>ACUUUG</u> 3'	335-343	6mer

IGFBP-3: Insulin-like growth factor binding protein-3; miR-17-5p: MicroRNA-17-5p.

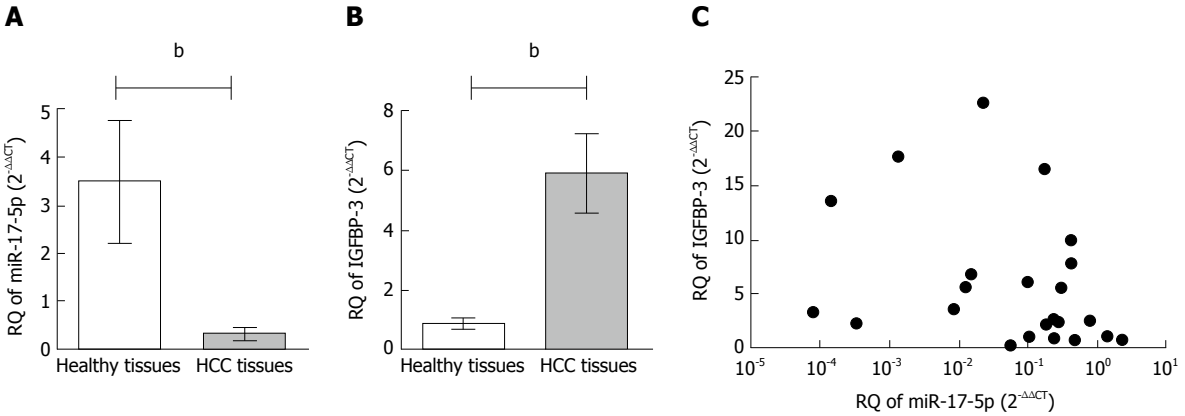

Figure 1 Expression profile of microRNA-17-5p and insulin-like growth factor binding protein-3 and their correlation in liver tissues. The expression of miR-17-5p and IGFBP-3 were investigated in 10 healthy and 23 HCC liver tissues using TaqMan qRT-PCR and normalized in each sample to RNU6B endogenous control for miR-17-5p and B2M for IGFBP-3. A: miR-17-5p expression was down-regulated in non-metastatic HCC patients compared to healthy liver tissues (P = 0.0012); B: Regarding IGFBP-3, its mRNA expression showed a significant higher expression in HCC tissues compared to healthy tissues (P = 0.0041). Statistical analysis was performed using the Mann-Whitney test; C: Relative quantitation (RQ) values of miR-17-5p and IGFBP-3 mRNA in HCC tissues were analyzed using Pearson's method of correlation. A non-significant inverse correlation was found with Pearson's r = -0.3244 (P = 0.1310). ^{b}P < 0.01. HCC: Hepatocellular carcinoma; IGFBP-3: Insulin-like growth factor binding protein-3; miR-17-5p: MicroRNA-17-5p; qRT-PCR: Real-time quantitative PCR.

is shown in bold and italic, while the target sequence of the 3'UTR of IGFBP-3 is underlined. The lines indicate complementarity between the binding region of the mRNA and the seed sequence of the microRNA, while the dots indicate mismatches or GU wobbles.

Expression profile of miR-17-5p and IGFBP-3 in non-metastatic HCC liver tissues

Expression of miR-17-5p in non-metastatic HCC tissues (n = 23) (0.318 ± 0.109) was significantly lower compared to healthy tissues (n = 10) (3.488 ± 1.267, P = 0.0012; Figure 1A). On the other hand, the expression of IGFBP-3 in the same non-metastatic HCC tissues (5.913 ± 1.294) was significantly higher compared to healthy tissues (1.352 ± 0.272, P = 0.0041; Figure 1B).

Correlation analysis between miR-17-5p and IGFBP-3 mRNA expression in HCC tissues

IGFBP-3 mRNA was quantified in all HCC tissues and correlated to miR-17-5p expression in the same patients. Using Pearson's statistical method of correlation, miR-17-5p expression was found to be moderately inversely correlated but not statistically significant with IGFBP-3 transcript levels in all HCC tissues studied (r = -0.3244, P = 0.1310; Figure 1C).

Impact of miR-17-5p on IGFBP-3 mRNA in HuH-7 cells

HuH-7 cells were transfected with miR-17-5p mimics and transfection efficiency was achieved with an observed 250 fold increase (P = 0.0470) in miR-17-5p levels in transfected cells (266.6 ± 113.2) compared to their respective untransfected mock cells (1.069 ± 0.1927) (Figure 2A). Mimicking of miR-17-5p in HuH-7 resulted in a significant downregulation of IGFBP-3 mRNA levels (0.6527 ± 0.1021) compared to mock untransfected cells (1.069 ± 0.1502, P = 0.0267). Conversely, inhibitors of miR-17-5p in HuH-7 cells showed a tendency of increase compared to mock untransfected HuH-7 cell lines (Figure 2B).

Impact of miR-17-5p on free IGF-II protein in HuH-7 cells

In miR-17-5p mimicked HuH-7 cells, there was a significant upregulation in the amount of the free IGF-Ⅱ protein (1.045 ± 0.05255) compared to mock untransfected HuH-7 cells (0.8344 ± 0.06783, P = 0.0339). Antagonizing the expression of miR-17-5p had no effect on the amount of the free IGF-Ⅱ protein compared to the mock HuH-7 cells (Figure 3).

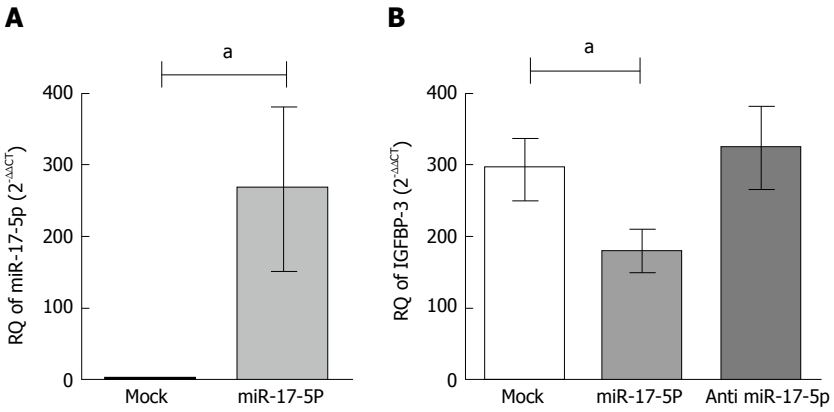

Figure 2 Impact of microRNA-17-5p on insulin-like growth factor binding protein-3 mRNA expression in HuH-7 cell line. A: The expression of miR-17-5p was determined by TaqMan qRT-PCR in HuH-7 cells transfected with oligonucleotide mimics of miR-17-5p, 48 h post-transfection, relative to their expression in mock untransfected HuH-7 cells. The expression of miR-17-5p was normalized to RNU6B endogenous control. A: Transfection of miR-17-5p mimics increased miR-17-5p levels in HuH-7 by 250 fold compared to mock cells (P = 0.0470). Unpaired t-test was performed; B: HuH-7 cells were transfected with miR-17-5p mimics or inhibitors, and the relative expression of IGFBP-3 was determined using TaqMan qRT-PCR, relative to mock untransfected cells, and gene expression was normalized to endogenous control B2M. IGFBP-3 mRNA expression was dramatically suppressed upon mimicking of miR-17-5p compared to mock cells (P = 0.0267), while inhibitors of miR-17-5p showed a tendency of increase compared to mock cells. Unpaired t-test was performed. [a]P < 0.05. IGFBP-3: Insulin-like growth factor binding protein-3; miR-17-5p: MicroRNA-17-5p; qRT-PCR: Real-time quantitative PCR.

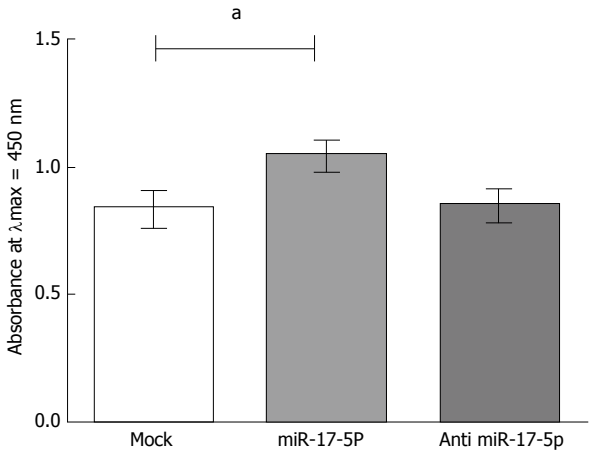

Figure 3 Impact of microRNA-17-5p on free insulin-like growth factor-Ⅱ protein in HuH-7 cells. HuH-7 cells were transfected with miR-17-5p mimics or inhibitors. The free IGF-Ⅱ protein was measured in media of mimicked and antagonized HuH-7 cells using an IGF-Ⅱ ELISA Kit. Free IGF-Ⅱ protein, measured at λmax = 450, was found to be significantly increased upon mimicking of miR-17-5p expression compared to mock untransfected cells (P = 0.0339), while inhibitors of miR-17-5p showed no effect on the levels of free IGF-Ⅱ protein levels compared to mock cells. Unpaired t-test was performed. [a]P < 0.05. IGF-Ⅱ: Insulin-like growth factor-Ⅱ; miR-17-5p: MicroRNA-17-5p.

