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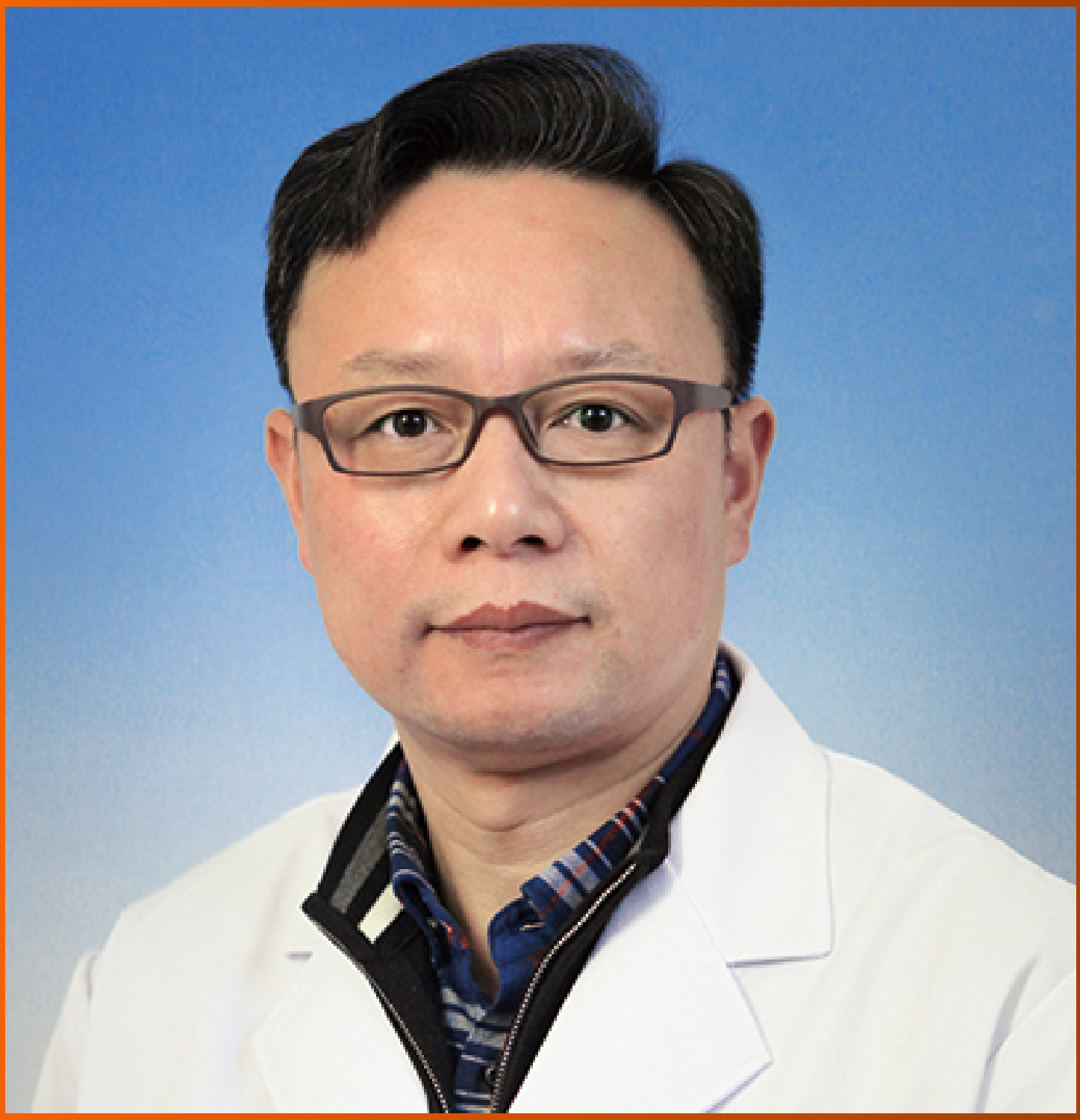

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Contents

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## Contents

## ABOUT COVER

Editorial Board Member of *World Journal of Hepatology*, Dr. Guang-Hua Luo is Director of the Clinical Medical Research Center in the Third Affiliated Hospital of Soochow University (China). He graduated from the Laboratory Medicine Department of Zhenjiang Medical College in 1996, obtained a PhD in 2013 at Soochow University, and was nominated to professorship in 2014. Currently, he is an editorial board member of Endocrine, Metabolic & Immune Disorders-Drug Targets. He has published more than 240 papers, 86 of which are included in SCI. Since 2009, four of his inventions have obtained patent authorization. His research activities mainly focus on molecular diagnostics in personalized medicine and on the regulatory role and mechanism of apolipoprotein M in liver diseases and chronic inflammatory diseases. (L-Editor: Filipodia)

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The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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*REVIEW*

# Molecular pathways of liver regeneration: A comprehensive review

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## Abstract

The liver is a unique parenchymal organ with a regenerative capacity allowing it to restore up to 70% of its volume. Although knowledge of this phenomenon dates back to Greek mythology (the story of Prometheus), many aspects of liver regeneration are still not understood. A variety of different factors, including inflammatory cytokines, growth factors, and bile acids, promote liver regeneration and control the final size of the organ during typical regeneration, which is performed by mature hepatocytes, and during alternative regeneration, which is performed by recently identified resident stem cells called "hepatic progenitor cells". Hepatic progenitor cells drive liver regeneration when hepatocytes are unable to restore the liver mass, such as in cases of chronic injury or excessive acute injury. In liver maintenance, the body mass ratio is essential for homeostasis because the liver has numerous functions; therefore, a greater understanding of this process will lead to better control of liver injuries, improved transplantation of small grafts and the discovery of new methods for the treatment of liver diseases. The current review sheds light on the key molecular pathways and cells involved in typical and progenitor-dependent liver mass regeneration after various acute or chronic injuries. Subsequent studies and a better understanding of liver regeneration will lead to the development of new therapeutic methods for liver diseases.

**Key Words:** Liver regeneration; Molecular pathways; Hepatic progenitor cells; Cytokines;

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**Core Tip:** The liver is a unique parenchymal organ with a regenerative capacity that can restore up to 70% of its volume. A variety of different factors and signaling pathways are involved in the process of liver mass regeneration during the priming, proliferative and termination phases. This review describes the types of liver regeneration, the phases of typical liver regeneration, the cell types involved in liver regeneration, the process of alternative liver regeneration, and the stem cells and micro ribonucleic acids that play roles in liver mass regeneration.

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## INTRODUCTION

The capacity of the liver to regenerate has been well known since the myths of Prometheus, who was banished from Olympus by Zeus. Legend has it that eagles pecked out half of his liver every day, but because the liver regrew during the night, the hero endured never-ending torture[1]. Today, many centuries after these events, liver regeneration is a universally known phenomenon that has been studied at the molecular and cellular levels. However, many aspects remain unclear[2].

Liver regeneration is a complex process regulated by the interaction between growth factors and cytokines secreted near the site of injury or transferred to the liver by the blood. This strictly orchestrated process is divided into 3 phases: Priming, proliferation, and termination[3]. The sum of all signals that sense the physiologically necessary liver mass is called the "hepatostat", which can initiate and terminate liver regeneration[4]. This phenomenon reflects the correlation between the needs of organisms and the organ mass that is required for homeostasis[5].

A better understanding of liver regeneration mechanisms will help improve the methods used to treat various organ diseases, prevent hepatic failure in high-risk patients, control liver grafts for transplantation, and more[6]. Importantly, the term "liver regeneration" is used improperly because during actual regeneration, not only the function of the organ but also the morphology is restored whereas only compensatory hypertrophy occurs in the liver. Second, mature hepatocytes are the source of new liver cells, not stem cells; however, stem cells play an important role in some cases of liver regeneration[7]. However, the term "liver regeneration" is widely accepted and the most commonly used term[8].

## TYPES OF LIVER REGENERATION

Until recently, it was believed that the liver mass after partial hepatectomy (PH) or injury recovers *via* hepatocyte proliferation for 1–2 cell cycles; however, recent studies have shown that different stimuli define the type of liver regeneration that occurs[9]. There are two known types of liver regeneration: The first is conducted through the hypertrophy and/or hyperplasia of hepatocytes and biliary epithelial cells (BECs) and is called typical regeneration. Typical regeneration is specific to a healthy liver that was exposed to resection or an acute liver injury; conversely, progenitor-dependent regeneration requires the reprogramming of specific hepatic cells, whose activation depends on the volume of the residual liver mass. Progenitor-dependent regeneration is specific to chronic liver diseases and massive acute liver injuries[10,11]. Thus, a 2/3 hepatectomy leads to the immediate hypertrophy of hepatocytes and further hyperplasia, whereas a 1/3 hepatectomy only triggers cell hypertrophy. Various chronic diseases and massive injuries initiate the activation of hepatic progenitor cells

(HPCs), which are responsible for liver regeneration[9]. Consequently, typical liver regeneration is driven by mature hepatocytes and BECs, whereas the alternative regeneration method is performed by HPCs[11].

## TYPICAL LIVER REGENERATION

PH causes a hemodynamic disturbance, expressed as a portal pressure escalation, which serves as a regeneration stimulus. Consequently, hepatocytes, BECs, Ito cells, Kupffer cells (KC) and sinusoid endothelial cells (SECs) are proliferated. Interestingly, hepatocytes proliferate first, whereas BECs start to proliferate only 2-3 d after PH. After a 2/3 PH, the hepatocytes go through one cycle of DNA synthesis, which is required for the restoration of 60% of the liver mass. In the following stages, several but not all hepatocytes continue to proliferate to achieve complete liver recovery. Afterward, apoptotic activity increases with the purpose of correcting an excessive regenerative response[12].

### *Phases of typical liver regeneration*

The beginning of each phase is initiated by a certain molecule set released in response to organ damage[13]. The earliest regeneration drivers are portal pressure changes and an increasing level of urokinase plasminogen activator (uPA)[8,14].

**Priming phase:** During the first phase of regeneration, hepatocytes, driven by various cytokines, simultaneously enter the $G_1$ phase of the cell cycle[10].

The increasing blood pressure in the hepatic sinusoids is conditioned by the incompatibility between the volume of the liver and the volume of inflowing venous blood[15], which results in a turbulent flow and mechanically stimulates SECs to secrete large amounts of uPA. uPA promotes plasminogen-plasmin transformation, leading to matrix metalloproteinase (MMP) activation and fibrinogen degradation. Plasmin and MMPs are involved in extracellular matrix (ECM) remodeling, resulting in the release of growth factors, such as hepatocyte growth factor (HGF)[8].

Two proinflammatory cytokines are the main mediators of the first phase: Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6); these cytokines are secreted primarily by liver macrophages under the influence of bacterial lipopolysaccharide and the C3a and C5a components of the complement system[16]. IL-6 drives the acute phase response and initiates cytoprotection and the proliferation of hepatocytes *via* the IL-6-IL-6R interaction and the activation of coreceptor glycoprotein 130 (gp130), which activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling pathways[5,17]. Although gp130 is present on the surface of most cells, IL-6R is primarily located on hepatocytes. However, there are also soluble IL-6Rs that initiate the trans-signaling pathway within cells lacking IL-6R and enhance the regenerative response of hepatocytes[3]. Fazel Modares *et al*[18] elicited the crucial role of the trans-signaling pathway in liver regeneration after PH because hepatocyte IL-6R activation alone was not sufficient to initiate cell proliferation. TNF-α has two main functions: It activates the NF-κβ signaling pathway through direct interaction with TNF-R1 on Kupffer cell surfaces and through the indirect induction of inhibitory KB kinase; it also stimulates hepatocyte c-Jun N-terminal kinase (JNK). JNK phosphorylates the c-Jun transcription factor in the nucleus to induce cyclin-dependent kinase 1 transcription, which activates hepatocyte proliferation[8].

The augmenter of liver regeneration (ALR) protein, which has three isoforms (15, 21 and 23 kDa) and is expressed primarily in the liver, testes, kidneys and brain, plays a crucial role in liver regeneration. Each isoform of ALR has a different location within the cell and thus plays a different role[19]. For example, mitochondrial long-form ALR translocates proteins and initiates MitoNEEt release, which leads to cell proliferation. Long-form ALR expression increases in cases of pathology and reduces liver damage, protects against oxidative stress and endoplasmic reticulum stress by decreasing Ca++ levels, and has an antimetastatic effect on hepatocellular carcinoma (HCC). Cytoplasmic short-form ALR enhances the hepatocyte response to IL-6 by inducing the phosphorylation of STAT3; it also has an antimetastatic effect on hepatoma by inhibiting the migrative and invasive capacity of cells[20]. After PH, the ALR concentration increases immediately and activates MAPK signaling; enhances IL-6, TNF-α and inducible nitricoxide synthase production by Kupffer cells; and inhibits NK cell activity. Short-form ALR protects hepatocytes by inhibiting apoptosis stimuli[21].

### *Proliferative phase*

During the second phase of liver regeneration, the G1/M phase transition occurs, which is driven by two groups of mitogens: Complete mitogens, including HGF, TGF-α, epidermal growth factor (EGF), and HB-EGF; and the stimulation of DNA synthesis and cell proliferation *via* Ras-MAPK and PI3K/AKT signaling activation and auxiliary mitogens, including bile acids, vascular endothelial growth factor (VEGF), noradrenalin, insulin-like growth factors (IGFs), estrogen and serotonin[3].

HGF is produced by mesenchymal liver cells and interacts with the methionine (MET) receptor, leading to PI3K and MAPK signaling protein phosphorylation followed by PI3K/AKT and extracellular-signal-regulated kinase 1/2 signaling activation. This process results in the proliferation, migration, and differentiation of liver cells and antiapoptotic effects[22,23]. Epidermal growth factor receptor (EGFR)-transmembrane receptors with tyrosine kinase activity interact with EGF, TGFa, amphiregulin (AR), epigen, and HB-EGF, leading to MAPK, PI3K/AKT–mammalian target of rapamycin (mTOR) and STAT signaling activation, which drives hepatocyte proliferation[24]. Natarajan *et al*[25] identified impaired liver regenerative capacity and delayed cyclin D1 expression in mice lacking EGFR.

Nuclear factor erythroid 2-related factor 2 (NRF2) transcription factors, which regulate a wide range of genes including antioxidant proteins and detoxifying enzymes, are activated in response to increased reactive oxygen species levels. The expression of this molecule increases in the earliest stages of liver regeneration as a result of cellular damage[26]. Zou *et al*[27] discovered the important role of Nrf2 in the regulation of cell cycle progression in mice. Nfr2 is a transcriptional suppressor of Cyclin A2 and a regulator of the Wee1/Cdc2/Cyclin B1 pathway, which controls the beginning of the M phase[27]. Nfr2 also regulates hepatocyte proliferation by modulating the insulin/IGF-1 and Notch1 signaling activities and facilitates the capability of hepatocyte nuclear factor 4 alpha (HNF4α) to keep newly formed hepatocytes in a differentiated state[28].