Confirming IGFBP-3 as a direct target of miR-17-5p

To confirm that miR-17-5p directly targets the 3'UTR of IGFBP-3, wild-type constructs (WT1 and WT2) were designed where each of the two predicted 3'UTR target regions were inserted downstream to a luciferase reporter gene in pmiRGLO vector. To assess that the effects were due to specific binding to these binding regions, a mutant construct for each binding site was also prepared in which 3 base pairs were deleted from the predicted binding sequence in the 3'UTR of IGFBP-3, to form mutant constructs MUT1 and MUT2, respectively. Also, in a set of cells, empty pmiRGLO vector was transfected as a control to ensure that miR-17-5p mimics have no effect on the vector itself. For each binding region, experiments were performed by transfecting HuH-7 cells with either the construct containing the WT 3'UTR binding region of IGFBP-3, or the construct containing the MUT 3'UTR binding region. Then miR-17-5p mimics were co-transfected with the vectors or constructs and luciferase reporter activity was assessed. In cells transfected with WT1 construct, luciferase activity was not affected upon co-transfection with miR-17-5p mimics (Figure 4A). On the other hand, luciferase activity was inhibited by 27.5% in cells co-transfected with miR-17-5p mimics and WT2 construct (72.48 ± 2.383) compared to cells transfected with the WT2 construct alone (100.0 ± 9.432, P = 0.0474) (Figure 4B). In contrast, in cells transfected with either MUT1 or MUT2, no change in luciferase activity was observed upon mimicking with miR-17-5p (Figure 4). The inhibition in the luciferase activity observed only in the WT2 construct indicates direct targeting and transcriptional inhibition of IGFBP-3 by miR-17-5p mimics through only one of the two predicted target regions.

DISCUSSION

The regulation of IGFBP-3 by microRNAs has not been extensively studied but recently our research group showed that the oncomiR miR-155 represses IGFBP-3 expression in HCC cell lines[18]. In addition, we showed an increased expression of IGFBP-3 upon forcing the expression of miR-96 and miR-182[17]. To the best of our knowledge the IGF-Ⅱ bioavailability has never been investigated after targeting IGFBPs with microRNAs, therefore in this study, we aimed at identifying a new microRNA which could regulate the IGFBP-3 and consequently the IGF-Ⅱ bioavailability, and hence influence HCC tumorigenesis. *In silico* analysis revealed IGFBP-3 as a potential downstream target for miR-17-5p (Table 4), a microRNA which we have previously shown to have

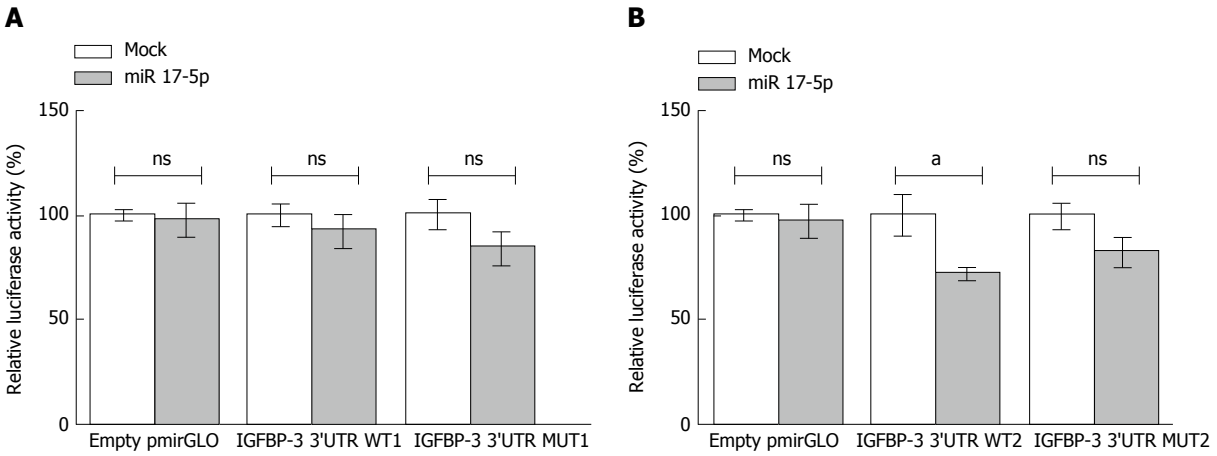

Figure 4 Insulin-like growth factor binding protein-3 is a direct target of miR-17-5p. For each target sequence, experiments were performed by transfecting HuH-7 cells with either empty pmiRGLO vector, or the construct with the wild-type (WT) miR-17-5p target region insert, or the construct with the mutant (MUT) miR-17-5p target region insert. Then miR-17-5p mimics were co-transfected with the vectors or constructs. A: Luciferase activity was not affected in cells co-transfected with miR-17-5p mimics and WT1 construct compared to cells transfected with the WT1 construct alone; B: On the other hand, luciferase activity was inhibited by 27.5%, in cells co-transfected with miR-17-5p mimics and WT2 construct compared to cells transfected with the WT2 construct alone (P = 0.0474). The cells transfected with either of the mutant constructs (MUT1 or MUT2) show no change in the luciferase activity upon mimicking with miR-17-5p. Unpaired t-test was performed. $^{a}P < 0.05$. NS: Not significant; IGFBP-3: Insulin-like growth factor binding protein-3; miR 17-5p: MicroRNA-17-5p.

oncogenic properties in HCC[20].

No correlation analysis was previously done between miR-17-5p and IGFBP-3 expression in HCC patients, therefore non-metastatic liver tissues of HCC patients were screened for that purpose. miR-17-5p was markedly downregulated (Figure 1A) while IGFBP-3 was significantly upregulated (Figure 1B) in the non-metastatic liver tissues of HCC patients compared to healthy controls. This goes in line with previous studies showing IGFBP-3 to be highly expressed in breast and esophageal cancer[21,22]. But on the other hand, it contradicts other studies that reported reduced IGFBP-3 mRNA expression and protein levels in metastatic HCC patients[12,13]. Moreover, the repression of miR-17-5p in HCC tissues (Figure 1A) corroborates our previous results that showed a significant downregulation of miR-17-5p expression in non-metasatic HCC patients[20], but nonetheless it contradicts other studies in metastatic HCC tissues[23]. These disparities can, however, be attributed to differences in the cohorts of patients included in the various studies, with regards to stage and etiology of the disease as well as other factors such as ethnicity, gender and age. Of note, the results of the correlation analysis revealed a moderate negative correlation between miR-17-5p and IGFBP-3 expression in HCC patients (Figure 1C), suggesting that IGFBP-3, as predicted by *in silico* analysis, may in fact be under the posttranscriptional regulation of miR-17-5p.

In order to investigate the effect of miR-17-5p on IGFBP-3, transfection experiments were performed by forcing miR-17-5p expression in HuH-7 cell lines and the expression of IGFBP-3 mRNA was assessed, where it was found that upon forcing miR-17-5p expression in HuH-7 cells, there was a significant downregulation in IGFBP-3 expression (Figure 2B). This finding further implies that miR-17-5p may target and regulate IGFBP-3 expression. As revealed by *in silico* analysis, the 3'UTR of the IGFBP-3 transcript contains two exclusive putative binding sites for miR-17-5p. In order to validate IGFBP-3 as a direct downstream target of miR-17-5p, a WT and a MUT luciferase reporter gene construct was prepared for each binding region on the 3'UTR of IGFBP-3. Using these microRNA-target expression constructs, it was demonstrated that forcing the expression of miR-17-5p significantly decreased luciferase activity only in the construct harboring the WT2 binding region of the 3' UTR of IGFBP-3 target gene (Figure 4). This interesting finding indicates that only one of the two putative binding sites is in fact functionally active and that miR-17-5p effectively targets and inhibits the transcription of IGFBP-3 by directly associating with this specific target region. This unusual observation has also been found in colon cancer where bioinformatic tools predicted two target sites on the oncogene Friend leukemia virus integration 1 (Fli-1) for the tumor suppressor miR-145; however, upon measuring the luciferase activity only the construct harboring one of these two predicted target sites of Fli-1 showed a decrease in luciferase activity by more than 50% upon miR-145 mimicking, while the other construct harboring the second target site did not respond to miR-145[24].

Since IGFBP-3 is a crucial negative regulator of the bioavailability of IGF-Ⅱ, therefore the levels of free IGF-Ⅱ protein were quantified in the media of miR-17-5p mimicked and mock untransfected HuH-7 cells. The results showed a significant increase in unbound IGF-Ⅱ in miR-17-5p mimicked HuH-7 cells compared to mock untransfected cells (Figure 3). This in turn confirms that miR-17-5p regulates IGF-Ⅱ bioavailability through direct targeting of IGFBP-3. In this regard, the biological function of miR-17-5p appears to simulate the effect of another regulator of the IGF pathway, the MMPs, whose

overexpression leads to the decrease in IGFBP-3 and subsequent increase in IGF-Ⅱ bioavailability[25].

In conclusion, the findings of this study shed light on the important role of the oncogenic miR-17-5p in hepatocarcinogenesis, where this microRNA was found to increase IGF-Ⅱ bioavailability by directly targeting and repressing IGFBP-3 expression. Hence, manipulating microRNA expression might be a compelling potential therapeutic approach in preventing HCC progression.

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COMMENTS

Background

Insulin-like growth factor-Ⅱ (IGF-Ⅱ) is a major activator of the oncogenic IGF axis, often overexpressed in hepatocellular carcinoma leading to the promotion of tumor cell migration, proliferation and metastasis. IGF-Ⅱ protein bioavailability is controlled by a class of insulin-like growth factor binding proteins (IGFBPs) 1-6 which regulate the binding of IGF-Ⅱ to its receptor, IGF-1 receptor. Very few studies have investigated the regulation of IGFBPs by microRNAs.

Research frontiers

Recently, microRNAs have entered the first clinical trials investigating their therapeutic potential in primary liver cancer.

Innovations and breakthroughs

This is the first study to investigate the effect of a microRNA on an IGFBP and consequently on the IGF-Ⅱ bioavailability.

Applications

microRNA-17-5p affected IGFBP-3 and consequently the level of free IGF-Ⅱ which could allow for the activation of the oncogenic IGF axis. This suggests that microRNAs can be manipulated to regulate the activation of this axis.

Terminology

microRNAs are approximately 22 nucleotide long single stranded, small, non-coding RNA sequences that post-transcriptionally regulate gene expression by binding to the 3'UTR of their target mRNA, suppressing its translation or causing its degradation.

Peer-review

The study is well planned involves proving of a concept by bioinformatics tools and then confirming *in vitro* and patient's tissues.

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ORIGINAL ARTICLE

Basic Study

Reversal of multidrug resistance of hepatocellular carcinoma cells by metformin through inhibiting *NF-κB* gene transcription

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Abstract

AIM: To interfere with the activation of nuclear factor-κB (NF-κB) with metformin and explore its effect in reversing multidrug resistance (MDR) of hepatocellular carcinoma (HCC) cells.

METHODS: Expression of P-glycoprotein (P-gp) and NF-κB in human HepG2 or HepG2/adriamycin (ADM) cells

treated with pCMV-NF-κB-small interference RNA (siRNA) with or without metformin, was analyzed by Western blot or fluorescence quantitative PCR. Cell viability was tested by CCK-8 assay. Cell cycle and apoptosis were measured by flow cytometry and Annexin-V-PE/7-AnnexinV apoptosis detection double staining assay, respectively.

RESULTS: P-gp overexpression in HepG2 and HepG2/ADM cells was closely related to mdr1 mRNA (3.310 ± 0.154) and NF-κB mRNA (2.580 ± 0.040) expression. *NF-κB* gene transcription was inhibited by specific siRNA with significant down-regulation of P-gp and enhanced HCC cell chemosensitivity to doxorubicin. After pretreatment with metformin, HepG2/ADM cells were sensitized to doxorubicin and P-gp was decreased through the NF-κB signaling pathway. The synergistic effect of metformin and NF-κB siRNA were found in HepG2/ADM cells with regard to proliferation inhibition, cell cycle arrest and inducing cell apoptosis.

CONCLUSION: Metformin *via* silencing NF-κB signaling could effectively reverse MDR of HCC cells by down-regulating MDR1/P-gp expression.

Key words: Metformin; Reversal; Multidrug resistance; Hepatocellular carcinoma

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Core tip: Metformin might target AMP-activated protein kinase mammalian target of rapamycin pathway, suppress hypoxia-inducible factor-1α and transcriptionally down-regulate P-glycoprotein (P-gp) and multidrug resistance (MDR)-associated protein 1, suggesting that metformin may reverse MDR by targeting the AMP-activated protein kinase/mammalian target of rapamycin/hypoxia-inducible factor-1α/P-gp and MDR-associated protein 1 pathways. In the present study, HepG2/ADM cells pretreated with metformin were sensitized to doxorubicin and P-gp was decreased through the nuclear factor-κB (NF-κB) signaling pathway. The synergistic effects were found in the cells with regard to proliferation inhibition, cell cycle arrest and inducing apoptosis, and inhibiting P-gp expression *via* the NF-κB signaling pathway effectively reversed MDR by down-regulating MDR1/P-gp expression.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and causes of cancer-related mortality worldwide[1-3]. Due to the lack of specific symptoms, the vast majority of HCCs are diagnosed at late and/or advanced stages[4,5]. Although recent advances in surgical techniques and interventional therapy have improved survival, the emergence of multidrug resistance (MDR) to a series of clinical chemotherapeutics with different structures or different target sites severely blocks the successful management of HCC[6,7]. The well recognized mechanism of classical MDR is the significant overexpression of human *MDR1* gene encoding MDR1/P-glycoprotein (P-gp) that acts as an efflux pump on cell surface[8,9]. Intracellular anti-cancer drugs increasingly flow from cells through the efflux pump, thus drug concentrations become lower and cancer cells become resistant to chemotherapeutic drugs such as doxorubicin[10,11].

Recently, some studies have found diverse anticancer effects of metformin in the cells of lung, gastric, endometrial, breast, and other types of cancer[12,13]. Metformin exhibits anti-proliferative effects in tumor cells *in vitro* and *in vivo*[14,15]. Metformin might target the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway[16,17], suppress the hypoxia-inducible factor-1α (HIF-1α)[18,19] and transcriptionally down-regulate P-gp and MDR-associated protein 1 (MRP1), suggesting that metformin may reverse MDR by targeting the AMPK/mTOR/HIF-1α/P-gp and MRP1 pathways[20,21]. In addition, the activation of nuclear factor-kappa B (NF-κB) pathway plays an important role in the development of HCC[22-24], but whether it is related to MDR and the underlying molecular mechanisms remain to be explored[25,26]. In this study, we silenced *NF-κB* gene transcription with specific small interference RNA (siRNA) in human resistant HepG2/adriamycin (HepG2/ADM) cells, and explored the impact of metformin and NF-κB silencing, alone or in combination, on MDR1 regulation and MDR in HCC cells.

MATERIALS AND METHODS

Cell culture

Human hepatoma cell line HepG2, HepG2/ADM cell line and hepatocyte cell line LO2 were purchased from Aibio Biotech Company (Shanghai, China). LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, KeyGen Biotech Co., Ltd, Nanjing, China) containing 10% fetal bovine serum (FBS, Invitrogen, United States), penicillin (100 U/mL)/streptomycin (100 U/mL), at 37 ℃ with 5% CO_2. HepG2 and HepG2/ADM cells were cultured in RPMI 1640 (KeyGen Biotech Co., Ltd, Nanjing, China) complete medium supplemented with 10% FBS, penicillin (100 U/mL)/streptomycin (100 U/mL) at 37 ℃ in a humidified incubator containing 5% CO_2.

Western blot

The cultured cells were washed with phosphate buffered saline (PBS) twice and lysed in phenylmethane sulfonyl fluoride (PMSF, Beyotime, Nantong, China) cell lysis buffer (1:1000), and the protein concentrations were determined with the bicinchoninic acid (BCA, Beyotime,

Nantong, China) protein assay kit. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF, Millipore, United States) membranes. After blocking with 5% skim milk in Tris-buffered saline with tween (TBST) at room temperature for 3 h, the membranes were incubated with the primary antibody overnight at 4 ℃. The primary antibodies were diluted as follows: p65 and P-p65 (rabbit anti-human, 1:1000, Cell Signaling, United States), MDR1 (rabbit anti-human, 1:500, Abcam, United States) and β-actin (mouse anti-human, 1:2000, internal reference, Proteintech, United States). Then the membranes were washed three times with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody (mouse or rabbit anti-human, 1:1000, Univ-bio, Nanjing, China) for 2.5 h at room temperature. Finally, the samples were detected with Quantity One software using the electrochemiluminescence kit (Millipore, United States). All Western blot experiments were repeated three times.

Real-time quantitative PCR

The cultured cells were digested with trypsin. Total RNA was extracted with TRIzol (Invitrogen, United States) reagent according to the protocol of the manufacturer. The quantity of total RNA was determined based on absorbance at 260 nm, and the purity of total RNA was analyzed based on the absorbance ratio at 260 and 280 nm ($A_{260/280}$). Reverse transcription of total RNA to complementary DNA (cDNA) was performed with RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, CA, United States). PCR was carried with an SYBR Premix Ex TaqTM Ⅱ kit (TaKaRa, Dalian, China), and GAPDH was used as an internal reference. The sequences of the primers used[27] were: NF-κB/p65 (forward: 5′-CTATCAGTCAGCGCATCCAG-3 and reverse: 5′-GCCAGAGTTTCGGTTCACTC-3′); mdr1 (forward: 5′-CCGGTT TGGAGCCTACTTG-3′ and reverse: 5′-TCCAA TGTGTTCGGCATTAG-3′); and GAPDH (forward: 5′-CAAGGTCATCCATGACAAC TTTG-3′ and reverse: 5′-GTCCACCACCCTGTTGCTGTAG-3′). Real-time PCR cycling parameters consisted of initial denaturation at 94 ℃ for 2 min and 40 cycles of 95 ℃ for 10 s, 55 ℃ for 30 s, and 70 ℃ for 45 s. The amplification specificity was confirmed by the melting curves. Ct values were calculated based on duplicates and normalized to GAPDH. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR experiments were repeated three times.