Bile acids are the main end products of cholesterol metabolism and are synthesized exclusively in the liver, where they function as signaling molecules that activate membrane G-protein-coupled BA receptor 1 (or TGR5) and nuclear farnesoid X receptor (FXR)[29]. After the loss of liver mass due to PH, the bile acids concentration increases during the first minute, which leads to FXR activation, resulting in inhibited BA synthesis and induction of the *FOXM1B* gene[30]. *FOXM1B* is a transcription factor that regulates DNA synthesis and mitosis *via* cyclin-dependent kinase 2 (CDK2) activation, which is required for the G1/S transition and CDK1 activation and is responsible for the S/M transition[31]. FXR activation also appears in enterocytes and leads to the induction of fibroblast growth factor (FGF)15/FGF19 expression. The Fgfr4/β-Klotho receptor, which is located on the hepatocyte surface, inhibits BA synthesis and activates the cell cycle *via FOXM1B* induction when activated[32]. Fgfr4/β-Klotho activation also regulates the termination of liver regeneration and terminal organ size. Kong *et al*[33] showed that mice with enhanced Fgf15 expression have the most active Hippo signaling pathway, which induces cellular senescence and suppresses transcriptional activation. TGR5, which is located on KC, SEC and BEC surfaces, leads to cAMP induction and nuclear factor kappa B (NF-κB)-signaling inhibition[34]. As a result, decreased proinflammatory cytokine synthesis occurs in KCs and bone marrow macrophages *via* the protein kinase B-dependent activation of the mTOR[35]. TGR5 protects the liver from BA overload by increasing its excretion with urine; it also enhances the secretion of $HCO_3^-$ and $Cl^-$ and controls BA polarity because inordinately hydrophobic molecules can damage the regenerating liver[36].

Wnt ligands are glycoproteins secreted by nonparenchymal liver cells, mostly KCs and SECs, and are crucial molecules of liver regeneration[37]. Wnt ligands lead to the integration of Axin into the cytoplasmic membrane through interaction with the Frizzled receptor and the coreceptors LRP5/6, resulting in impaired function of the β-catenin degradation complex. Therefore, Wnt ligands lead to β-catenin accumulation, followed by its translocation to the nucleus and interaction with members of the transcriptional T cell factor family, resulting in target gene transcription, for example, of cyclin D1, leading to hepatocyte proliferation[38]. Preziosi *et al*[39] identified the constitutional secretion of Wnt2 and Wnt9b by central vein endotheliocytes and the essential role of these molecules in the basal activation of β-catenin and metabolic zonation of hepatocytes. PH leads to increased Wnt2 and Wnt4 expressions within all zones of the hepatic acinus and the additional secretion of Wnt9b and Wnt5b within the pericentral zone during the first 12 h. This leads to a 7–8-fold increase in cyclin D1 expression within the periportal and intermediate zones and 20-and 100-fold increases in glutamine synthetase expression within the intermediate zone and the pericentral zone, respectively. The role of increased glutamine synthetase expression remains

unknown but is thought to be an enhancer of pericentral detoxification since the other 2/3 of hepatocytes restore organ mass[39].

The Hedgehog (Hh) signaling pathway is a morphogenic pathway that regulates embryonic development and is implicated in homeostasis maintenance[40,41]. Among vertebrates, this pathway is activated within a special organelle, the primary cilium (PC), *via* the interaction of Hh ligands Sonic hedgehog, Indian hedgehog and Desert hedgehog and the Ptched receptor[42]. After that, phosphatidylinositol 4-phosphate[43], sumoylated molecules and cholesterol[44] form a complex with smoothened (Smo), which leads to its activation. Activated Smo dislocates to the apex of the PC and activates Glis (including Gli1, Gli2 and Gli3), which then translate to the nucleus and regulate gene transcription[45]. The said pathway is canonical, but there are also different types of noncanonical Hh signaling pathways; for example, the Smo-free activation of Glis or the Hh pathway arises beyond the PC[45-47]. Ochoa *et al*[48] identified a meaningful role of Hh signaling in liver regeneration. PH leads to Hip inhibition, thus activating the Hh pathway *via* an increase in the Indian hedgehog level in the replicative period and an increase in the Sonic hedgehog level in the postreplicative period[48]. Platelet-derived growth factor, TGF-β, and EGF are secreted in response to liver damage induced by JNK-dependent Hh ligand synthesis[49,50]. Hh signaling activation occurs within hepatocytes, Ito cells[51] and BECs[52], leading to ECM remodeling, progenitor cell expansion and liver epithelial cell proliferation[45]. Additionally, Hh signaling controls Yes-associated protein 1 (YAP) of activated Ito cells[53]. The Hh-YAP signaling pathway induces the glutaminolysis required for Ito cell activation to regulate liver regeneration[54]. Furthermore, Hh signaling facilitates cell survival *via* inhibiting hepatocytes, BECs, Ito cells and progenitor cell apoptosis[55].

Notch signaling is an important pathway in embryonic development, homeostasis maintenance, and liver regeneration[56]. Mammals have 4 types of receptors for this pathway (Notch1, Notch2, Notch3, and Notch4); Notch1 and Notch2 are located primarily on BECs and HPCs whereas Notch3 and Notch4 are expressed by the mesenchymal compartment of the liver and are poorly represented on epithelial liver cells. JAG-1 and DLL-4 are ligands of Notch signaling that are expressed in the liver[57]. The main role of this pathway in liver development is the JAG1-NOTCH2 interaction, which results in the differentiation of hepatoblasts to BECs and the development of the intrahepatic biliary tree[58]. Lu *et al*[59] showed that the role of the Notch–RBPJ interaction is to drive HPC differentiation to BECs *via* Yap inactivation after PH in mice. The direction of HPC differentiation is defined by the balance of NOTCH signaling and Wnt ligands[60]. Ortica *et al*[61] pointed out the important role of Notch3 in HPC differentiation to hepatocytes. Zhang *et al*[62] elicited the regulatory role of Notch signaling in hepatocyte proliferation *via* the NICD/Akt/Hif-1α pathway after PH, whereas its inhibition leads to delayed S phase entry, impaired S phase and M phase progression, and the loss of the hepatocyte mitotic rhythm due to cyclin E1, A2 and B1 dysregulation. Yang *et al*[63] demonstrated the involvement of Notch signaling in the regeneration of 8 types of liver cells, which is performed by the activity of 9 different pathways and regulates cellular proliferation, apoptosis, the cell cycle, *etc.*

### *Termination phase*

When the needed liver mass: Body mass ratio is achieved, cellular proliferation stops due to inhibitory molecules that control the rapidity and direction of liver regeneration. Among the inhibitors of cell proliferation, IL-1, which is synthesized by nonparenchymal liver cells, inhibits the DNA synthesis induced by HGF, EGF and TGF-α. IL-6 is multifunctional and plays a role as both a liver regeneration inducer and inhibitor; its effect depends on the time and dose of the molecule. The IL-6-dependent inhibition of proliferation is likely to occur by increasing p21 expression[64]. The JAK/STAT signaling pathway is inhibited by 8 members of the SOCS family of proteins; hereafter, only SOCS1 and SOCS3 contain the extended SH2 and kinase inhibitory region. SOCS1 directly binds and inhibits JAK, whereas SOCS3 binds to cytokine receptors, forms a complex with JAK and inhibits the STAT3 signaling pathway. SOCS3 is the main suppressor of the signaling pathway activated by IL-6; it inhibits the phosphorylation of coreceptor gp130, JAK and STAT3. SOCS1 negatively regulates the hepatocyte proliferation induced by HGF *via* c-MET signaling inhibition and likely regulates the TNF-α levels because it interacts with toll-interleukin 1 receptor domain-containing adaptor protein, which drives the synthesis of a current mediator[65].

Some TGF-β family members function as inhibitors of proliferation. In particular, TGF-β1 plays a special role in binding to receptor types 1 and 2 and inducing cell apoptosis to correct an excessive liver mass. Outside of the liver, TGF-β1 is synthesized in platelets and the spleen. The spleen might be involved in the termination phase for

it inhibits HGF and its c-MET receptor expression. In this regard, splenectomy leads to increased hepatocyte proliferation in the first 48 h after PH. Other members of the TGF-β family are involved in the termination phase of liver regeneration, including activin A-hepatocyte proliferation inhibitors and bone morphogenetic proteins (BMPs)[3]. BMP9 is expressed exclusively by liver tissues and in part by hepatocytes. BMP9 regulates a variety of biological functions such as glucose and lipid metabolism, angiogenesis, oncogenesis, and fibrogenesis, and it affects liver regeneration after acute injuries[66]. Addante *et al*[67] reported a regulatory function of BMP9 over HPCs that is affected by anaplastic lymphoma kinase 2 type I receptor activation, resulting in SMAD 1, 5, and 8 induction, HPC apoptosis stimulation, and a reduction in HPCs. Apart from its negative influence on liver regeneration, BMP9 also has profibrogenic activity and promotes HCC proliferation and invasion. Additionally, BMP9 enhances the expression of TLR4 on the SEC surface, leading to inflammatory cell recruitment. Therapy with anti-BMP-9/ anaplastic lymphoma kinase 1 can potentially enhance hepatocyte proliferation among patients with chronic liver diseases and decrease the probability of fibrosis and HCC development[68].

HNF4-α regulates hepatocyte differentiation and, according to Huck *et al*[69], promotes the termination of liver regeneration. The expression of the current molecule significantly decreased during the priming phase and increased during the following phases, which is necessary for termination and hepatocyte function recovery after PH[69]. HNF4-α is a YAP and TGF-β/SMAD3 antagonist; therefore, decreased expression of this molecule stimulates promitogenic functions and activates connective tissue growth factor. Increased HNF4-α expression during the subsequent phases of regeneration prevents the excessive synthesis of connective tissue and therefore fibrosis[70]. Hnf4-α also leads to the inhibition of HPC proliferation and migration in rats[71].

Integrin-linked kinase is a suppressor of hepatocyte proliferation that is located under the cytoplasmic membrane and is associated with a3/b1 integrins of the ECM. Interruption of this connection results in hepatostat imbalance and excessive liver mass. Focal adhesion kinase is also associated with a3/b1 integrin and promotes hepatocyte proliferation[72].

The Hippo signaling pathway is a crucial regulator of the terminal organ size within mammals. The key component of the mammalian Hippo pathway is a kinase cascade in which the Ste20-like kinases 1/2 phosphorylate and activate large tumor suppressor 1/2, its adapter protein Mps one binder 1, and the transcriptional coactivators Yap and Taz[73]. Phosphorylated Yap and Taz emerge from the nucleus, where they are bound to transcription factors that control the proliferation and differentiation of cells, such as TEAD family members[74]. The Hippo/Yap signaling pathway is likely an integrator of a large number of alternative growth factor signaling pathways and regulates liver size by balancing negative and positive regulatory signals[72]. The Hippo signaling pathway does not have any specific receptors and is regulated by molecules that control cellular polarity and morphology, intercellular adhesion and other processes. The activity of this pathway is modulated in response to mechanical deformation and intercellular adhesion defects and cell adhesion to the intercellular matrix. Consequently, Hippo signaling senses cellular and tissue integrity[75]. Intranuclear Yap is located in periportal hepatocytes and BECs, whereas pericentral hepatocytes contain few current molecules, which is exactly the opposite of the constitutive Wnt ligand content; therefore, current pathways inhibit one another[76]. Table 1 summarizes the main molecular factors in liver regeneration.

### *Cells involved in liver regeneration*

The hepatic acinus is the structural and functional unit of the liver. It consists of three zones. The hepatocytes in the first zone have a periportal location and are specialized in gluconeogenesis and the beta-oxidation of fatty acids; conversely, hepatocytes in the third zone lie pericentral and perform glycolysis, lipogenesis and detoxification. Therefore, hepatocytes are functionally heterogeneous and express various genes depending on their localization[77]. Experiments performed on mice have mainly investigated diploid hepatocyte populations within the third zone, which express the early progenitor cell markers Tbx3 and Axin2 and can proliferate twice as fast as other hepatocytes. This ability depends on Wnt ligand expression in nearby SECs[78]. Sun *et al*[79] reported that Axin2$^+$ pericentral hepatocytes are not confined to the liver stem cell compartment and do not have an enhanced capacity for proliferation. Cells of every zone participate in cellular homeostasis. The authors further controverted the opinion that Axin2$^+$ pericentral hepatocytes translocate to the periportal zone. Axin2$^+$ induction was identified in every zone of the acinus during regeneration[79].

Hybrid hepatocytes, which account for 5% of all hepatocytes, were found in the first

**Table 1 The main factors driving liver regeneration**

| Factor of regeneration | Influence on LR |
|---|---|
| TNF-α | Induction of CDK-1 |
| IL-6 | Activation of the JAK/STAT, MAPK, and PI3K/AKT signaling pathways |
| Hh signaling pathway | ECM remodulation; induction of progenitor cell and liver epithelial cell expansion; induction of glutaminolysis; inhibition of hepatocyte, BEC, Ito cell and progenitor cell apoptosis |
| ALR | lfALR: Enhancement of the hepatocyte response to IL-6 and STAT3 phosphorylation induction. MAPK signaling pathway activation; NK cells inhibition; increase in IL-6, TNFα and iNOS production by Kupffer cells, sfALR: Inhibition of proapoptotic stimuli |
| NRF2 | Regulation of M phase entry, hepatocyte proliferation, maintenance of newly formed hepatocytes in a differentiated state |
| Growth factors (HGF, TGF-α, EGF, HB-EGF) | Stimulation of DNA synthesis and cell proliferation *via* Ras-MAPK and PI3K/AKT signaling pathway activation |
| BAs | Activation of CDK2, cell cycle, regulation of termination phase and terminate liver size, decrease in the inflammatory cytokine production, enhancement of BA excretion and $HCO3^-$, $Cl^-$ secretion, control of BA polarity |
| Wnt-β-catenin | Hepatocyte proliferation induction |
| Notch signaling pathway | Modulation of HPC differentiation toward BECs, regulation of hepatocyte proliferation, mitotic rhythms, cyclin E1, A2 and B1 |
| IL-1 | DNA synthesis inhibitor |
| SOCSs | c-MET and JAK-STAT signaling pathway inhibition |
| TGF-β1, activin A, BMPs | Induction of apoptosis to correct excessive liver mass |
| HNF4 | Regulation of hepatocyte differentiation, initiation of the termination phase, antagonism YAP and TGF-β/SMAD3, prevention of excessive connective tissue synthesis, inhibition of HPC proliferation and migration |
| Hippo/YAP signaling pathway | Terminal liver size control |

LR: Liver regeneration; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; CDK-1: Cyclin-dependent kinase 1; JAK/STAT: Janus kinase/signal transducer and activator of transcription; Hh: Hedgehog; ECM: Extracellular matrix; MAPK: Mitogen-activated protein kinase; ALR: Augmenter of liver regeneration; lfALR: Long-form ALR; sfALR: Short-form ALR; iNOS: Inducible nitric oxide synthase; NRF2: Nuclear factor erythroid 2-related factor 2; BA: Bile acids; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; HPC: Hepatic progenitor cells; BEC: Biliary epithelial cells; MET: Methionine; BMP: Bone morphogenetic proteins; HNF4 : Hepatocyte nuclear factor 4 alpha; YAP: Yes-associated protein.

zone of acinus. These cells express the hepatic transcription factors Hnf4a and sulfur oxide (Sox) 9, which are active in BECs. Hybrid hepatocytes are capable of differentiation into BECs and hepatocytes and help recover liver mass after various chronic diseases[80].