Cell viability assay

Cell viability was evaluated with CCK-8 kit (Dojindo, Japan). Cells were divided into blank, negative control and experimental groups. Briefly, cells in logarithmic growth phase were digested with trypsin, and the cell suspension liquid (100 μL) was seeded in 96-well plates. Toxicity tests were performed with different concentrations of ADM added to 96-well plates in the experimental group. The micro-plates were pre-cultured at 37 ℃ in a humidified incubator containing 5% CO_2, and liquid was changed at a fixed time interval. Then 10 μL/well CCK-8 solution was added and incubated at 37 ℃ for 4 h. The absorbance (A) was measured with a microplate reader at a wavelength of 450 nm. Cell survival rate was calculated as $A_{exp}/A_{con} \times 100\%$. Values of IC_{50} were evaluated with the Graphpad Prism5 software. Each individual experiment was performed at least three times.

Metformin treatment

HepG2/ADM cells were divided into three groups: Blank, control and experiment. The experimental group was treated with 1 μmol/L metformin for 24 h, and then continued to be cultured for 48 h with 1.5 μmol/L doxorubicin. The control group was only treated with doxorubicin, and the blank group did not undergo any treatment.

Analysis of cell apoptosis

HepG2/ADM cells were treated with drugs for 48 h, and then continued to be cultured for 24 h with another culture solution. Cells were harvested using trypsin without EDTA and washed with cold PBS twice. Cell cycle and apoptosis ($n = 3$) were measured by flow cytometry and Annexin-V-PE/7-AnnexinV apoptosis detection double staining assay (BD, United States), respectively.

Plasmid construction and cell transfection

NF-κB-siRNAs were designed according to the previously reported sequences[28] and synthesized by the Biomics Company (Nantong, China) according to Rel A sequence obtained from Gene ID 5970. The sequences of siRNAs were: NF-κB/p65 siRNA (forward, 5′-TGCTGTTCATCTCCTG AAAGGAGGCCGTTTTGGCCACTGACTGACGGCCTCCT CAGGAGATGAA-3′ and reverse, 5′-CCTGTTCATCTCCT GAGGAGGCCGTCAGTCAGTGGCCAAAACGGCCTCC TTTCAGGAGATGAAC-3′; and negative-siRNA (forward, 5′-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCA CTGACTGACGTCTCCACGCAGTACATTT-3′ and reverse, 5′-CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCC AAAACGTCTCCACGCGCAGTACATTTC-3′. Each siRNA was inserted to a pcDNA™ 6.2-GW/EmGFPmiR vector (Invitrogen, United States). HepG2/ADM cells were divided into blank control, negative siRNA control and NF-κB/p65 siRNA transfection groups. After cells were planted into microwell plates at a density of 70%, the plasmids were transfected into cells for incubation for 24 h according to the manufacturer's instructions. The medium was removed on another day and replaced with the fresh one, and the transfection efficiency was observed with a fluorescence microscope. These experiments were performed in triplicate.

Statistical analysis

Data are expressed as the mean ± SD. Statistical analyses were done using the SPSS21.0 software package. Differences between groups were assessed using analysis of variance or *t*-test. $P \leqslant 0.05$ was regarded as

Table 1 Absorbance values (n = 3, mean ± SD) of HepG2/adriamycin cells treated with different concentrations of metformin

Time (h)	0 (blank)	0.1 mmol/L	0.3 mmol/L	1 mmol/L	3 mmol/L	10 mmol/L
24	1.242 ± 0.03	1.233 ± 0.04	1.221 ± 0.02	1.195 ± 0.00	1.189 ± 0.02	1.101 ± 0.02[a]
48	1.744 ± 0.01	1.734 ± 0.02	1.718 ± 0.04	1.703 ± 0.03	1.583 ± 0.03[a]	1.483 ± 0.01[a]
72	1.692 ± 0.04	1.677 ± 0.01	1.650 ± 0.06	1.583 ± 0.06	1.420 ± 0.06[a]	1.300 ± 0.04[a]

[a]P < 0.05 *vs* the blank group.

Table 2 Effect of adriamycin combined with metformin on the proliferation of HepG2/adriamycin cells (n = 3, mean ± SD)

Adriamycin (μmol/L)	24 h		48 h		72 h	
	Control	Metformin	Control	Metformin	Control	Metformin
0	1.434 ± 0.03	1.327 ± 0.04[a]	1.477 ± 0.08	1.357 ± 0.01	1.695 ± 0.08	1.507 ± 0.05[a]
0.01	1.280 ± 0.06	1.160 ± 0.01[a]	1.489 ± 0.03	1.314 ± 0.03[a]	1.505 ± 0.01	1.378 ± 0.07[a]
0.1	1.194 ± 0.10	1.111 ± 0.09	1.418 ± 0.01	1.213 ± 0.02[a]	1.453 ± 0.02	1.249 ± 0.04[a]
1	0.847 ± 0.02	0.662 ± 0.02[a]	0.661 ± 0.01	0.661 ± 0.06	0.753 ± 0.04	0.508 ± 0.04[a]
5	0.628 ± 0.08	0.458 ± 0.02[a]	0.358 ± 0.02	0.208 ± 0.03[a]	0.347 ± 0.03	0.194 ± 0.03[a]
10	0.531 ± 0.00	0.399 ± 0.01[a]	0.162 ± 0.01	0.062 ± 0.01[a]	0.122 ± 0.01	0.049 ± 0.01[a]
20	0.284 ± 0.01	0.162 ± 0.01[a]	0.143 ± 0.01	0.051 ± 0.00[a]	0.084 ± 0.01	0.027 ± 0.00[a]

[a]P < 0.05 *vs* the control group. The proliferation of HepG2/adriamycin cells calculated with SPSS21.0 is presented as mean ± SD from CCK-8 assay in triplicate.

statistically significant.

RESULTS

Expression of P-gp, mdr1, and NF-kB in different liver cell lines

The levels of P-gp, mdr1, and NF-κB expression in different liver cell lines are shown in Figure 1. The proliferation of HepG2 and HepG2/ADM cells was decreased along with the increase of the concentration of doxorubicin, and the ability of proliferation was higher in HepG2/ADM cells than in HepG2 cells. At 24, 48 and 72 h, the IC_{50} values of doxorubicin against HepG2 cells were 0.489, 0.221 and 0.224 μmol/L, respectively, and the IC_{50} values of doxorubicin against HepG2/ADM cells were 4.166, 1.522 and 1.380 μmol/L, respectively. The resistance index (RI, μmol/L) of HepG2/ADM cells was 8.519 at 24 h, 6.874 at 48 h and 6.166 at 72 h. There was almost no P-gp expression in LO2 cells. Different degrees of expression of P-gp protein were observed in HepG2 and HepG2/ADM cells, but the P-gp expression in HepG2/ADM cells was significantly higher than that in HepG2 cells (Figure 1A and B). The p-p65 expression was significantly increased, while the expression of p65 was significantly decreased in HepG2/ADM cells (Figure 1C and D). The levels of mdr1 mRNA and NF-κB mRNA were 3.310 ± 0.154 and 2.580 ± 0.040, respectively, in HepG2/ADM cells, and 0.084 ± 0.038 and 0.607 ± 0.032, respectively, in HepG2 cells; the former was significantly higher than the latter (P < 0.01). Relative transcript levels ($2^{-\Delta\Delta ct}$) of mdr1 mRNA and NF-κB mRNA were 9.381 ± 0.750 and 3.927 ± 0.069, respectively (Figure 1E).

Effect of metformin on HepG2/ADM cells

The effect of metformin on the proliferation of HepG2/ADM cells was concentration- and time-dependent (Table 1). Metformin showed no significant effect on HepG2/ADM cells when its concentration was less than 3 mmol/L, but had different degrees of inhibition on the proliferation of HepG2/ADM cells when its concentration was between 3-10 mmol/L (P < 0.05). The HepG2/ADM cells were divided into experimental and control groups. After pretreatment with metformin, the experimental group cells were treated with different concentrations of doxorubicin. The effect of adriamycin combined with metformin on the proliferation of HepG2/ADM cells is shown in Table 2. After treatment with metformin, HepG2/ADM cells were more sensitive to adriamycin.

Metformin promotes HepG2/ADM cell apoptosis

The levels of HepG2/ADM cell apoptosis in the experimental (treated with metformin plus adriamycin), control (only treated with adriamycin) and blank (without adriamycin or metformin) groups are shown in Figure 2. After the cells were pretreated with 1 mmol/L metformin for 24 h, adriamycin was added. MDR1 in HepG2/ADM cells was down-regulated, the cell cycle was blocked at G_0/G_1 phase, and apoptosis was enhanced. Significant differences in the apoptosis rates were found among different groups (F = 3726.97, P < 0.001), and the apoptosis rate was significantly higher in the experimental group (22.17% ± 0.37%) than in the control group (14.86% ± 0.21%) or the blank group (4.17% ± 0.13%).

Metformin reverses MDR via the NF-κB signaling pathway

Metformin reversed the MDR of HCC cells *via* the NF-κB signaling pathway (Figure 3). The levels of P-gp expression in the HepG2/ADM cells were decreased with

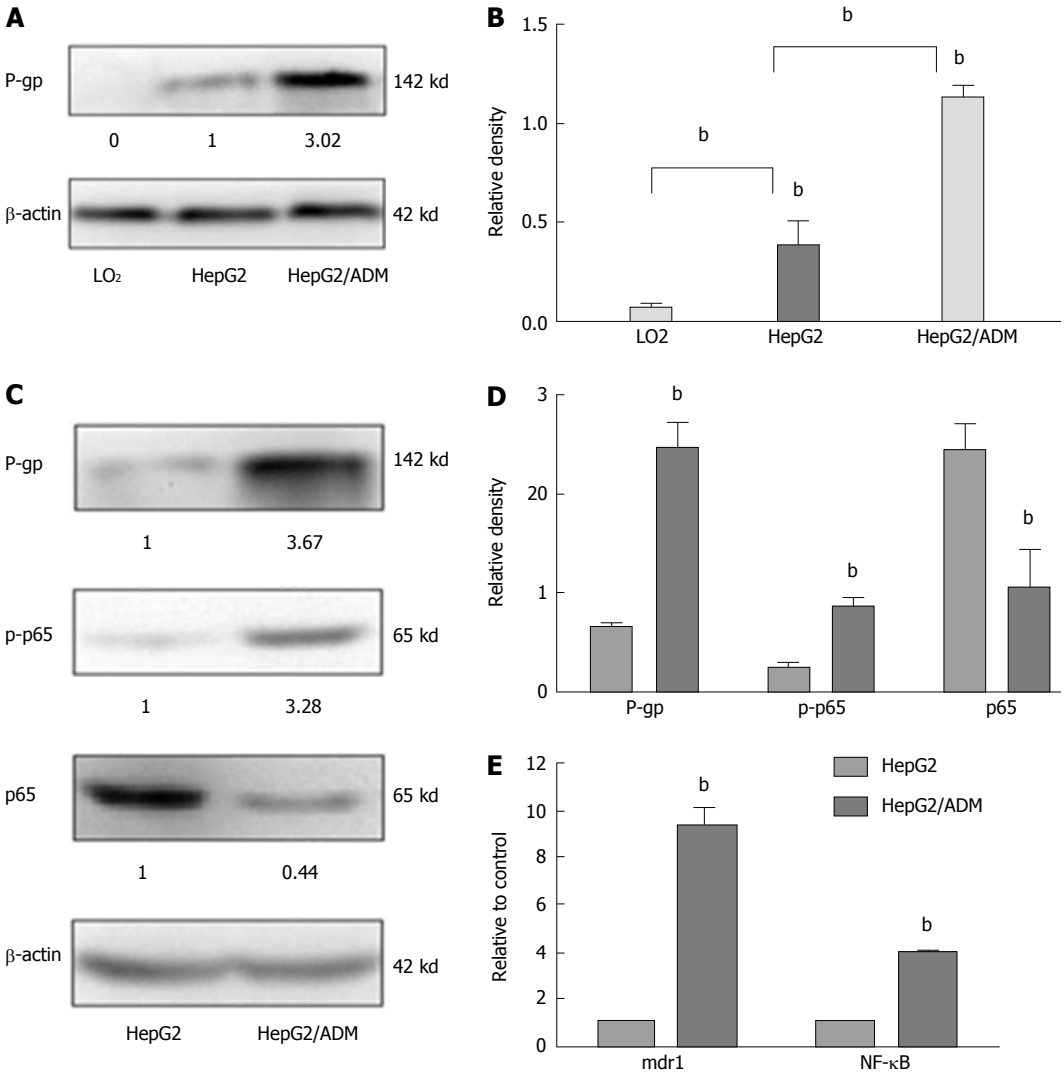

Figure 1 The levels of P-glycoprotein, mdr1 and nuclear factor-κB expression in different cell lines. A and C: The levels of P-gp and NF-κB expression in HepG2 or HepG2/ADM cells were determined by Western blot. The number indicates the ratio of HepG2/ADM cells to HepG2 cells (n = 3, mean ± SD); B and D: The gray intensity images of Figure 1A and Figure 1C, respectively; E: The levels of mdr1 and NF-κB mRNA expression were determined by qRT-PCR. $^{b}P < 0.01$ (n = 3, mean ± SD), compared with hepG2 or LO2 cell line. P-gp: P-glycoprotein; NF-κB: Nuclear factor-κB; ADM: Adriamycin.

the increasing dose of metformin, and the phosphorylated p65 expression in the nucleus was also decreased. Metformin could down-regulate P-gp expression by inhibiting NF-κB activation in a dose- and time-dependent manner.

Synergistic effect of metformin plus NF-κB-siRNA

The synergistic effects of metformin combined with NF-κB siRNA in reversing MDR are shown in Figure 4. HepG2/ADM cells were divided into three groups: Untreated cells, cells treated with metformin alone and those treated with metformin combined with NF-κB-siRNA. In the NF-κB-siRNA group, NF-κB-siRNA was transfected into HepG2/ADM cells for 24 h, and then cells were treated with 1 mmol/L metformin for 48 h. The levels of P-gp expression were 0.91 ± 0.24, 0.63 ± 0.13 and 0.22 ± 0.02 (F = 14.47, P = 0.005) in untreated, metformin and the metformin combined with NF-κB-siRNA groups, respectively. The expression of P-gp was significantly reduced in cells treated with metformin plus NF-κB-siRNA compared with that in cells only treated with metformin (t = 5.39, P = 0.006).

DISCUSSION

Recent advances in surgical techniques and interventional therapy have improved survival of HCC patients[6,7,29]. However, the emergence of MDR to a series of clinical chemotherapeutics with different structures or target sites severely blocks the successful management of HCC and still is a difficult problem to be solved in clinical practice[30,31]. MDR in HCC could result from several biochemical mechanisms including decreased drug influx, increased drug efflux, altered cell cycle checkpoints, altered drug targets, increased drug metabolism and/or resistance to drug-induced apoptosis. Therefore, it is very important to find safe and effective MDR reversal agents for HCC[32]. In the present study, metformin with

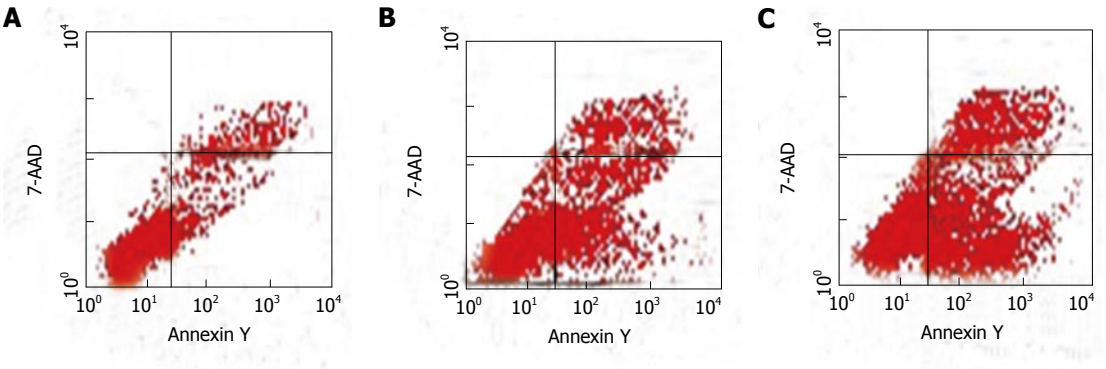

Figure 2 Metformin enhances adriamycin-induced apoptosis of HepG2/adriamycin cells. Cell early apoptosis was measured by Annexin-V-PE/7-AAD double staining assay in triplicate. A: The blank group (untreated); B: The control group (only treated with adriamycin); C: The experiment group (treated with metformin plus adriamycin). AAD: AnnexinV apoptosis detection.