In contrast with the regenerative pool of the third zone, cells of the first zone do not proliferate in the absence of functional damage. The specific marker of periportal hepatocytes is the major facilitator superfamily domain-containing 2a[9]. Liver regeneration in the homeostatic state was performed *via* hepatocyte self-renewal within each acinus zone. Pu *et al*[81] elicited the capacity of major facilitator superfamily domain-containing 2a+ hepatocytes to proliferate more actively after PH and to completely replace pericentral hepatocytes during $CCl4^-$ induced chronic injury. Therefore, depending on the type and duration of damage, liver regeneration occurs *via* different pools of cells[81].

Immune cells, including Kupffer cells, circulating monocytes and lymphocytes, play an important role in liver regeneration. Kupffer cells secrete mediators of proliferation, such as HGF, IL-6, TNF-α and Wnts, stimulating angiogenesis *via* VEGF-A secretion. Circulating monocytes play an important role in the first hours of liver regeneration, as indicated by the significantly increased number of adhesion molecules on the SEC surface. Monocytes play a role because it takes time for Kupffer cells to reach the sinusoids from the space of Disse[82]. Organ damage initiates the release of chemoattractants, such as osteopontin, monocyte chemoattractant protein-1, and intercellular adhesion molecule 1, resulting in macrophage recruitment to the liver where lipopolysaccharide and the C3a and C5a components of the complement system activate the NF-κB signaling pathway and the synthesis of IL-6 and TNF-α[83]. Apart from macrophage activation, components of the complement system directly influence liver regeneration. C5a binds to C5aR1, whose expression on hepatocyte surfaces

significantly increases during regeneration, inducing cellular growth. C3a and C3b might facilitate liver regeneration since organ recovery within C3-deficient mice (C3–/–) was disturbed in a previous experiment[84].

NK and natural killer T cells inhibit regenerative processes *via* interferon (IFN)-γ secretion, which stimulates the synthesis of antiproliferative proteins, such as STAT1, IRF-1, and p21CIP1/WAF1, by hepatocytes. The influence of natural killer T cells on liver regeneration is significantly lower[83,85]. The medium limitation of NK cell activation is required for normal liver regeneration, which provides the increased expression of the coinhibitory receptor T cell Ig and ITIM domain (TIGIT) on these cell surfaces. TIGIT binds to the poliovirus receptor (PVR), which is located on hepatocyte and Kupffer cell surfaces and results in the inhibition of INF-γ secretion by NK cells. Hepatocyte expression of PVR significantly increased because the current protein is not only a ligand of NK cell receptors but is also a mediator of cellular growth, adhesion, migration and immunomodulation. However, the main role of PVR in liver regeneration seems to be interaction with TIGIT[86]. Eosinophils are key cells that secrete IL-4, which stimulates the G1 phase entrance of hepatocytes by binding to IL4Rα[87].

Hepatic stellate cells (HSCs, Ito cells) are located in the space of Disse and function in retinoid storage during the inactive stage[88,89]. Ito cells have regenerative potential and, in addition to growth factor secretion, can exhibit stem cell properties. Thus, stellate cells have the capacity to differentiate into HPCs, hepatocytes and BECs based on the influence of certain cytokines[90,91]. Ito cells demonstrate this capacity, as described above, in the case of chronic liver diseases, including cirrhosis[92]. Swiderska-Syn *et al*[93] demonstrated that hepatocytes require modulation of the epithelial-mesenchymal transition in multipotent progenitors derived from HSCs. A crucial role in this process is canonical Hh signaling. Although Ito cells have characteristics of multipotent cells, they improve the supportive role of each progenitor pool rather than nullify the importance of other liver progenitor populations[93]. HPC expansion and infiltration are correlated with ECM remodeling. HSCs engage in the degradation of collagen, forming an HPC niche that is rich in laminin, hyaluronic acid (HA) and collagen III, which are necessary for the development of the undifferentiated HPC phenotype. Collagen type I and fibronectin promote cell cycle arrest and HPC differentiation into hepatocytes and BECs[94]. Several studies have contradicted the capacity of HSCs to give rise to an epithelial pool of liver cells in various models of liver injury and in isolated cell cultures. The sources of hepatocytes and BECs are mature hepatocytes and bipotential liver progenitor cells[95,96]. Kordes *et al*[97] showed that the pancreatic stellate cells of rats express stem cell markers, such as CD133 and nestin, and have the possibility to display the β-catenin-dependent Wnt and Notch signaling pathways, which are required for stem cell maintenance and expansion. Transplantation of these cells after the surgical removal of 70% of the liver mass and the inhibition of hepatocyte proliferation (2AAF/PHX) led to the transdifferentiation of current cells into Hnf4α$^+$ hepatocytes and panCK$^+$ BECs[97]. Further studies in the given field are required because the role of HSCs in liver regeneration is significant. Research by Mabuchi *et al*[98] defined the importance of HSC and hepatocyte interactions in the early phases of liver regeneration, resulting in HSC activation[98]. Activated stellate cells transdifferentiate into myofibroblasts, secreting ECM components and cytokines, which drive the proliferation and differentiation of liver cells[92]. Among the cytokines secreted by Ito cells, HGF, lymphotoxin-beta, FGF, IL-6, NOTCH, delta-like noncanonical Notch ligand 1 and TGF-β1 play important roles[92,99]. HSCs regulate HPC proliferation *via* the antiproliferative effect of TGF-β1, which controls the termination phase of liver regeneration. Ito cells regulate the cytokine profile, affecting various phases of liver mass restoration[100]. Konishi *et al*[73] demonstrated the intensification of hepatocyte proliferation *via* HSC activation after ischemia-reperfusion injury (IRI). Herein, activated YAP and TAZ served as the inductors of HSC proliferation in the postischemic liver[73].

In addition to playing a role in thrombogenesis, platelets are involved in the development of inflammation and several syndromes; they also lead to the metastasis of some tumors and are required for liver regeneration. Previous studies have elicited impaired regeneration after PH under conditions of thrombocytopenia, whereas an elevated level of platelets was associated with enhanced regeneration since platelets produce HGF, Platelet-derived growth factor and TGF-β[101]. Partial resection or chronic liver injury leads to platelet accumulation in the sinusoids and space of Disse, likely *via* von Willebrand factor (vWF) secretion by SECs[102]. vWF plays a crucial role in the early stages of liver regeneration by promoting platelet adhesion, which is significantly decreased when anti-vWF antibodies are present. After the initiation of regeneration, the secretion of vWF antigens increases. The postoperative level of vWF

antigens may be used to predict the survival prognosis[103]. Platelets secrete various growth factors that positively influence liver regeneration. The most important secreted cytokines are HGF, IGF and serotonin, which promote hepatocyte proliferation[104]. Human platelets do not secrete a considerable amount of HGF; therefore, the primary platelet mediator of liver regeneration is IGF-1. Apart from hepatocytes, platelets also interact with SECs and Kupffer cells and thus positively affect liver regeneration. Sphingosine-1-phosphate is secreted by platelets and stimulates SEC proliferation and IL-6 secretion, which drives DNA synthesis within hepatocytes. The interaction between platelets and Kupffer cells leads to the activation of both cells[105]. Platelets enhance the Kupffer cell secretion of mediators, *i.e.*, TNF-α and IL-6, that are required for liver regeneration[101]. Platelets can either activate angiogenesis or inhibit it, depending on the mediator secreted from α-granules. Thus, thrombospondin 1 is an antiangiogenic mediator, whereas VEGF has a proangiogenic function. As long as platelets secrete both of these mediators, the PH outcome depends on the pattern of α-granule secretion[106]. Since platelets secrete many mitogens, the transfusion of blood enriched with platelets promotes liver regeneration after PH; however, it may lead to complications, including fatality[3].

A general scheme of the molecular processes involved in the various phases of typical liver mass regeneration is shown in Figure 1.

## PROGENITOR-DEPENDENT LIVER REGENERATION

The mechanisms described above are specific for healthy livers and occur among living liver donors. However, in most cases, liver resection occurs within patients with impaired liver function, and subsequent regeneration proceeds in a nonstandard way, which can lead to hepatic failure and death[107].

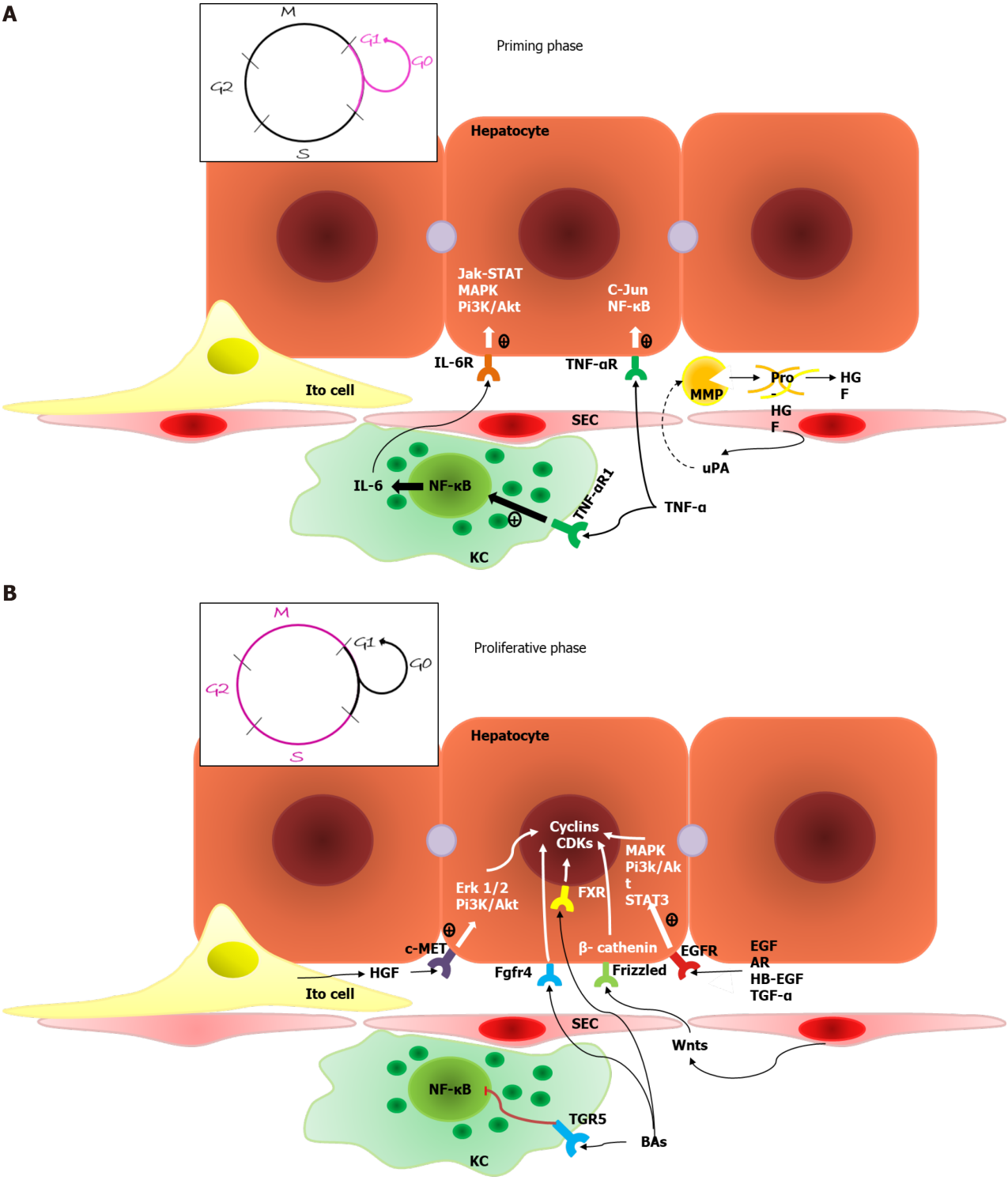
Acute liver failure caused by intoxication, viral hepatitis A, B or E, autoimmune liver disease, *etc.*, is often followed by widespread necrotic and apoptotic zones, and adequate liver regeneration becomes impossible[107]. During acute liver failure, the main regenerative role is given to HPCs, as indicated by the increased level of alpha-fetoprotein (AFP). Therefore, a high AFP level is correlated with a positive prognosis after acetaminophen-induced liver damage[12]. The immune system regulates liver regeneration *via* necrotic cell phagocytosis and controls inflammatory reactions in response to injury. The number of proliferative macrophages in the liver significantly increases after organ damage, and monocytes are recruited from the bloodstream and differentiate into macrophages in response to increasing the colony-stimulating factor 1 levels. Colony-stimulating factor 1 injection promotes liver regeneration after PH; conversely, a low level of the current factor is correlated with a negative patient prognosis[11].

Liver steatosis is associated with an impaired regenerative function, in which GADD34 plays an important role since its increased expression promotes liver regeneration within mice. IRI often complicates the posttransplantation period and impairs typical liver regeneration. The current complication is followed by increased receptor for advanced glycation end product levels, which might be a therapeutic target. Thus, receptor for advanced glycation end product inhibitor injection leads to a reduction in organ damage and the induction of liver regeneration. The excessive synthesis of ECM components by activated HSCs inhibits hepatocyte proliferation, and if macrophage MMPs do not promote connective tissue restitution, the angioarchitecture of hepatic lobules is impaired, resulting in cirrhosis[107]. In the liver, damage due to cirrhosis and hepatitis B or C often reveals hepatocytes with BEC markers, such as epithelial cell adhesion molecule (EpCAM), on their surface. The presence of these intermediate hepatobiliary cells is thought to be explained by their origin from biliary compartment progenitors[108].

Hepatocytes are the main cells driving typical liver regeneration, whereas alternative liver regeneration is performed by HPCs[92]. The process of progenitor-dependent liver regeneration is shown in Figure 2.