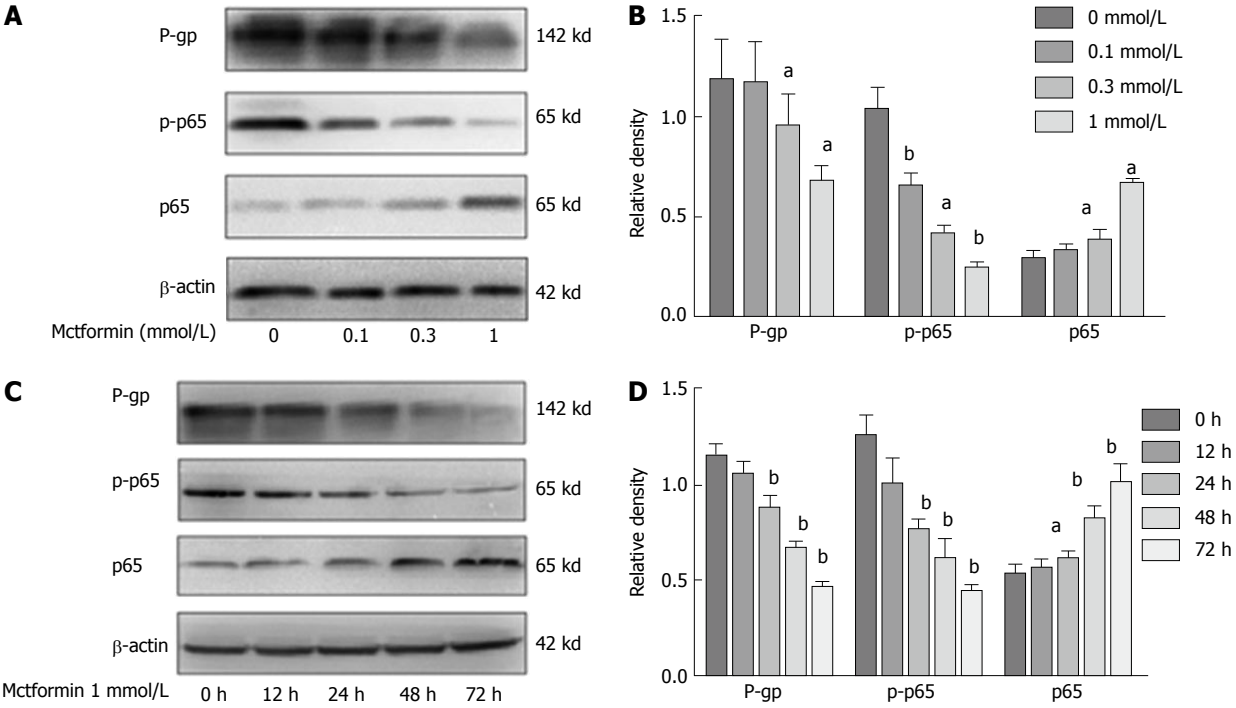

Figure 3 Metformin down-regulates P-glycoprotein expression *via* the nuclear factor-κB signaling pathway. A: After HepG2/ADM cells were treated with different doses of metformin for 24 h, the levels of P-gp and p-p65 expression analyzed by Western blot were decreased in a dose-dependent manner, and the cytoplasma p65 increased in a dose-dependent manner; B: The gray intensity images of Figure 3A. $^aP < 0.05$, $^bP < 0.01$ *vs* the blank group ($n = 3$, mean ± SD); C: After HepG2/ADM cells were treated with 1 mmol/L metformin for different time periods, the levels of P-gp and p-p65 expression analyzed by Western blot were decreased in a time-dependent manner, and the cytoplasma p65 increased in a time-dependent manner; D: The gray intensity images of Figure 3C. $^aP < 0.05$, $^bP < 0.01$ *vs* the blank group ($n = 3$, mean ± SD). P-gp: P-glycoprotein; NF-κB: Nuclear factor-κB; ADM: Adriamycin.

silencing *NF-κB* gene transcription was used to reverse MDR of HepG2/ADM cells with high NF-κB expression.

Anti-cancer drug efflux is one of the most common mechanisms of MDR of HCC cells, and it is mediated by ATP-binding cassette transporters[33,34], such as P-gp encoded by *MDR1* gene, which is located downstream of the NF-κB signaling pathway. P-gp expression regulated by MDR1 is the most important and common cause of MDR, and weakened the apoptosis of cancer cells induced by chemotherapeutic drugs. Both P-gp expression and NF-κB activation are linked closely with HCC progression[35]. Usually NF-κB takes part in gene transcription by means of homodimers or heterodimers, such as p50/p65, p65/p65, and p65/Rel. In quiescent cells, they are predominantly cytoplasmic, associating with members of inhibitory IκB family and forming NF-κB/IκB complexes without activity. Both P-gp and NF-κB at the protein or transcriptional level were significantly higher (Figure 1), with p65 expression decreasing in HepG2/ADM cells, indicating that abnormal P-gp and NF-κB expression could associate with the MDR formation of HCC cells[20].

Metformin is a safe, low-cost drug, and therefore remains one of the most commonly prescribed drugs worldwide[16,36]. The anticancer effects of metformin indicate the possibility that certain diabetes-associated types of

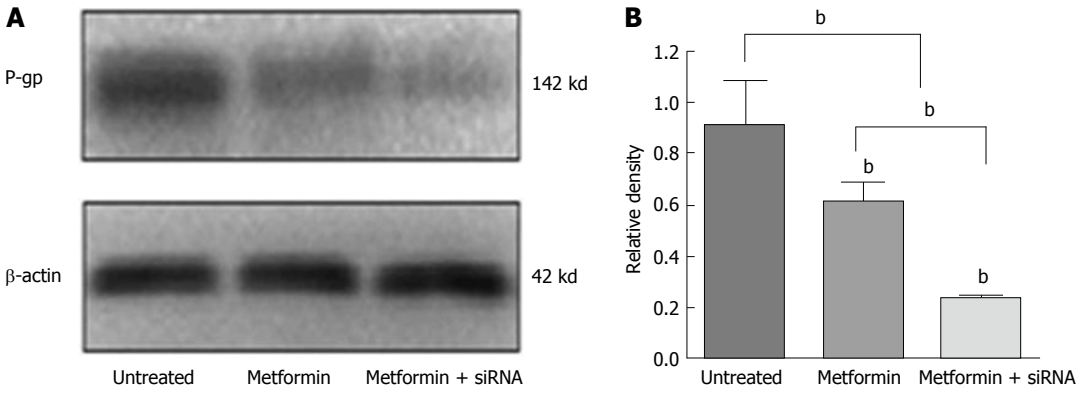

Figure 4 Alteration of P-glycoprotein expression in cells treated with metformin plus nuclear factor-κB-small interference RNA. A: Alteration of P-gp expression in different groups of cells. A significant decrease of P-gp expression analyzed by Western blot was found in HepG2/ADM cells treated with the metformin plus NF-κB-siRNA; B: The gray intensity images of Figure 4A. $^{b}P < 0.01$ *vs* the blank group ($n = 3$, mean ± SD). P-gp: P-glycoprotein; NF-κB: Nuclear factor-κB; ADM: Adriamycin; siRNA: Small interference RNA.

cancer[37,38] may be circumvented, and metformin has anti-proliferative potential against cancer cells or reversing MDR *in vitro* and *in vivo*[39,40]. However, the precise molecular mechanisms whereby metformin works in cancer prevention remain multi-factorial and ill-defined. Metformin affected HepG2/ADM cell proliferation in a dose- and time-dependent manner (Table 1). Metformin at < 3 mmol/L had no significant impact on HepG2/ADM cells, but the cells treated with metformin between 3-10 mmol/L were more sensitive to adriamycin with regard to promoting cell apoptosis (Figure 2) and inhibiting cell proliferation (Table 2), suggesting that metformin could increase the sensitivity of HepG2/ADM cells to anti-cancer drugs.

There are few studies on the effect of metformin on MDR of HCC cells. siRNA strategy is a powerful technique to inhibit specific gene expression, which has highlighted the potential use of siRNA molecules to study gene function or explore new HCC therapeutic agents[41,42]. The expression of *NF-κB* gene transcription was inhibited by specific siRNA, which significantly down-regulated P-gp and enhanced the chemosensitivity of HCC cells to doxorubicin, confirming the mechanism of decreasing P-gp *via* the NF-κB signaling pathway. The synergistic effects of metformin and NF-κB siRNA were found in HepG2/ADM cells with regard to cell proliferation inhibition, cell cycle arrest, and inducing cell apoptosis. These data confirm that the metformin could enhance the HepG2/ADM cells sensitivity to adriamycin and reverse MDR *via* the NF-κB signaling pathway (Figure 4).

In conclusion, the development of MDR still is one of major causes of HCC chemotherapy failure[43,44]. Although specific NF-κB siRNA is a powerful small molecule reagent designed to silence expression of NF-κB and MDR1/P-gp related to MDR to increase tumor cell sensitivity to anti-cancer drugs, how to apply metformin plus interfering NF-κB activation for effective reversal of MDR of HCC cells still needs to be further explored.

ACKNOWLEDGMENTS

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COMMENTS

Background

Hepatocellular carcinoma (HCC) multidrug resistance (MDR) to a series of clinical chemotherapeutics with different structures or different target sites severely blocks the successful management of HCC. The mechanism of classical MDR is the significant overexpression of MDR1/P-glycoprotein (P-gp) that acts as an efflux pump on cell surface. Intracellular anti-cancer drugs increasingly flow from cells through the efflux pump, thus drug concentrations become lower and cancer cells become resistant to chemotherapeutic drugs such as doxorubicin.

Research frontiers

Metformin could target AMP-activated protein kinase mammalian target of rapamycin pathway, suppress hypoxia-inducible factor-1α (HIF-1α) and transcriptionally down-regulate P-gp and MDR-associated protein 1, suggesting that metformin may reverse MDR by targeting the AMP-activated protein kinase/mammalian target of rapamycin/HIF-1α/P-gp and MDR-associated protein 1 pathways. However, whether metformin plus nuclear factor-κB (NF-κB) inhibition might effectively reverse MDR of HCC cells remains to be explored.

Innovations and breakthroughs

Recently, there are few studies on the effects of metformin on MDR of HCC cells. In this study, the data suggested that the abnormal expression of MDR1/P-gp and NF-κB activation during HCC development were related to MDR formation, which might be down-regulated through inhibiting activation of the NF-κB signaling pathway with specific small interference RNA (siRNA). The combination of metformin with interfering *NF-κB* gene transcription could effectively reverse the MDR of HCC cells.

Applications

The abnormal expression of MDR1/P-gp in HCC was related to MDR formation, which could be down-regulated through inhibiting activation of the NF-κB signaling pathway with specific siRNA and increasing sensitivity of HCC cells to chemotherapy drugs. Interfering NF-κB activation with metformin is effective to reverse MDR of HCC cells. However, how to apply metformin plus interfering NF-κB activation for effective reversal of MDR of HCC cells still needs to be explored.

Terminology

Metformin is a safe, low-cost drug. The anticancer effects of metformin indicate the possibility that certain diabetes-associated types of cancer may be circumvented. Indeed, many retrospective meta-analyses have shown that metformin possesses anti-cancer activities and decreases the incidence of primary cancer development in those taking metformin routinely, and a multitude of clinical cancer trials are actively assessing its benefits in non-diabetic population who have already developed cancer. However, the precise molecular

mechanisms whereby metformin works in cancer prevention remain multi-factorial and ill-defined.

Peer-review

Authors have done excellent work in this present study. They have explored the effect of metformin and interfering *NF-κB* gene transcription with specific siRNA, alone or in combination, on *MDR1* gene regulation. The application of interfering NF-κB activation with metformin was more effective to reverse MDR of HCC cells.

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CASE REPORT

Metastatic recurrence to a solitary lymph node four years after hepatic lobectomy for primary hepatocellular carcinoma

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Abstract

This report describes a patient that developed recurrent metastatic hepatocellular carcinoma (HCC) to a supra-pancreatic lymph node four years after being treated for primary HCC *via* complete left hepatectomy. Metastatic HCC was proven by pathologic confirmation. The report addresses the role of surgical resection as a treatment modality for recurrent HCC to solitary lymph nodes. The role of biological chemotherapy as adjuvant treatment is also addressed.

Key words: Hepatocellular carcinoma; Lymph node; Recurrence; Metastatic; Extrahepatic

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Core tip: Recurrence of primary hepatocellular carcinoma to a solitary extracellular site is a rare occurrence, especially after complete hepatic lobectomy for the

primary tumor. In this report we describe a case of recurrence to a solitary suprapancreatic lymph node four years after initial resection. This is the only report to describe such a recurrence this long after the primary resection.

Caparelli ML, Roberts NJ, Braverman TS, Stevens RM, Broun ER, Allamaneni S. Metastatic recurrence to a solitary lymph node four years after hepatic lobectomy for primary hepatocellular carcinoma. *World J Hepatol* 2016; 8(23): 994-998 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i23/994.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i23.994

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with the highest prevalence rates occurring in the eastern hemisphere. However, there has been a rise in prevalence in the Western hemisphere. It has been postulated that this pattern is due to higher incidence of hepatitis B and C virus seen outside of the United States[1]. Tumor staging and strategies for treatment of HCC have been well described with current guidelines following the recommendations of the 2010 AHPBA/SSO/SSAT consensus conference on HCC[2]. Current guidelines are primarily geared toward patients with primary resectable and non-resectable HCC. However, data is lacking with regard to the treatment of recurrent extrahepatic HCC. Systemic chemotherapy has proven to be of minimal benefit for patients with advanced, and recurrent extrahepatic HCC. There are current studies being conducted that support the use of multikinase inhibitors, including Sorafenib, as a viable option for patients with advanced and extrahepatic HCC[3].

It is well known that the most common type of recurrence of HCC is intrahepatic. The most common sites for hematogenous spread are the lung, followed by the adrenal gland, and bone[4]. Metastases of HCC to lymph nodes (LN) are quite rare. In one report that included a subset of Japanese patients who underwent hepatic resection, the prevalence of lymphatic involvement was as low as 2.2%[5]. Another study showed that the 5-year survival rate for patients with lymph node metastasis is approximately 20%[6]. There have been few reports describing metastasis to LN that have been treated with surgical resection, and their results have been varied[7-11]. With this in mind, the importance of surgical resection of extrahepatic HCC recurrent to lymph nodes cannot be understated as a viable treatment modality. Interestingly, this is the first reported case where isolated lymph node metastasis has occurred greater than 3 years after initial hepatic resection. We describe a case of HCC recurrent to a solitary suprapancreatic lymph node treated by complete surgical resection.

CASE REPORT

The patient is a 67-year-old woman who presented with a suprapancreatic mass on magnetic resonance imaging (MRI). She initially presented 4 years prior with HCC of the left lobe of the liver measuring 10.8 cm × 7.4 cm × 9.5 cm. She was asymptomatic at the time of the discovery and the tumor was found due to imaging studies prior to a recent thoracic aortic aneurysm repair. Interestingly she did not have known risk factors for developing HCC such as cirrhosis, chronic hepatitis, tobacco use, diabetes, nonalcoholic fatty liver disease, hemochromatosis, or alpha-1 antitrypsin deficiency. Laboratory findings at that time showed a alpha fetoprotein (AFP) level of 119000 ng/mL. She subsequently underwent complete left hepatic lobectomy and had no complications post procedure. The patient was in remission for almost 4 years, but had a steady increase in AFP, 177-883 ng/mL, from year 3 to 4. Serial computed tomography (CT) imaging showed no evidence of recurrence over that time period. Subsequent MRI showed a soft tissue mass medial to the right hepatic lobe/porta hepatis measuring 4.6 cm × 5.6 cm (Figure 1). CT guided biopsy of the mass revealed a poorly differentiated malignant neoplasm, favoring HCC. The patient had no history of viral hepatitis, alcoholic liver disease, jaundice, abdominal pain, weight loss, chronic cough, bloody stools, bone pain, or any other signs to suggest metastatic disease. She was subsequently taken to the operating room for *en bloc* resection of a large suprapancreatic retroperitoneal mass, celiac and portal lymphadenectomy. Pathology showed the suprapancreatic mass to be consistent with HCC, high grade within a lymph node structure. Portal and celiac axis lymph nodes were negative for metastasis. Interestingly, immunohistochemical stains for the recurrent carcinoma showed not only tumor markers that confirm hepatocellular origin, but might suggest a more aggressive tumor - staining positive for cytokeratin 19 (CK19), glypican 3 (G3) and hepatocyte paraffin 1 (HP1). Microscopic pathologic figures are shown in Figures 2 and 3. The patient's post-operative course has been uncomplicated and at eight months post op she is disease free. Current AFP level is 2.2 ng/mL.

DISCUSSION

The recurrence of HCC can be classified as early or late phase[12]. Early phase recurrence typically occurs within the first two years post-resection, and is related to aggressive features of the primary tumor such as high tumor grade, local invasion, and multifocal tumors. Late recurrence occurs more than two years after resection and is related to *de novo* tumor formation, typically in patients with cirrhotic liver disease. The fact that our patient recurred to an extrahepatic LN nearly four years post-surgery is remarkable, and of the first to be reported this late, post-resection. The initial tumor was without aggressive characteristics, as it was moderately

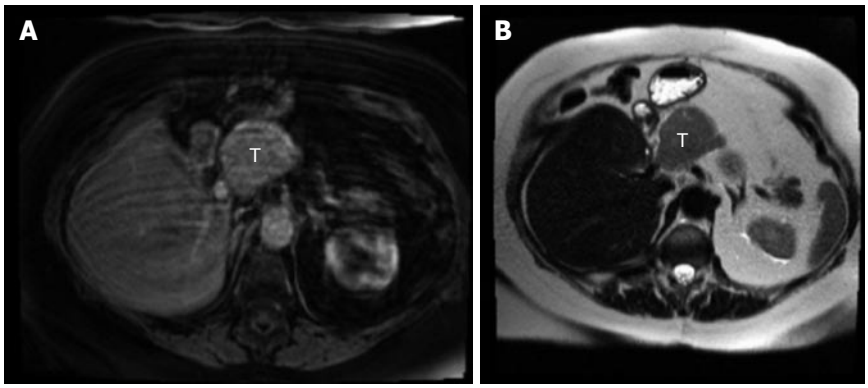

Figure 1 Suprapancreatic mass. The left panel (A) shows an axial post contrast T1 fat suppressed sequence that demonstrates an arterial phase enhancing mass medial to the liver; the right panel (B) shows an axial T2 HASTE sequence demonstrating a mass with increased T2 signal medial to the liver. T: Tumor.