## RESIDENT STEM CELLS OF THE LIVER

Therefore, the liver regenerative capacity is significantly impaired during chronic liver diseases due to the accumulation of senescent hepatocytes[109,110]. In this case, liver mass restoration is performed by HPCs[111]. HPCs are located in the canals of Hering and have a bipotential nature; in other words, they can differentiate into both hepatocytes


A
M
G1
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Priming phase
Hepatocyte
Jak-STAT
MAPK
Pi3K/Akt
C-Jun
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IL-6R
TNF-αR
Ito cell
MMP
Pro
HG
F
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SEC
uPA
IL-6
NF-κB
TNF-αR1
TNF-α
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G1
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Proliferative phase
Hepatocyte
Cyclins
CDKs
MAPK
Pi3k/Ak
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STAT3
Erk 1/2
Pi3K/Akt
FXR
c-MET
β- cathenin
EGFR
EGF
AR
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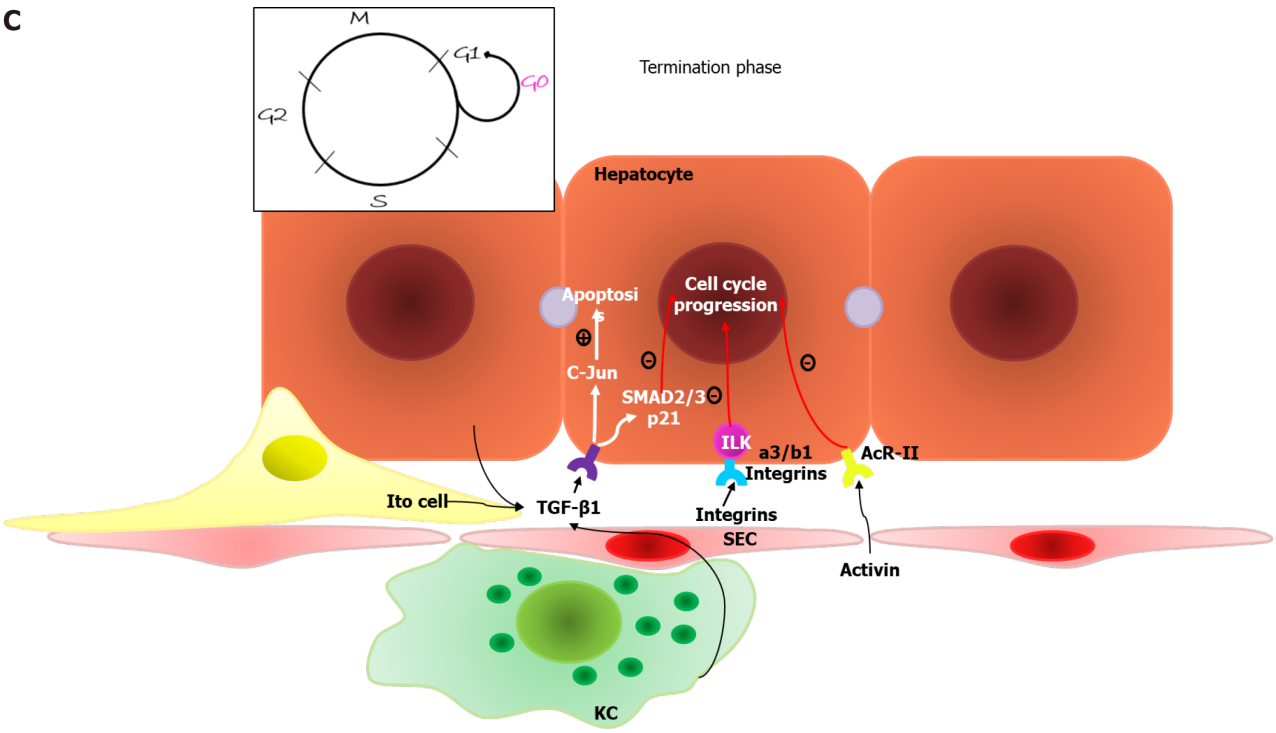


**Figure 1 Typical liver regeneration**. A: Priming phase. Mature hepatocytes undergo the G0-G1 transition driven by interleukin-6 and tumor necrosis factor-α. Sinusoid endothelial cells produce urokinase plasminogen activator in response to increased blood pressure. Urokinase plasminogen activator activates matrix metalloproteinase, resulting in extracellular matrix remodeling and the release of growth factors; B: Proliferative phase. Numerous factors, including Wnt-ligands, growth factors and bile acids, lead to the transcription of cyclin-dependent kinase and cyclins, resulting in the S-M transition and hepatocyte proliferation. Bile acids also suppress the synthesis of inflammatory cytokines by Kupffer cells; C: Termination phase. Different factors, primarily tumor necrosis factor-β family members, initiate the cell cycle arrest of hepatocytes and reversion to the G0 phase and cause the apoptosis of newly formed cells to correct the excessive regenerative response. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; MMP: Matrix metalloproteinase; KC: Kupffer cells; SEC: Sinusoid endothelial cells; HGF: Hepatocyte growth factor; EGFR: Epidermal growth factor receptor; MAPK: Mitogen-activated protein kinase.

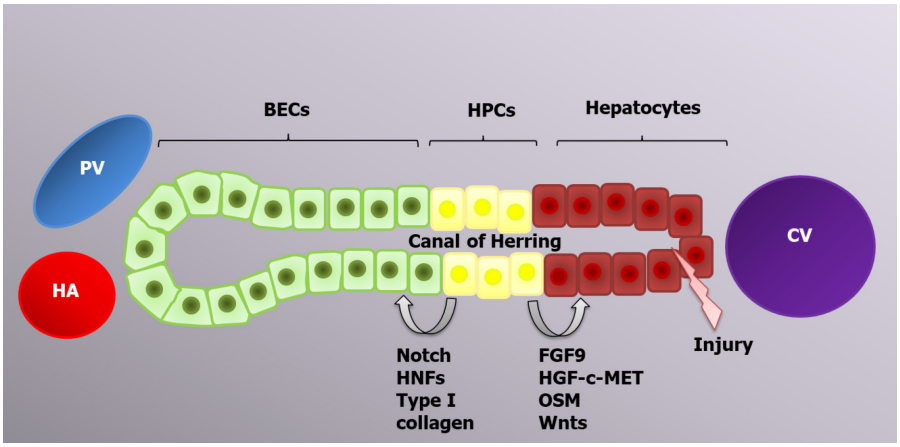


**Figure 2 Progenitor-dependent liver regeneration**. In case of excessive acute injury or chronic liver diseases, hepatic progenitor cell activation occurs in response to different inflammatory cytokines, including tumor necrosis factor-like weak inducer of apoptosis. Depending on the type of stimulus, hepatic progenitor cells can differentiate into biliary epithelial cells or hepatocytes to restore the liver mass. PV: Portal vein; HA: Hepatic artery; CV: Central vein; BECs: Biliary epithelial cells; HPCs: Hepatic progenitor cells; HNFs: Hepatocyte nuclear factors.

and BECs, the choice of which is determined by the activation of certain genes[12,108]. The canals of Hering connect the hepatocyte canalicular system and the biliary tree, and such a location of HPCs is consistent with their bipotential features[94]. Transplantation of current cells leads to liver regeneration enhancement *via* HPC proliferation and differentiation, which can be applicable for the treatment of certain liver diseases[112-115]. CK19, EpCAM and CD133 are markers common to both HPCs and BECs. Trop2 (Tacstd2) is a transmembrane molecule that is present on the HPC surface and absent on BECs; therefore, it can play a role as a specific marker, similar to

Foxl1[116].

The origin of HPCs is still being researched. Many scientists think that HPCs arise from mature differentiated BECs due to the presence of similar markers and cell localization. The expression of hepatocyte markers, such as albumin, AFP and HNF4α, appears earlier than HPC expansion. Newly formed HPCs have various markers on their surface, including the BEC markers HNF1b and CK19, which are maintained until the HPCs differentiate into mature hepatocytes[117]. Hepatocytes and BECs are formed from common cells, called hepatoblasts, during the second trimester of embryonic development. Consequently, the possibility of hepatocyte to BEC transdifferentiation and vice versa is genetically feasible and might be programmed to form a facultative pool of progenitors[12].

HPC compartment activation in the human liver is called ductular reaction because of the role of ductular epithelium activation. In the niche, HPCs are surrounded by epithelial and nonparenchymal cells, immune cells, and the components of the ECM, which transport activating signals[118]. As long as HPCs drive the regeneration of massive or chronic damage facilitated by immune cells, inflammatory cytokines, such as TNF-α, lymphotoxin-β, interferon-γ and IL-6, will play a crucial role in HPC activation. TNF-like weak inducer of apoptosis (TWEAK) is a TNF superfamily member and the main inducer of HPC activation[119]. Macrophages and NK cells are primary sources of TWEAK ligands. The interaction with target cells is realized by FGF-inducible 14 receptors. The TWEAK/ FGF-inducible 14 interaction leads to ductular reaction initiation *via* activation of the NF-κB signaling pathway[120]. HPC regulation is also performed by free oxygen radicals, which act as second messengers, realizing the balance between self-renewal and the differentiation of current cells. Low reactive oxygen species levels promote HPC proliferation *via* extracellular-signal-regulated kinase 1/2, Jun 1/2, Wnt and NF-κB signaling[121].

HPC differentiation into hepatocytes and BECs is regulated by a variety of signaling pathways. Thus, FGF9, the HGF-c-MET signaling pathway[122] and oncostatin M activate AKT and STAT3, which are required for HPC differentiation into hepatocytes, whereas HNF-6, HNF-1β and NOTCH signaling lead to BEC development[123,124]. All-trans retinoic acid is a significant active metabolite of vitamin A that is involved in HPC differentiation by increasing miR-200a expression, which regulates cell autophagy[125]. Ma *et al*[126] demonstrated the regulatory function of autophagy in HPC differentiation into hepatocytes *via* activation of the Wnt/β-catenin signaling pathway. Autophagy can also regulate HPC differentiation into BECs since it inhibits the Notch1 signaling pathway, which is required for the development of biliary duct cells. Therefore, autophagy is decreased during the early stages of liver regeneration[127].

Recently, a new pool of multipotential biliary progenitor cells, which can differentiate into hepatocytes, BECs and the islets of Langerhans cells, was identified in peribiliary glands, which are epithelial invaginations of extrahepatic and large intrahepatic biliary ducts[108]. This pool was named biliary tree stem/progenitor cells (BTSCs). BTSCs express stem cell markers such as Sox17, Pdx1, Sox9, EpCAM, Sall4 and Lgr5 on their surface. BTSCs are primarily involved in biliary epithelium regeneration in chronic diseases such as primary sclerosing cholangitis, cholangiocarcinoma, nonanastomotic strictures and biliary atresia[128].

## MICRO RIBONUCLEIC ACIDS AND LIVER REGENERATION

Micro ribonucleic acids (MiRNAs) are short molecules of 19–25 nucleotides in length that regulate the posttranscriptional silencing of target genes. One miRNA molecule can regulate hundreds of mRNAs, thus controlling the expression of various genes[129,130]. After PH, miRNA expression is primarily decreased (miR-16, miR-22, miR-23, miR-24, miR-26a, miR-29, miR-30, miR-31, miR-33, miR-122a, miR-126, miR-127, miR-145, miR-150 and miR-378); however, the expression of certain miRNAs increases (miR-21, miR-26b, miR-192, miR-194, MiR34a, miR-122, miR-203 and miR-221), thus affecting the hepatocyte cell cycle[131]. Table 2 summarizes the significant miRNAs in liver regeneration after PH.

Castro *et al*[132] demonstrated the crucial role of miRNAs in liver regeneration after PH. Thus, it was demonstrated that the expression of 26 different miRNAs changes during regeneration, notably in both increasing and decreasing ways. The expressions of miR-19a, -21, and-214 were significantly increased. MiR-21 transcription is activated by activator protein 1 (AP-1), which is also required for the activation of the important Stat3 and TGF-β signaling pathways[132]. Ng *et al*[133] pointed out the regulatory role of miR-21 in hepatocyte cell cycle events preceding the S phase *via* the indirect induction

**Table 2 Main micro ribonucleic acids influencing liver regeneration**

| miRNA | Expression change | Target genes | Influence on LR |
|---|---|---|---|
| miR-21 | Increased | Rhob, Sox7, Crebl2, Bcl-2, Btg2, Timp3, Reck, Pdcd4, Tgfbi, Smad7, PTEN | Induction |
| miR -19a | Increased | PTEN | Induction |
| miR-214 | Increased | PTEN | Induction |
| miR-203 | Increased | SOCS3 | Induction |
| miR-27a/b | Increased | Tmub1 | Induction |
| miR-503 | Decreased | Cyclin D1, Cyclin E2, CDC25A, CDKN1B, CHK1 | Suppression |
| miR-23a | Decreased | TNF-α, c-Myc CCNL2, HNF4G MET | Suppression |
| miR-150 | Decreased | TNF-α, survivin, FoxP1, c-Myb | Suppression |
| miR-663 | Decreased | TGF-β1, AP-1, Jun-B, Jun-D | Suppression |
| miR-378 | Decreased | Odc1 | Suppression |
| miR-34a | Decreased | INHBB | Suppression |
| miR-33 | Decreased | CDK6, EEF1A1, RAP2A | Suppression |
| miR-26a | Decreased | MAP3K2, MXI1, SENP5, CCND2, CCNE2 | Suppression |

miRNA: Micro ribonucleic acids; PTEN: Phosphatase and tensin homolog; LR: Liver regeneration; TNF-α: Tumor necrosis factor-α; CDK-6: Cyclin-dependent kinase 6.

of cyclin D1 translation, which occurs due to a reduction in cell cycle inhibitor expression. MiR-21 has a binding site on Ras homolog gene family member B, whose expression leads to the suppression of Akt1 activation, thus regulating cyclin D1 expression *via* mTORC1[133]. Additionally, miR-21 plays a significant role in decreasing phosphatase and tensin homolog expression, resulting in increased Akt and mTOR activities[134]. MiR-203 induces liver regeneration *via* IL-6/STAT3 signaling enhancement and SOCS3 expression inhibition[135]. MiR-27a/b regulates hepatocyte proliferation during regeneration because it suppresses Tmub1 expression[136], which suppresses the IL-6/STAT3 signaling pathway[137].

The decreased expression occurs within molecules such as miRs-503, -23a, -150, -663, -654 and is associated with their negative influence on liver regeneration. Thus, miR-150 inhibits TNF-α expression, which is essential for liver regeneration[138]. Increased miR-503 expression leads to the enhancement of essential cell cycle gene expression, including that of cyclin D1, E1, E2, F, Wee1, CDC25A and CHK1[139]. The AP-1 transcription factors, including the Jun and Fos family members, are the target genes of miR-663[140]. The c-Jun/AP-1 signaling pathway controls hepatocyte proliferation and has antiapoptotic activity *via* p-53-dependent pathway suppression[141]. An important negative regulator of hepatocyte epithelial-mesenchymal transition is miR-378, whose expression is decreased by Smo during liver regeneration, resulting in Hh-pathway activation and the transdifferentiation of hepatocytes and BECs into myofibroblasts[142]. MiR-34a expression is significantly decreased during the first days after PH, whereas the expression of its target genes (Notch1, Notch 4 and Hes1) is increased, leading to hepatocyte differentiation and growth enhancement[143]. MiRs inhibiting liver regeneration are also important because they prevent excessive regeneration. Among these molecules, for example, miR-33 suppresses CDK6 and CCND1[144], and miR-26a targets CCND2 and CCNE2[145].

A further understanding of the miRNAs involved in normal and progenitor-dependent liver regeneration can improve the use of miRNAs for the diagnosis of different liver diseases, control the adequacy of liver regeneration and act as a potential therapy for insufficient liver regeneration.

## STIMULATION OF INSUFFICIENT LIVER REGENERATION

Therapeutic methods for insufficient liver regeneration treatment are lacking, although many studies have focused on the efficiency of various molecules in promoting liver regeneration. Shi *et al*[146] determined that baicalin can stimulate liver regeneration after

acetaminophen-induced acute liver injury in mice *via* inducing hepatocyte proliferating cell nuclear antigen, increasing cyclin D1 expression and Nrf2 cytosolic accumulation, and enhancing IL-18 Levels, leading to the upregulation of hepatocyte proliferation. So *et al*[147] showed the promotion of liver regeneration after the inhibition of EGFR or MEK/extracellular signal-regulated kinase (ERK) and the genetic suppression of the EGFR-ERK-SOX9 axis *via* inducing HPC-to-hepatocyte differentiation in zebrafish. The research of Xiang *et al*[148] noted the therapeutic effect of IL-22Fc in inducing liver regeneration in acute-on-chronic liver failure patients due to the shift from anti-regenerative IFN-γ/STAT1 to the pro-regenerative IL-6/STAT3 pathway. Li *et al*[149] reported that aldose reductase (AR) is a new potential therapeutic target for enhancing normal and fatty liver regeneration after surgery and IRI because the knockout of AR leads to enhanced oxisome proliferator activated receptor-α and oxisome proliferator activated receptor-γ expression, thus improving energy metabolism in the liver. The research of Loforese *et al*[150] revealed that the inhibition of MST1 and MST2 with si-RNA resulted in improved hepatocyte proliferation in aged mice after PH; therefore, Ste20-like kinases 1/2 may be a potential therapeutic target. Many other molecules and molecular pathways have been shown to enhance liver regeneration in experimental models. Further studies would help implicate the potential therapy in the clinic and improve the survival of patients with different liver diseases in the near future.