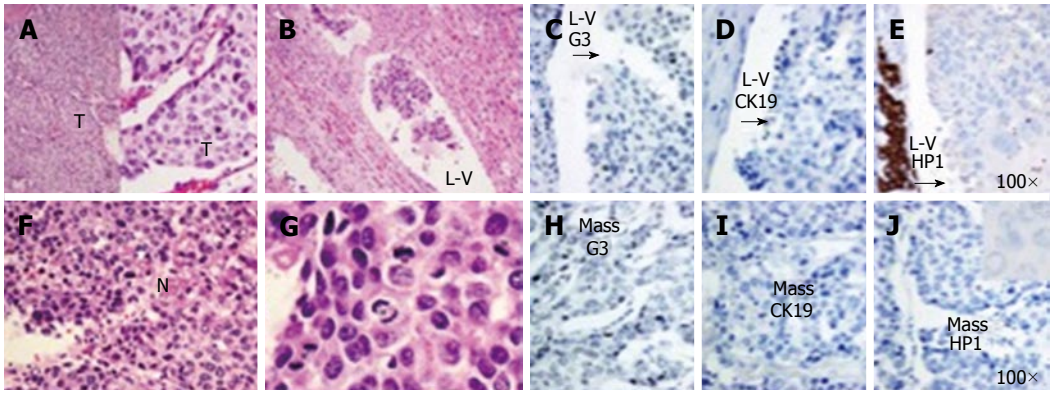

Figure 2 Initial hepatic lobectomy. On the left-hand set of H and E-stained quarter panel images, the upper left two-image combination quarter panel (A) shows the trabecular architecture, the trabeculae as "T"; the upper right H and E quarter panel (B) shows L-V; the lower H and E quarter panels show high power views of solid pattern tumor with focal geographic necrosis [lower left (F) as "N"] and numerous mitoses [lower right (G) with "M" times 4]; on the right-hand set of IHC-stained split full-height images, the mass, along the bottom (H-J) show negative G3 (H), CK19 (I), HP1 (J); the L-V, along the top (C-E) with a similar pattern, but with weak but convincing HP1 positivity. Both HP1 stains are inset with 100 × high power images (100 ×). Arrows on the L-V IHC (C and D) stains indicate the tumor (opposite vessel wall). L-V: Lymph-vascular tumor; Mass: Main mass; LN: Lymph node; G3: Glypican 3; CK19: Cytokeratin 19; HP1: Hepatocyte paraffin 1; T: Tumor.

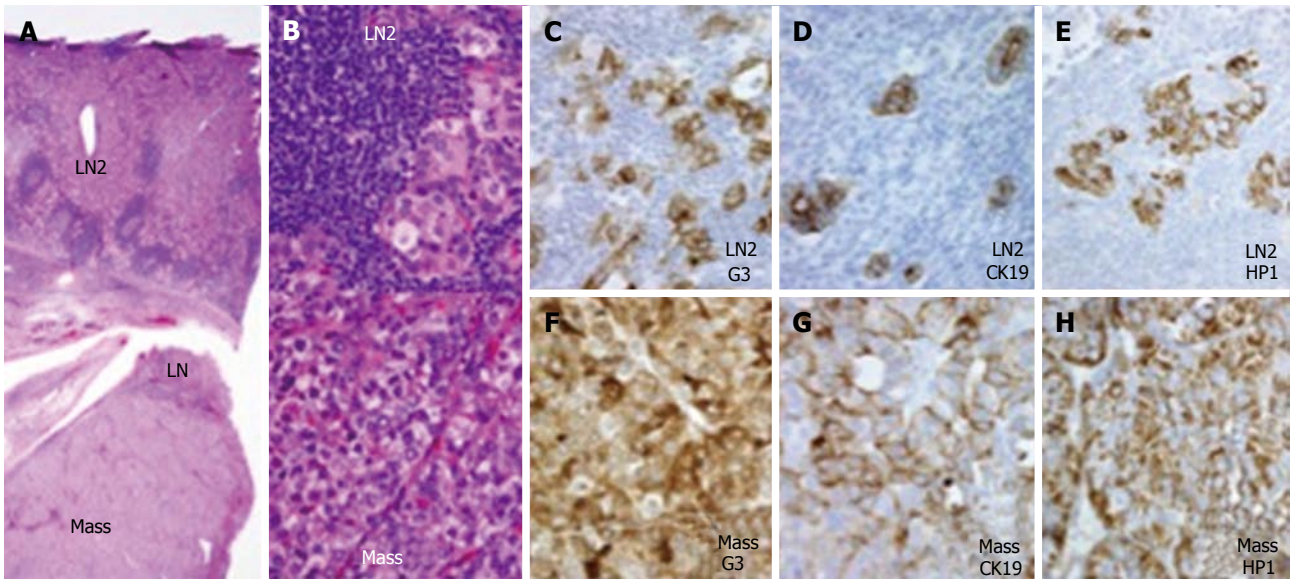

Figure 3 Resected recurrence. A: The H and E-stained shows a tiny area of likely LN at one end; B: Shows obvious tumor cells nests; On the right-hand set of IHC stained split full-height images, both the main mass (F-H); and obvious nodal tumor (LN2) (C-E), are strongly positive on G3 (C), CK19 (D), and HP1 (E) stains. Mass: Main mass; LN: Lymph node; G3: Glypican 3; CK19: Cytokeratin 19; HP1: Hepatocyte paraffin 1.

differentiated and without local invasion. Additionally, the initial tumor stained negative for CK19, G3 and was only weakly positive for HP1 in the lympho-vascular invasive sample as seen in Figure 1. Interestingly, the recurrent tumor was positive for these three biomarkers, suggesting hepatocellular origin and a more aggressive tumor[13]. Clonal selection, therapeutic selection, or possibly both may explain this finding.

LN status is essential to the staging of cancers, including HCC. The presence of LN metastasis is associated with poorer survival and higher risk of tumor recurrence[4]. Although the most common intra- and extra-hepatic recurrence is to liver and lung respectively, metastases to LNs are not that uncommon. There have been two reports that showed LN metastases in 28% and 25% of autopsied cases of HCC, respectively[14,15]. However, a more recent study of surgical patients in Japan showed only 2.2% LN involvement in patients that underwent hepatic resection[5]. This discrepancy may be due to the fact that more advanced HCC cases that are more likely to have extrahepatic metastases are less likely to undergo resection. This finding illuminates the importance LN dissection in hepatic surgery. LN dissection is not the current standard when performing hepatic resection for HCC. In a study by Ercolani *et al*[16] the role of lymphadenectomy was addressed. In 40 patients with HCC the incidence of LN metastases was 7.5%. It was also found that the most common site of LN metastases from HCC is the hepatic pedicle node, followed by the retropancreatic space, and common hepatic artery station. The authors concluded that regional lymphadenectomy is a safe procedure after liver resection; however, this is yet to become common practice.

Several case reports have been published on the findings of metastatic HCC to LNs[7-11]. Patients in these reports often had cirrhosis, and all but one of these patients underwent resection with varied short-term survival results. One report described a patient with a solitary suprapancreatic LN mestastasis that underwent pancreaticoduodenectomy and had reported disease free survival for 27 mo. Another patient with LN metastases to two paraaortic mediastinal LNs underwent complete resection, but had recurrence and died 13 mo later[10]. It is reasonable to argue liver disease, and multiple LN involvement may be factors for worse prognosis post LN resection.

Our patient appears to be an excellent candidate for resection, as she had a solitary LN, and is without cirrhotic, viral or alcoholic liver disease. In addition, adjuvant treatment with sorafenib - an oral multikinase inhibitor that has been shown to suppress tumor growth and angiogenesis by inhibiting the Raf/MEK/ERK signaling pathway and receptor kinases, such as VEGFR-1, VEGFR-2, VEGFR-3, and PDGFβ - should be considered[3]. Sorafenib was shown to increase survival in patients with advanced HCC in the SHARP (Sorafenib HCC Assessment Randomized Protocol) trial. However, data is lacking on whether this multikinase inhibitor is useful in the treatment of recurrent extrahepatic HCC. One recent study showed that the therapeutic effect of sorafenib was comparable in advanced HCC with or without extrahepatic metastasis[3]. It may be beneficial to initiate adjuvant treatment in patients with recurrent LN involvement, but further studies need to be performed prior to this becoming standard.

COMMENTS

Case characteristic

A 67-year-old woman who presented with a suprapancreatic mass on magnetic resonance imaging (MRI). The patient was asymptomatic at the time of presentation. Imaging studies were performed because of increased serum alpha fetoprotein levels led to increase suspicion for recurrence of primary hepatocellular carcinoma (HCC) resected four years prior.

Clinical diagnosis

The patient was asymptomatic at the time of presentation.

Differential diagnosis

Recurrent primary HCC, metastatic cancer, reactive lymphadenopathy, primary tumor of unknown origin, lymphoma.

Laboratory diagnosis

Elevated alpha fetoprotein level of 883 ng/mL.

Imaging diagnosis

MRI showed a soft tissue mass medial to the right hepatic lobe/porta hepatis measuring 4.6 cm × 5.6 cm.

Pathological diagnosis

HCC, high grade within a lymph node structure.

Treatment

Surgical resection of lesion.

Related reports

HCC is a primary liver cancer. HCC typically does not recur to an extrahepatic solitary lymph node after primary resection.

Term explanation

HCC is a primary liver cancer. It is the fifth most common human cancer worldwide.

Experiences and lessons

Surgical resection of HCC recurrence to a solitary lymph node is a viable option and may also be curative. Long term follow-up of this patient will further illuminate the possibility of cure.

Peer-review

An interesting case presentation with a long period disease-free up to 4 years. It should be benefit to the knowledge of the hepatologists and keep in mind for the importance of clinical follow-up after extensive hepatectomy.

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