Mesenchymal stem cells (MSCs) have a self-renewal capacity and are derived from the bone marrow, adipose tissue, umbilical cord, *etc*. They are the subject of focus in regenerative medicine and serve as a potential therapy for different liver diseases[151,152]. MSCs were shown to improve liver regeneration in patients with cirrhosis by elevating anti-apoptotic factors, such as HGF and IGF-1, and angiogenic and mitogenic factors. In acute liver failure animal models, MSCs have been shown to promote liver regeneration mostly by suppressing the oxidative stress and inflammation *via* reducing TNF-α, IFN-γ and IL-4 Levels and stimulating liver regeneration with various released factors such as $PGE_2$ and delta-like 4[153,154]. MSCs can also stimulate liver regeneration after PH by upregulating hepatic cell proliferation and downregulating fat accumulation and HGF, IL-6, IL-10 and TNF-α serum levels[155].

Further studies in this field can help determine how to prevent hepatic failure after surgical interventions and acute and chronic injuries *via* improving liver regeneration.

## CONCLUSION

Liver regeneration is driven by multiple molecular processes. Biomolecular factors permit the possibility of targeted therapy to prevent serious complications, such as liver failure due to a decreased cellular regenerative potential.

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*MINIREVIEWS*

# Hepatitis D virus and liver transplantation: Indications and outcomes

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## Abstract

Hepatitis D virus (HDV) is a dependent virus that relies on hepatitis B virus for its replication and transmission. Chronic hepatitis D is a severe form of viral hepatitis that can result in end stage liver disease. Currently, pegylated interferon alpha is the only approved therapy for chronic HDV infection and is associated with significant side effects. Liver transplantation (LT) is the only treatment option for patients with end-stage liver disease, hepatocellular carcinoma, or fulminant hepatitis due to coinfection with HDV. As LT for HDV and hepatitis B virus coinfection is uncommon in the United States, most data on the long-term impact of LT on HDV are from international centers. In this review, we discuss the indications and results of LT with treatment options in HDV patients.

**Key Words:** Hepatitis delta virus; Liver transplant; Hepatitis B immunoglobins; Hepatocellular carcinoma



**Core Tip:** Hepatitis D virus (HDV) is a dependent virus and relies on hepatitis B virus

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(HBV) to synthesize the pathogenic genomes. Therefore, it can only survive as a coinfection with HBV or as a superinfection. Chronic HDV infection results in rapid liver damage and can result in end stage liver disease. Currently, pegylated interferon alpha is the only approved therapy for chronic HDV infection and is associated with significant side effects. Thus, liver transplant remains the only option for patients with end-stage liver disease, hepatocellular carcinoma due to coinfection or superinfection with HDV and HBV, fulminant liver failure and those who cannot be treated with interferon-based therapies. Post transplantation reinfection with HDV/HBV is an undesirable outcome. Though, there is a consensus that hepatitis B immune globulin in combination with a potent nucleoside/nucleotide analogue have shown promising results. In addition, there is ongoing research for newer treatment drugs. This review article focuses on liver transplant in patients as a result of hepatitis D virus. We have discussed the epidemiology, pathogenesis, clinical presentation, indication of liver transplantation, treatment options and the outcomes. New therapy trials have been also discussed in the treatment section. We believe that this topic is an area of knowledge gap and this article will cover the basics.

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## INTRODUCTION

Hepatitis D virus (HDV) was discovered in 1970s by Rizzetto *et al*[1]. It is formed by 1678 nucleotide single stranded ribonucleic acid (RNA) virus which is circular in shape and contains two viral proteins that are p24 and p27[2,3]. There is a total of 8 genotypes of HDV in the world[4]. HDV is not able to make its own proteins and relies on hepatitis B virus (HBV) to synthesize the pathogenic genomes. Therefore, it can only survive as a coinfection with HBV or as a superinfection. Around 5% of HBV carriers worldwide have been exposed to HDV and the prevalence of HDV coinfection in United States is reported to be 12%[5,6]. Chronic HDV infection results in rapid liver damage compared to patients infected with HBV alone. In addition, incidence of cirrhosis is almost three times with HBV/HDV chronic coinfection and associated with increased rate of early decompensation leading to hepatocellular carcinoma (HCC)[7]. As HDV uses host polymerase for replication, HBV polymerase inhibitors are not effective against it[8]. Therefore, the only widely accepted treatment is interferon at high doses which has a success rate of 25% to 30%, which is defined as virological response after one year of conventional or PEG-INFa treatment with most studies measuring virological response after 6 mo of treatment[9]. Thus, liver transplant (LT) remains the only option for patients with end-stage liver disease, HCC due to coinfection or superinfection with HDV and HBV, fulminant liver failure and those who cannot be treated with interferon-based therapies. In this review we discuss the indications of liver transplantation and its outcome in patients with HDV.

## EPIDEMIOLOGY

There are about 240 million people worldwide who have positive hepatitis B surface antigen (HBsAg). Amongst them 2% to 8% are co-infected with HDV resulting in approximately 20 to 40 million suffering from HDV[10,11]. However, recent studies have estimated the coinfection number to be higher as up to 72 million[12]. HDV is endemic in the Middle East, Mediterranean Area, Amazon Region, and African countries[13]. In Europe, HDV is mainly a problem in Eastern European immigrant populations and amongst intravenous drug users (IVDU)[14,15]. There are 8 different HDV genotypes and genotype 1 is the most common in North America[16]. Testing for HDV has not been widespread in the United States and the prevalence has been underestimated. There has been a 3.4% HDV seropositive rate reported in the veteran population positive

with HBsAg[17]. In comparison, National Health and Nutrition Examination Survey data (1999-2012) showed a significantly lower rate of HDV prevalence (0.02%) in the civilian population[18]. It increased to 0.11% in a repeat National Health and Nutrition Examination Survey (2011-2016) study[19]. Both these studies are limited as they excluded homeless, incarcerated, and other high-risk individuals. However, a study done among patients with IVDU in Baltimore by Kucirka *et al*[20] reported 11% prevalence of HDV in 2005-2006. Similarly, Gish *et al*[21] conducted a study in California on chronic HBV patients reporting a coinfection rate of 8%. This variability warrants routine testing of HDV in HBV carriers with specific recommendations for screening, treatment and follow-up. This will aid risk stratification of patients and allow for early discovery of complications, which in turn may improve outcomes.

## PATHOGENESIS, CLINICAL FEATURES AND DIAGNOSIS

HDV is parenterally transmitted and has variable clinical manifestations. There are two major patterns of infection that are described in literature. Notably, coinfection of HBV with HDV and superinfection of HDV in chronic HBV-infected patients. HDV develops innate and adaptive immunity and there are specific markers such as HDV RNA, hepatitis D antigen and anti-HDV antibodies, such as IgM and IgG, which help to detect and differentiate the chronicity of the disease[22].

As HDV's virulence is dependent on HBV, coinfection results from simultaneous acute HBV and HDV. It is usually transient and cannot be clinically distinguished from HBV infection[23]. HDV has incubation period of approximately 1 mo resulting in clinical symptoms of fatigue, loss of appetite and nausea. It is accompanied with a rise in liver enzymes, including serum alanine aminotransferase and aspartate aminotransferase. Then comes the jaundice phase with increase in bilirubin levels. As it is usually self-limiting and most patients recover completely with only 2% leading to chronic infection[7,24].

Superinfection with HDV can also result in acute hepatitis which is more severe than seen with co-infection. This is because HBV has already set the ground for more aggressive disease progression. It can lead to acute liver failure with clinical symptoms starting as nausea and progressing to coagulopathy, encephalopathy and coma[25]. About 80% to 90% of patients progress to chronic hepatitis. Amongst them, some dated studies have reported up to 70% to 80% progress to cirrhosis within 5 to 10 years[26]. However, newer studies suggested a 4% annual progression to cirrhosis[27]. This variability might be due to the different genotypes of HDV. Although there is controversy in the literature over whether HDV has oncogenic properties, cirrhosis from HDV does increase the risk of HCC, which is the second most common cause of cancer deaths in men worldwide[28].

HBsAg is necessary before other markers for HDV are investigated to establish the diagnosis. One important distinguishing test is IgM anti-HBc, which is only present in acute HDV/HBV coinfection and not in acute HDV superinfection. Likewise, HDV RNA is a sensitive marker for acute infection and reaches a very high quantitative value in chronic patients. Similarly, the presence of anti-HDV IgM or high anti-HDV IgG titer can differentiate between current and past infections. Therefore, knowing these markers helps to differentiate the disease pattern (Table 1)[23].

## INDICATIONS FOR LIVER TRANSPLANTATION

The global disease burden of HBV/HDV coinfection is increasing with 10.6% of HBsAg carriers without high risk sexual behavior or IVDU are HDV[12]. HDV can lead to a more severe form of viral hepatitis than in HBV mono-infection[29]. Irrespective of whether being coinfected with HBV or as superinfection, HDV can cause fulminant hepatitis[30]. The clinical course of fulminant hepatitis D is 4 to 30 d and transplant free survival is as low as 20%[7,31].

Chronic HDV results in rapid liver fibrosis, earlier decompensation, higher risk of HCC development and annual mortality rate between 7% to 9%[32]. Mortality rates of greater than 50% at 15 years follow up have been reported in Taiwan[33]. Though direct oncogenic properties of HDV is not clearly described, higher rates of cirrhosis in HDV patients can lead to increased rates of HCC[34]. Rates of HCC are variable across the globe with studies showing anti-HDV antibodies ranging from 4% to 23% in HBsAg positive HCC patients[35,36]. Treatment option for HDV has limited success. Therefore, the only definitive therapy for patients with end-stage liver disease, HCC, or

**Table 1 Summarizes markers specific for coinfection and superinfection**

| | Coinfection | Superinfection |
|---|---|---|
| HDV infection | Acute | Acute or chronic |
| IgM anti-HBc | Positive | Negative |
| Serum HDV RNA | Transient | Persistent and high |
| IgM anti-HDV | Transient | Persistent |
| IgG anti-HDV | Late appearance and low | Persistent and high |

HDV: Hepatitis D virus; RNA: Ribonucleic acid; HBc: Hepatitis B core.

fulminant hepatitis due to HDV is liver transplant[13].

## TREATMENT OPTIONS

Currently there is no United States Food and Drug Administration approved treatment for HDV[37]. However, PEG-IFNa is commonly used and is also recommended by major liver societies such as American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver[38,39]. Pegylated form requires only weekly dosing and metanalysis has shown increased suppression (29%) of HDV RNA at 6 mo compared to standard IFN alpha (19%)[40]. In addition, PEG-IFNa is also associated with lower rates of side effects such as anorexia, nausea, weight loss, alopecia, leukopenia and thrombocytopenia[41,42]. Although there is no definite treatment duration, negative HDV RNA at 24 wk is being considered a reference for virological response and treatment for 48 wk is recommended[38,43]. Pegylated IFN has been studied in combination with nucleosides. The Hep-Net/International Delta Hepatitis Intervention Trial randomized 90 patients to adefovir, peginterferon, or the combination arm. Approximately 25% achieved virological response at 24 wk in the peginterferon and combination group and none in the adefovir group[44]. Similarly, the HIDIT-2 trial (which replaced adefovir with tenofovir) yielded similar results to the trial[21] and did not show a response in the nucleoside alone[45]. Thus, combination of interferon with nucleosides or increasing the duration of treatment has shown no additional benefits.

In addition, newer experimental treatments are currently underway. One such example is the use of oral prenylation inhibitor lonafarnib (LNF). Prenylation inhibitors have been shown to abolish HDV-like particle production *in vitro* and *in vivo* [46]. LNF interferes with the HDV cycle and targets the virion assembly step in the hepatocyte cytoplasm, where the nascent HDV nucleoprotein complex is enveloped by HBsAg[47]. To explore this, the LOWR HDV-1 [Lonafarnib with and without Ritonavir (RTV) in HDV-1] phase two clinical trial was conducted by Yurdaydin *et al*[47] with the intention to study optimal LNF dosing while assessing tolerability and viral response when combined with P450 3A4 inhibitor RTV or PEG-IFNa. Results showed that LNF, whether as monotherapy or as combination with PEG-IFNa, led to HDV-RNA viral load decline in all patients. All treated patients in different treatment regimens reported GI adverse effects consisting of anorexia and weight loss. Higher dose of LNF, 300 mg peroral BID, was associated with increased adverse effects. RTV helped to lower LNF dose (100 mg per oral BID dosing) while still achieving better antiviral results. Similarly, LNF 100 mg BID with PEG-IFNa helped in more substantial and rapid HDV-RNA reduction, compared to PEG-IFNa alone[47]. Such trials have opened the door to further explore newer treatment options for HDV.

PEG-IFNa is only used amongst patients with compensated liver disease. For those who undergo LT, long-term survival depends on the prevention of allograft reinfection. LT for HDV is not common in United States and studies in literature are mostly from other countries[48,49]. Due to antivirals and with hepatitis B immune globulin (HBIG), rates of HBV/HDV reinfection after LT has decreased[50]. Currently there is no specific prophylaxis for HDV. However, as its growth is dependent on HBV, the focus should be on preventing HBV infection.

Levels of HBV DNA (> 105 copies/mL) strongly predict HBV reinfection in HBsAg positive LT recipients[51]. As per AASLD recommendations, all HBsAg-positive recipients should receive prophylactic nucleoside/nucleotide analogs with or without

HBIG post-LT. In addition, pretransplant hepatitis B e-antigen/HBV-DNA levels should not be taken into consideration and HBIG monotherapy should not be used. They further suggest that entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide should be the preferred antivirals and continued indefinitely post-LT[38]. The use of HBIG depends on the recipient and virologic factors. In medically adherent HBV mono-infected recipients with undetectable or low-level viremia at the time of LT and no evidence of concurrent infection, no HBIG or a very short course (5 d) of HBIG post-LT combined with long-term antiviral therapy is highly effective in preventing HBV recurrence[38]. On the other hand, in HBV/HDV co-infected recipients, the combination of long-term HBIG and antiviral therapy may be the best approach in preventing HBV and HDV recurrence[38].

Data regarding the dosage of HBIG therapy varies across transplant centers. In previous studies HBIG has been given as either high (≥ 10000 IU/mL) or low (< 10000 IU/mL) dose for either a fixed duration (median of 6 mo) or indefinitely post-LT[50]. It is administered either intravenous or intramuscularly during an hepatic phase, followed by daily doses during the first week, with subsequent doses given monthly or by following anti-HBs titers based on the transplant center protocol[50]. A trough anti-HBs titer of at least 100 IU/L is thought to be protective and reinfection rate can be further reduced by maintaining anti-HBs titers consistently above 500 IU/L[52] (Figure 1)[48].

## LIVER TRANSPLANT OUTCOMES

Long-term survival following LT for viral hepatitis depends on prevention of allograft reinfection[53]. This is a well-known concept for HBV as well as HCV and can be applied for HDV related LT as well. LT for HDV started in the late 1980s from Europe. One of the earliest reporting was from Rizzetto *et al*[54] from Italy, on 7 patients who underwent LT due to HDV cirrhosis. It resulted in reinfection rate of 70% with HDV and milder forms of hepatitis were reported in 40% of the cases. This encouraged others to believe that LT was a feasible option for ESLD from HDV. Ottobrelli *et al*[55] reported a larger series of 22 patients, which showed 80% reinfection rate and 73% survival rate at one year. Although the reinfection rate was high, the clinical course was mild, therefore giving hope to the patients that LT was the possible cure for HDV. At that time, it was unclear whether administration of HBIG will be beneficial in preventing reinfection. Therefore, a multicenter study was done in Europe and amongst 110 patients who underwent LT due to HDV cirrhosis, the three-year actuarial risk of HBV recurrence after transplantation was reported as 70% ± 14% in the group who received no HBIG and 17% ± 6% in patients who received HBIG for > 6 mo[56]. The actuarial three-year survival was reported as 83%. In that study, long-term administration of HBIG (RR: 2.22; 95% confidence interval: 1.13-4.33; $P < 0.001$) and HDV superinfection (RR: 6.25; 95% confidence interval: 3.13-12.42; $P < 0.001$) were reported as independent predictors of better survival[56]. With the passage of time and development of new antivirals which when used in combination with HBIG, post-LT HBV/HDV reinfection has significantly decreased. In a retrospective study Adil *et al*[57] reported HBV recurrence rate of 5.1% and no HDV recurrence among 255 patients, after a mean follow-up of 30 mo. Similarly, study by Idilman *et al*[58] endorsed this, showing that amongst 90 patients with delta co-infection-related cirrhosis who underwent LT, only one recipient (who received lamivudine and HBIG combination), had HBV recurrence upon follow up. Moreover, in an another study with 104 HDV patients, with a longer follow up of 82 mo, the survival and HBV recurrence rates were 97% and 13.4% respectively[59]. Thus, it was confirmed that it is very important for survival and viability of the graft that the patients remain HBsAg-negative after transplantation.

Interestingly, studies have shown that presence of HDV infection appears to provide a protective effect against HBV reinfection in LT patients, possibly *via* suppression of HBV replication resulting in longer survival rates[49]. Recently a study published on LT patients in Brazil showed significantly higher 4-year survival rate of 95% in HDV group ($n = 29$), compared to 75% in HBV group ($n = 40$)[60]. One of the largest series involving hepatic transplantation in patients with HDV ($n = 76$), identified 88% survival after 5 years[61]. This is likely because of low HBV recurrence rate in these series.

HDV leading to HCC has also been treated with LT. Romeo *et al*[27] performed a retrospective study where 29 of 299 patients diagnosed with HBV/HDV had liver transplant; amongst these 29 patients, 10 patients (34%) had HCC. After transplant, 5

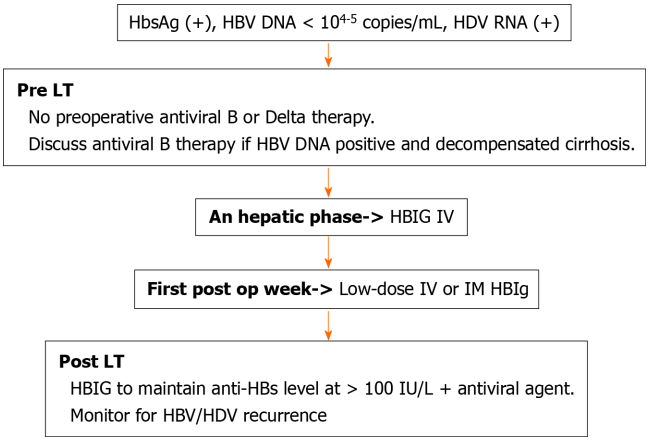


**Figure 1 Possible treatment flowchart in hepatitis D virus liver transplant patients.** HbsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; HBIG: Hepatitis B immune globulin; LT: Liver transplantation.

patients died (3 with primary graft failure, 1 with tumor recurrence, and 1 with non-liver–cancer-related reasons). Similarly, a retrospective study was conducted in Turkey amongst 25 live donor LT recipients with chronic HBV/HDV, 11 of which had HCC. The cumulative 5-year survival was 74%. In the HCC group, 7 of 11 tumors matched the Milan criteria and 4 patients did not (in whom 2 patients had HCC recurrence after 2 years which was treated by ablation techniques)[32]. Thus, results from our review supports the AASLD guideline, that using HBIG in conjunction with oral antivirals post-transplantation, changes the natural history of the liver disease even among recipients with HCC.

## CONCLUSION

HDV presents a severe health burden with liver transplantation as the only treatment for patients with End-stage Liver Disease, hepatocellular carcinoma, or fulminant hepatitis. Post transplantation reinfection with HDV/hepatitis B virus is an undesirable outcome as it affects survival. While transplant centers across the world have their own protocols, there is a consensus that hepatitis B immune globulin in combination with a potent nucleoside/nucleotide analogue have shown promising results. In the future, with the potential approval of the pipeline drugs for HDV treatment, their role in the post-transplant setting also needs to be explored. Currently, the data on liver transplant due to HDV is limited and more randomized controlled trials investigating the duration and frequency of hepatitis B immune globulin as well as the specific anti-HBs titer level are needed to optimize the pre- and post-transplant treatment plans.

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*MINIREVIEWS*

# Lymphatic dysfunction in advanced cirrhosis: Contextual perspective and clinical implications

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## Abstract

The lymphatic system plays a very important role in body fluid homeostasis, adaptive immunity, and the transportation of lipid and waste products. In patients with liver cirrhosis, capillary filtration markedly increases, primarily due to a rise in hydrostatic pressure, leading to enhanced production of lymph. Initially, lymphatic vasculature expansion helps to prevent fluid from accumulating by returning it back to the systemic circulation. However, the lymphatic functions become compromised with the progression of cirrhosis and, consequently, the lymphatic compensatory mechanism gets overwhelmed, contributing to the development and eventual worsening of ascites and edema. Neurohormonal changes, low-grade chronic inflammation, and compounding effects of predisposing factors such as old age, obesity, and metabolic syndrome appear to play a significant role in the lymphatic dysfunction of cirrhosis. Sustained portal hypertension can contribute to the development of intestinal lymphangiectasia, which may rupture into the intestinal lumen, resulting in the loss of protein, chylomicrons, and lymphocyte, with many clinical consequences. Rarely, due to high pressure, the rupture of the subserosal lymphatics into the abdomen results in the formation of chylous ascites. Despite being highly significant, lymphatic dysfunctions in cirrhosis have largely been ignored; its mechanistic pathogenesis and clinical implications have not been studied in depth. No recommendation exists for the diagnostic evaluation and therapeutic strategies, with respect to lymphatic dysfunction in patients with cirrhosis. This article discusses the perspectives and clinical implications, and provides insights into the management strategies for lymphatic dysfunction in patients with cirrhosis.

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**Core Tip:** Lymphatic dysfunction appears to play a significant role in the pathophysiology of advanced cirrhosis. Sustained portal hypertension, neurohormonal changes, and low-grade chronic inflammation have been implicated in causing lymphatic dysfunction in advanced cirrhosis, leading to worsening of ascites, lymphedema, and abnormal lipid transport; it also results in increased susceptibility to infections. Chylous ascites and intestinal lymphangiectasia are the rare manifestations of lymphatic dysfunction in cirrhosis, leading to loss of protein, fat, lymphocytes, and immunoglobins, with several clinical consequences. Lymphatic dysfunctions in cirrhosis have been ignored to date; hence, new exploratory research must be undertaken to gain insight into this important subject.

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## INTRODUCTION

The lymphatic system consists of capillaries located inside the tissue that are highly permeable and are needed to transport lymph containing cellular proteins, lymphocytes, and lipoproteins[1-4]. It is essential for maintaining homeostasis of tissue *via* interstitial fluid reabsorption, immune cell trafficking, and the transport of lipids[3-5]. The lymphatic system removes interstitial fluid from tissues and returns it to the bloodstream. When this interstitial fluid gets into lymphatic capillaries, it is called lymph. The liver is the largest organ generating lymph, and liver lymphatics are believed to play a vital role in maintaining normal hepatic function by helping to eliminate protein, cholesterol, and immune infiltrates[5]. In the absence of normal lymphatic function, interstitial fluid accumulation may contribute to clinical manifestations such as lymphedema and ascites[6]. In patients with early cirrhosis, the lymphatic system helps to prevent development of ascites by reabsorbing excess fluid in the hepatic and splanchnic areas. As a result, lymph flow is enhanced, which promotes hepatic lymphangiogenesis[7,8]. However, in advanced cirrhosis patients, this compensatory mechanism is not adequate to prevent the development of ascites. Moreover, there appears to be an impaired lymphatic pump function in patients with an advanced liver disease[9]. Despite its significant clinical value, the literature on lymphatic dysfunction in cirrhosis is very limited, and the area remains open for new investigations. This article summarizes the current knowledge regarding dysfunctions of lymphatic system in patients diagnosed with liver cirrhosis, with special attention to pathophysiology, clinical implications, and insights into management strategies.

## LYMPHATIC VASCULAR SYSTEM

The lymphatic system consists of a large network of lymphatic vessels, with lymphoid organs and tissues. Lymphatic vessels are classified anatomically into capillaries and collecting vessels. Further, the lymphatic capillaries are closed-ended and composed of a single layer of lymphatic endothelial cells (LECs). The initial lymphatics are highly permeable for transport of interstitial fluid macromolecules and immune cells. LECs have anchoring filaments that contract and relax, which enable them to "flap" open to allow interstitial fluid uptake[10,11]. The lymphatics capillaries merge into larger collecting lymphatic vessels, which possess a continuous basement membrane and have unidirectional bicuspid valves with contractile smooth muscle cells' (SMCs) covering for assisting the flow of lymph. Similar to lymphatic capillaries, the liver has

sinusoids, consisting of a single layer of liver sinusoidal endothelial cells (LSECs), without the basement membranes[12]. Hepatic lymph is produced by plasma components filtered through the LSECs into the space of Disse. In the gastrointestinal tract, lymphatics are present in mucosal, submucosal, and muscular layers; they merge with collecting lymphatic vessels near the mesenteric border. The lymphatics present in the center of each intestinal villus are referred to as lacteals, which have a structure similar to the lymphatic capillaries elsewhere, consisting of a single layer of LECs, without a basement membrane[13].

There is constant filtration of plasma into the interstitial space during the passage of blood through the capillaries. The rate of filtration is primarily dictated by the hydrostatic pressure and plasma oncotic pressure in the capillaries. Due to the change in interstitial pressure, interstitial fluid enters the lymphatic capillaries, as lymph, and moves towards larger lymphatic vessels[14]. The contractile activity of SMCs, of the collecting lymphatic vessels, is believed to be one of the major driving forces of lymphatic circulation[15]. The $Ca^{2+}$ channels of SMCs and nitric oxide (NO) produced in LECs is thought to contribute to the regulation of lymphatic flows, by modulating the contractility of SMCs[16]. In liver, most of the lymph from space of Disse drains into lymphatic vessels in the area near portal triads. Some part of the lymph also circulates into the interstitium around the central vein or underneath the Glisson's capsule. Finally, all the liver lymphatic vessels converge into the hepatic hilum and flow into the lymph nodes arranged in the lesser omentum along the hepatic vessels and hepatic ducts[5,17]. The collecting lymphatic vessels, from all organs, connect to one or more lymph nodes and, finally, lymph trunks, which ultimately drain into the subclavian vein *via* thoracic duct or right lymph trunk (Figure 1). Thus, interstitial fluid, collected as lymph, is finally returned to the blood circulation through the lymphatic vessels. It is estimated that approximately 3 L to 5 L of lymph fluid travel through the thoracic duct each day, of which 50% to 90% comes from the intestines and liver[18]. Being capillary ultrafiltrate, all plasma proteins are present in lymph. However, several proteins derived from extracellular matrix, cellular metabolism, and cell death are enriched in lymph instead of the plasma[19]. Therefore, the composition of the lymph arising from various areas varies to a degree.

## FUNCTIONS OF LYMPHATIC SYSTEM

The lymphatic system plays an important role in maintaining tissue homeostasis, by transporting interstitial fluid, serum protein, and lipids from tissues to the systemic circulation. After plasma filtration through the capillaries, the only way the fluid can be returned to blood circulation is *via* the lymphatic system[20]. When there is a mismatch between capillaries filtration and lymphatic removal, fluid accumulation occurs in the extravascular space. Lymphatic system plays a key role in adaptive immunity. It delivers antigen and antigen-presenting cells to the regional lymph nodes, where they evoke immune responses. Lymphatics also play a role in controlling the inflammatory response, by influencing the drainage of extravasated fluid and inflammatory mediators, and by facilitating the discharge of infiltrated immune cells from inflamed sites[21,22]. Moreover, lymphatic vessels are essential for the removal of cholesterol from peripheral tissues[23]. LECs are known to take up cholesterol carried by high-density lipoprotein, and dysfunctional LECs can lead to the development of hepatic steatosis[24]. Furthermore, intestinal lacteals play important role in the absorption of fat and fat-soluble vitamins as chylomicrons.

## LYMPHATIC SYSTEM CHANGES IN CIRRHOSIS

In patients with cirrhosis, capillary filtration increases steadily and gradually, primarily due to an increase in hydrostatic pressure. This contributes to an enhanced lymph production, with consequent lymphatic compensatory responses, such as an increase in the number and size of lymphatic vasculature, to enhance the drainage of interstitial fluid[8,25,26]. Several structural and functional changes in the lymphatic system have been reported in patients with cirrhosis.

### *Increase in the lymph flow*

An increased architectural distortion in cirrhosis causes resistance to sinusoidal blood flow, increased hydrostatic pressure in the sinusoid, and increased filtration of plasma.

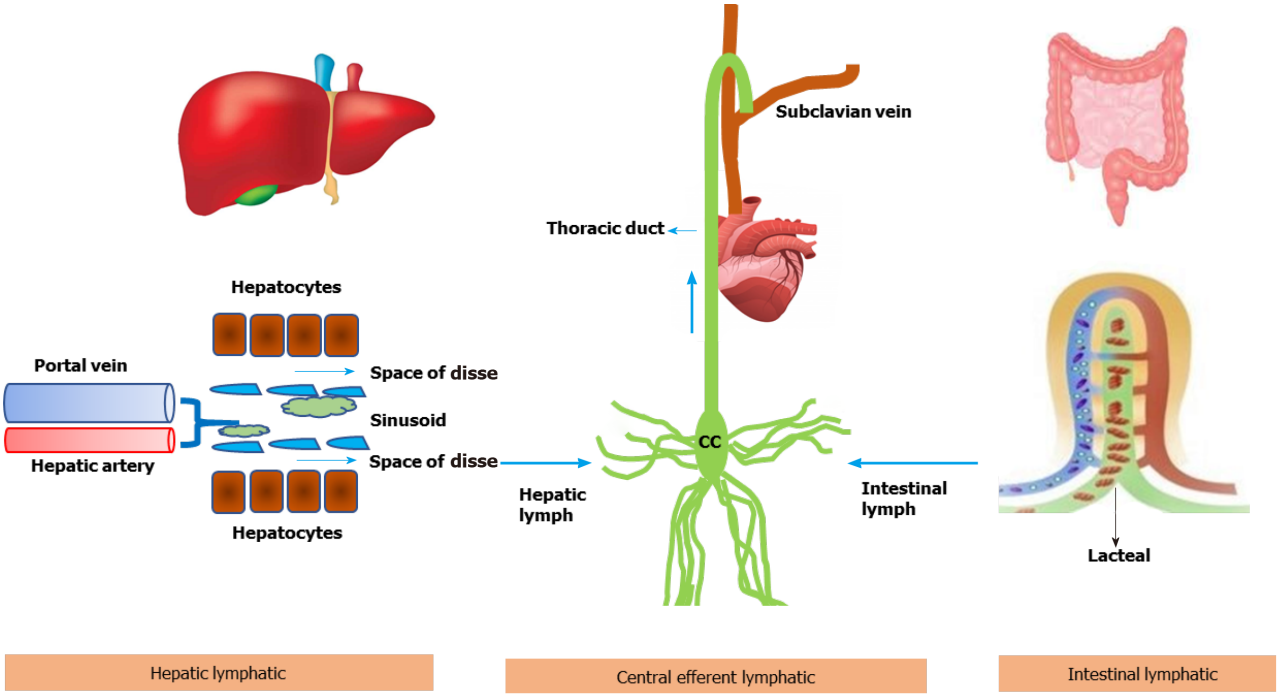


**Figure 1 Schematic diagram showing lymph flow kinetics from liver and intestine to the systemic circulation.** The capillary filtrate enters the lymphatic capillaries, as lymph, and moves towards larger lymphatic vessels. In liver, lymph is produced by filtration of plasma through the sinusoidal endothelial cells into the space of Disse. The collecting lymphatic vessels from all organs connect to one or more lymph nodes, and finally to the lymph trunks which ultimately drain into subclavian vein *via* cysterna chyli and thoracic duct. Approximately 80% of thoracic duct lymph comes from the intestines and liver.

This process may be further enhanced by concomitant hypoalbuminemia and increased capillary permeability under certain circumstances. Thus, lymph production and flow is greatly increased (up to 30 folds) in patients with cirrhosis[27,28]. Witte *et al*[7] demonstrated that lymph in the thoracic duct of cirrhosis patients had a high protein concentration. Because the protein concentration of hepatic lymph is higher (50%-80% of plasma), such overproduction of lymph in cirrhosis appears to come primarily from the liver. However, with advancement of cirrhosis, the protein content of hepatic lymph also decreases because of a dysfunctional lymphatic transport system. In an animal study of cirrhotic livers, a positive correlation between hepatic lymph flow and increasing portal pressures was found. Moreover, this study also demonstrated a compromised functional capacity of lymphatic vessels to absorb interstitial fluid[29].

### *Increase in the number and density of lymphatic vessels*

Dumont and Mulholland[30] were the first to describe an increased diameter and lymph flow in the thoracic duct, in patients with cirrhosis. Such expansion of lymphatic vasculature has also been reported by Sadek *et al*[31] on computed tomography and Shimada[32] on laparoscopy. The expansion of lymphatic density correlates positively with the severity of fibrosis around the portal tracts of human liver. Yamauchi *et al*[26] found that the intrahepatic lymphatic vessels remain stable during the early stages of liver disease, but when it progresses to advanced cirrhosis, it increases significantly. In addition, Yokomori *et al*[33] recently calculated the density of lymph vessels by immunohistochemistry in patient specimens and found that the density increased with the progression of liver disease, peaking at the most advanced stages of cirrhosis. In cirrhotic livers, a substantial increase in vascular endothelial growth factors (VEGF)-D expression, an inducer of lymphangiogenesis, was observed and in addition, VEGF-D expression was found to be positively associated with liver fibrosis progression[8]. This lymphangiogenic response may help to enhance the drainage of increased interstitial fluid.

### *Lymphatic oversaturation and flow dysfunction*

The lymphatic system keeps tissue edema free, by returning excess tissue fluid back to the bloodstream. In cirrhotic patients, when interstitial fluid is increased, expansion of lymphatics and increased lymphatic flow initially tries to prevent development of ascites and edema[7]. However, it is not clear as to what extent the lymphatic

vasculature may compensate for enhanced lymph production. In a sustained increase of the hydrostatic pressure, fall in plasma oncotic pressure, compounding effects of capillarization/defenestration of sinusoidal endothelium, and neurohormonal changes, the compensatory mechanism is gradually overwhelmed, resulting in fluid accumulation in the extravascular space[34,35]. In the splanchnic circulation of cirrhosis patients, arteriolar vasodilation occurs; it increases the production of splanchnic lymph beyond the ability of the lymphatic system to transport and, thus, triggers lymph leakage into the peritoneal cavity. Moreover, an increased splanchnic vascular permeability and chronic retention of renal sodium and water plays a major role in the sustained development of ascites[36,37]. Over time, increased pressure and flow stasis in the intestinal lymphatic channels may lead to lymphangiectasia, followed by the rupturing of dilated lacteals and intestinal loss of protein, chylomicrons, and lymphocyte[38]. Rarely, the rupture of subserosal lymphatic, secondary to a sustained high pressure, results in the development of CA[39].

Apart from lymphatic oversaturation, functional defect in the lymphatic transport system has also been reported in patients with cirrhosis. Henriksen[40] have described a model of lymphatic conductivity (flow rate per unit pressure difference), based on protein kinetic and hemodynamic measurement in patients with cirrhosis. They found that lymphatic conductance in the thoracic duct was three times higher than normal in patients without ascites, while in patients with tense ascites, these values were close to normal. Moreover, conductance in the right lymphatic duct system was ten times below that of thoracic duct of cirrhotic patients with ascites. The results of this study suggest that a relatively insufficient lymphatic drainage plays an important role in the accumulation of ascites in decompensated cirrhosis. Recently, the functionality of the splanchnic and peripheral lymphatic system was studied by fluorescent lymphangiography, in an experimental model of rats exposed to chemokine ligand 4 (CCL4). A substantial decrease in fluorescence-labeled lymphatics was observed in cirrhotic rats, in both peripheral and splanchnic regions, indicating a deficiency in lymphatic drainage[9].

## PATHOPHYSIOLOGY OF LYMPHATIC DYSFUNCTION IN CIRRHOSIS

The pathophysiological mechanism behind lymphatic dysfunction in cirrhosis is an area yet to be explored at cellular and molecular level (Figure 2). In a study on cirrhotic rats with ascites, Ribera *et al*[9] found that an impaired lymphatic drainage in the splanchnic and peripheral regions was accompanied by increased activity of endothelial nitric oxide synthase (eNOS) and production of NO by LECs. In addition, SMC coverage of lymphatic vessels was found to be significantly decreased. Interestingly, when cirrhotic rats were treated with inhibitor of eNOS activity (L-NG-methyl-L-arginine, L-NMMA), a significant improvement of lymphatic drainage, reduction in ascetic fluid volume, and an increase in lymphatic smooth muscles were seen. Therefore, this study demonstrated a role of NO in the lymphatic dysfunction of cirrhotic rats. Whether the same applies for human cirrhosis remains to be seen. Lymphangiogenesis observed in cirrhosis appears to be due to increased expression of several induces of lymphogenesis, such as VEGF-D and VEGF-C. Their levels have been found to be significantly elevated during hepatic fibrosis and positively correlated with fibrosis progression[8,41]. Study on cirrhotic rat has found a four-fold increase in VEGF-D, in the endothelial cells. Additionally, the receptor of this VEGF (VEGR-3) was found to be overexpressed in the LECs of cirrhotic rats[42]. It has recently been shown that autonomic nervous system is a key modulator of the lymphatic vessels' function[43].

Lymphatic function, in general and in patients with cirrhosis, can be modulated by numerous factors including age, obesity, diabetes, dyslipidemia, neurohormonal alterations, and chronic inflammation. Neurohormonal changes are known to occur in advanced cirrhosis, and the levels of a number of vasoactive substances such as noradrenaline, histamine, substance P, prostaglandins, and endothelin are altered, which can affect contractility of lymphatic vessels[44-46]. Intestinal motility plays an important role in the propulsive motion of intestinal lymph, and by inducing VEGF-C, intestinal microbiota is an important regulator of intestinal lacteal integrity[13,47]. Therefore, the intestinal dysmotility and intestinal dysbiosis that are frequently seen in advanced cirrhosis may interfere with intestinal lymphatic function. Moreover, Cirrhosis and portal hypertension (PHT) is known to create a state of low-grade chronic inflammation[48]. Furthermore, gut dysbiosis, bacterial translocation, and release of Inflammatory cytokines such as tumor necrosis factor alpha, and

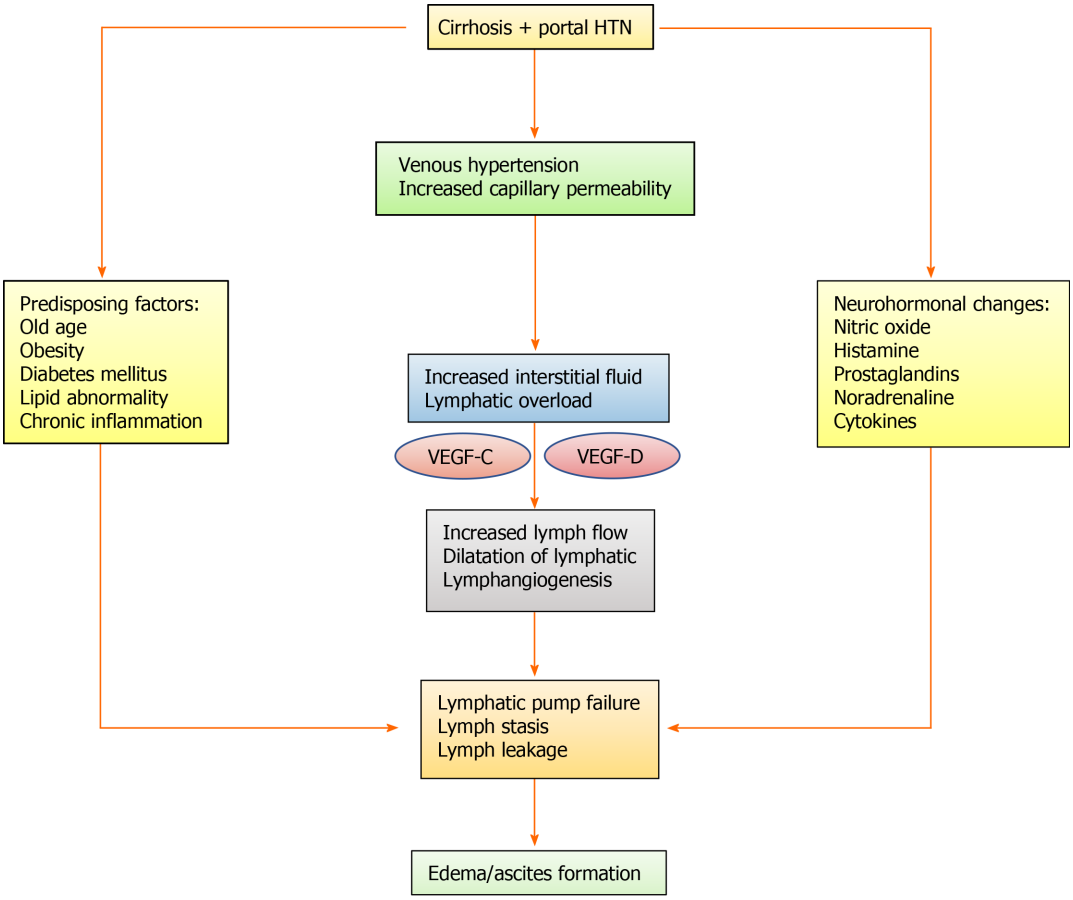


**Figure 2 Flow diagram showing the possible pathophysiological mechanism behind lymphatic abnormalities in cirrhosis patients leading to fluid imbalance.** The exact pathophysiological mechanism, at cellular and molecular level, is poorly understood in human cirrhosis. Some of the information has been derived from the experimental study on animal. VEGF: Vascular endothelial growth factor; HTN: Hypertension.

interleukin-1β occur in cirrhosis[49]. Consequently, chronic inflammation and neurohormonal disturbances, in advanced cirrhosis, can lead to structural and physiologic changes in the lymphatic system. Dysfunctional lymphatics, with lymph stasis, can impair lipid transport and stimulate adipogenesis in the affected area[50,51].

Old age and obesity also affect lymphatic functions. Aging induces structural changes in the lymphatic vessels, such as loss of extracellular matrix, reduced contractile protein expression, and changes in eNOS and histamine gradients, which tend to decrease the lymphatic transport of interstitial fluids[52,53]. Obesity results in several structural and physiological changes in the lymphatic system, including increased lymphatic leakiness, decreased contractility of the collecting vessel, and changes in the architecture of the lymph node, which significantly affect lymphatic transport functions[54,55]. Notably, most cirrhosis patients belong to the old age group, and obesity is presently a growing cause of non-alcoholic fatty liver disease (NAFLD)-related cirrhosis. Given that obesity is a growing cause of NAFLD-related cirrhosis and that most patients with cirrhosis are older, they may be at a higher risk of developing lymphatic dysfunction.

## CLINICAL IMPLICATIONS OF LYMPHATIC DYSFUNCTION

Lymphatic dysfunctions have been aptly described in patients with cirrhosis; however, little has been described about the clinical consequences of such dysfunctions. Given the role of lymphatic vasculature in the body fluid homeostasis, adaptive immunity, and the transport of lipid and waste materials, it is tempting to speculate that lymphatic dysfunctions, in cirrhosis, may have several clinical implications, particularly with regard to the body fluid homeostasis.

### Edema and ascites

In advanced cirrhosis, the activation of compensatory vasoconstrictor pathways compromises glomerular filtration, causing greater renal retention of sodium and water. This further increases the production of lymph, burdening the already inefficient lymphatic system with the responsibility for drainage. Moreover, inability of the lymphatic system to recirculate extravasated albumin may worsen pre-existing hypoalbuminemia, leading to a change in the transcapillary oncotic pressure gradient and worsening of fluid imbalance. Additionally, serum albumin is also required for furosemide to work properly[56]. Therefore, severe lymphatic dysfunction can lead to the development of refractory edema and ascites in patients with cirrhosis.

Lymphedema should be fairly common in patients with advanced cirrhosis for obvious reasons; however, its description is lacking in existing literature. Lymphedema is deposition of protein-rich lymph fluid within the tissues, as a consequence of lymphatic leak and an imbalance between the rate of lymph production and drainage. Recent evidences suggest that lymphedema can also occur as an immune response secondary to lymphatic injury or metabolic derangements, including adiposity and infection[57]. Furthermore, fat deposition is present in lymphedema due to failure of lipid transport and stimulation of adipogenesis[50,51]. Clinically, a diagnosis of lymphedema can be made by physical characteristics, including pitting edema, peau-d'orange appearance, and a positive Stemmer sign. Patients with lymphedema are often susceptible to various skin infections, such as cellulitis.

### Intestinal lymphangiectasia

An increase in lymphatic pressure secondary to PHT may lead to dilatation of the intestinal lymphatics, known as intestinal lymphangiectasia[58]. A sustained rise in lymph pressure leads to the rupture of lymphangiectasia and lymph leakage into the lumen of the intestines, with many clinical consequences (Figure 3). As intestinal lymph contains many proteins, lipoproteins, and lymphocytes, its loss would result in hypoproteinemia, hypoalbuminemia, lymphocytopenia, and hypogammaglobulinemia[59,60]. Hence, in patients with advanced cirrhosis, lymphangiectasia can lead to worsening of ascites, by causing severe hypoalbuminemia. The disruption of lymphatic flow, in lymphangiectasia, leads to malabsorption of fats and fat-soluble vitamins (vitamins A, D, E, and K), which may cause steatorrhea, vision problems, muscles weakness, osteopenia, and coagulopathy in cirrhosis patients. In addition, loss of lymphocytes may contribute to an increased susceptibility to infection in cirrhosis[60].

### Chylous ascites

Chylous ascites (CA) results from the leakage of lipid-containing lymph (chyle) into the peritoneal cavity[61]. Elevated lymphatic pressure secondary to PHT can rarely cause rupture of dilated subserosal intestinal lymphatics, leading to the formation of CA[39]. Intestinal lymph, which constitutes 50%-75% of intra-abdominal lymph, contains fat droplets rich in triglyceride and appears to be milky in color. CA is found in 0.5%-1% of patients with cirrhosis, and cirrhosis is responsible for 11% of cases of atraumatic CA[62,63]. In patients with cirrhosis, CA may also develop due to complications of shunt surgery, sclerotherapy-related thoracic duct injury, or hepatocellular carcinoma[62,64]. A diagnosis of CA is made when triglyceride concentration of fluid is ≥ 110 mg/dL. It is to be noted that a rupture of hepatic lymph, which drains 25%-50% of abdominal lymph, does not produce CA, as hepatic lymph is devoid of fat droplets.

### Other clinical implications

Patients with lymphatic dysfunction often exhibit impaired immune function predisposing them to a variety of infections[65,66]. Recurrent cellulitis/erysipelas and interdigital fungal infections are common in presence of lymphedema. The lymphatic vasculature is preferential route for the spread of cancer cells. Therefore, lymphangiogenesis can promote tumor metastasis if patients with cirrhosis have hepatocellular carcinoma[67]. Moreover, lymphatic dysfunction may interfere with the removal of inorganic material, dying cells, and mutant cells from the body, but such adverse effects are unknown in patients with cirrhosis. Furthermore, lymphatic dysfunction can affect oral bioavailability of lipophilic drugs, which require functional intestinal lacteals for absorption.

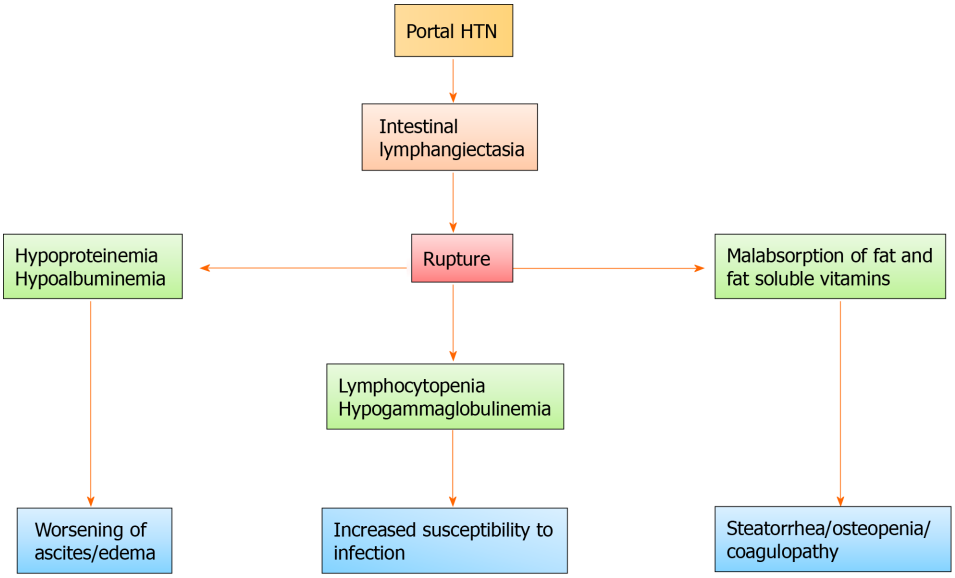


**Figure 3 Flow diagram showing clinical consequences arising from the rupture of intestinal lymphangiectasia.** HTN: Hypertension.

## ASSESSMENT OF LYMPHATIC DYSFUNCTIONS IN CIRRHOSIS

No recommendation exists with regard to the diagnosis and assessment of lymphatic dysfunction in patients with cirrhosis. Table 1 provides a rational overview of the assessment of lymphatic dysfunction in cirrhosis patients. Techniques to evaluate the lymphatic system radiologically are still evolving[68]. There are various imaging techniques available, such as X-ray or magnetic resonance lymphography, lymphoscintigraphy, and duplex ultrasonography. The gold standard that offers insight into the lymphatic anatomy as well as lymph flow dynamics is lymphangioscintigraphy. However, these imaging modalities are often limited by sub-optimal resolution, lack of standardization, invasiveness, risk of radiation exposure, and low availability[69]. Therefore, as of now, no recommendation can be made with respect to the use of a radiological technique for assessment of lymphatic dysfunction in patients with cirrhosis.

Lymphatic dysfunction, especially in elderly cirrhosis with diabetes and dyslipidemia, should be considered when there is severe generalized edema, sctrotopenile swelling, diuretic-resistant ascites, and peripheral lymphedema. On blood investigation, the presence of disproportionate hypoproteinaemia, combined with severe lymphocytopenia, may also suggest lymphatic dysfunction. Intestinal lymphangiectasia is an endoscopic manifestation of lymphatic abnormality in cirrhosis. It is characterized by swollen mucosa with scattered white spots, white villi, and chyle-like substances covering the mucosa (Figure 4). This must be confirmed *via* histopathological examination, which should reveal dilated intestinal lacteals in the lamina propria region of the intestinal villi. Morphologically, it is often difficult to distinguish lymphatic vessels from blood vessels. Therefore, use of specific lymphatic endothelium markers may be necessary for accurate identification of lymphatic vessels on pathological specimens[25,70]. These markers include LYVE-1 (lymphatic vessel endothelial hyaluronan receptor), Prox-1 (a transcription factor), and podoplanin or D2-40 (lymphatic vessel endothelial hyaluronic acid receptor-1). However, even these markers may not be exclusive to lymphatic vessels. Mouta Carreira *et al*[25] found that LYVE-1 is also present in Kupffer cells and normal LSECs. Therefore, a combination of lymphatic markers should be used for accurate identification. Finally, presence of CA, as evident by milky appearance of ascitic fluid with triglyceride levels > 110 mg/dL, indicates lymphatic abnormality related to cirrhosis, after exclusion of alternative causes such as malignancy, tuberculosis, post-operative or post-radiation status, and cardiac diseases.

## THERAPEUTIC PERSPECTIVE

From a pathophysiological point of view, a number of therapeutic options are available for lymphatic dysfunctions, but no adequate evidence is available for the use

**Table 1 Assessment of risk factors, clinical markers and investigations for lymphatic dysfunction in cirrhosis**

| Parameters | Findings that support or indicate lymphatic dysfunction |
|---|---|
| Risk factors | (1) Old age; (2) metabolic syndrome (obesity, diabetes, dyslipidemia); and (3) concomitant inflammatory disorders |
| Clinical examination | (1) Diuretic-resistant ascites; (2) severe generalised edema, scrotal/penile swelling; (3) lymphedema: Peau-d'orange appearance and a positive stemmer sign; (4) frequent cellulitis/lymphangitis of affected limbs; and (5) hyperkeratotic skin lesions, yellow nail |
| Blood investigations | (1) Hypoproteinaemia and hypoalbuminemia; (2) lymphocytopenia; and (3) hypogammaglobulinemia |
| Ascitic fluid analysis | Chylous ascites: Milky appearance, fluid triglyceride level ≥ 110mg/dL |
| Upper endoscopy | Intestinal lymphangiectasia: Whitish congested villi in duodenum |
| Radiological imaging: (lymphography, lymphoscintigraphy) | Abnormal lymphatic structure and/or lymph flow dynamics: Dilated lymphatic vessels, lymph stasis, lymph leakage |
| Histopathological examination (liver/intestine) | (1) Increase in number and size of lymphatic structures; and (2) specific lymphatic endothelial markers for accurate identification: Prox-1, podoplanin, LYVE-1 |

LYVE-1: Lymphatic vessel endothelial hyaluronan receptor.

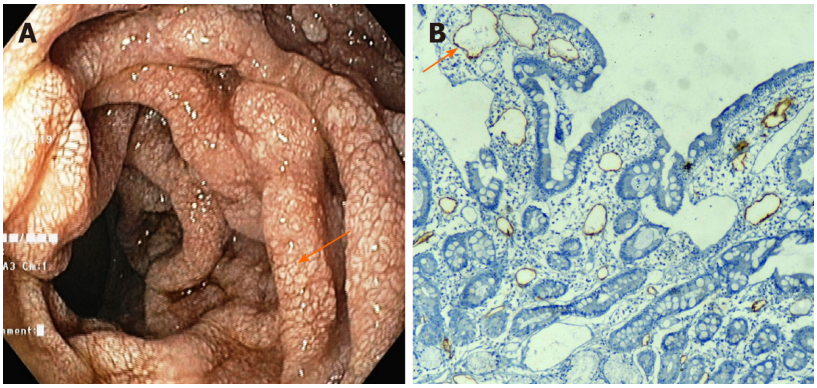


**Figure 4 Intestinal lymphangiectasia in a patient with cirrhosis.** A: Upper gastrointestinal endoscopy of a patient showing whitish swollen villi in the duodenum, suggestive of intestinal lymphangiectasia; B: On immunohistochemistry (× 10), markedly dilated vessels were seen in the lamina which showed strong D2-40 positivity indicating dilated lymphatics.

of several of them in patients with cirrhosis (Table 2). The mobilization of fluid is particularly difficult in cirrhosis patients with lymphatic dysfunction. An effort should be made to minimize capillary filtration into the interstitial space. Local skincare and compression therapy remains the cornerstone for lymphedema affecting limbs. Common infections, such as cellulitis, should be vigorously treated, as they can deteriorate lymphedema very rapidly. Limb elevation may facilitate lymphatic drainage and prevent the transfer of tissue fluid to an affected limb due to gravity. Pressure effect of compression therapy with elastic stockings/gloves or bandages may help to minimize capillary leakage, reduce lymph regurgitation, and avoid the movement of fluid related to gravity[71]. However, compression therapy should be avoided when cellulitis, venous thrombosis, and congestive heart failure are present. Obesity and salt consumption may worsen lymphedema; therefore, salt and calorie diet should be restricted. Role of conventional diuretic therapy in lymphatic edema, per se, is limited; however, it may be beneficial in mixed-origin edema which occurs in cirrhosis patients. In addition, diuretics may also render lymphedema worse by removing fluid and increasing lymph protein concentration, resulting in a reversed gradient of oncotic pressure and increased vulnerability to infection. The role of newer molecules with diuretic activity, such as V2-receptor antagonist and sodium-glucose cotransporter 2 (SGLT2) inhibitors, needs to be explored in cirrhosis patients with lymphatic dysfunction. Tolvaptan is an oral selective V2-receptor antagonist and a novel water diuretic. Unlike loop diuretics, tolvaptan has a different effect on fluid distribution, and it can ameliorate fluid retention with a low risk of a worsening renal function[72,73]. SGLT2 inhibitors are the new class of antihyperglycemic agents with a good safety profile in cirrhosis patients. SGLT2 inhibitors have been shown to have

**Table 2 Possible therapeutic strategies for treatment of lymphatic dysfunction in cirrhosis**

| | |
|---|---|
| **To decrease formation of lymph** | |
| Decrease water retention | Low salt diet, diuretic therapy |
| Control of portal hypertension | Beta-blocker, octreotide, transjugular intrahepatic portosystemic shunt |
| Increase interstitial pressure | Compression therapy |
| **To promote lymphatic drainage** | |
| Facilitate fluid movement into the lymphatic vessels | Compression therapy, limb elevation, diuretic therapy (limited role) |
| Increase contractility of the lymphatic vessels | Nor-adrenaline, phenylephrine, nitric oxide-inhibitors (experimental) |
| Facilitate lysis of interstitial protein | Benzopyrones (coumarin and flavoids) |
| Promote lymphangiogenesis | Prostaglandins E2 (experimental), vascular endothelial growth factor-C (experimental) |
| **To control aggravating factors for lymphatic dysfunction** | |
| Care of lymphedema | Control of infection (aggressive use of antibiotics), avoidance of trauma, hot bath and other heat-producing treatment |
| Control risk factors | Control of diabetes, dyslipidemia and obesity |
| **To decrease leakage of lymph** | |
| Decrease stimulants of intestinal lymph flow | Low fat diet, octreotide |
| Decrease leakage of lymph by intervention | Compression therapy, antiplasmin (tranexamic acid); radiological intervention to obliterate the site of leak |
| **To correct underlying condition** | |
| Definitive therapy of cirrhosis | Liver transplantation |

significant diuretic effects and, interestingly, without altering the intravascular volume, they can induce interstitial fluid clearance[74]. In addition to inducing glycosuria and natriuresis, these agents have beneficial effects on neurohormonal regulation and hepatorenal fibrosis[75]. Given that DM is also a risk factor for lymphatic dysfunction, SGLT2 inhibitors may be potentially helpful in diabetic patients with cirrhosis, with lymphatic dysfunction.

The contractile function of lymphatic vessels is very important for the reabsorption of extravascular fluid. While lymphatic vessels can modulate their contractile function in response to various neural, hormonal endothelial and humoral factors, no specific therapeutic agent has been approved for this purpose. In an animal study, intravenous adrenaline infusion has been found to increase the frequency of lymphatic contraction and lymph flow in efferent lymphatic vessels[76]. In an experimental study, significant improvements were observed in lymphatic vessels' contractility and lymphatic drainage, when treated with an eNOS inhibitor[9]. Inhibition of eNOS can, therefore, be a useful therapeutic target for lymphatic dysfunction in cirrhosis. However, any attempt to inhibit NO must take into account the fact that inhibition of intrahepatic NO may increase intrahepatic pressure, so that the resulting increased lymph production may negate its impact on improving the drainage of the lymph. As a result, to target only eNOS of extra-hepatic lymphatic vessels, a tissue-specific delivery strategy is required. Benzopyrones (flavonoids and coumarin) have been found to be effective in lymphatic edema treatment[77]. These drugs facilitate removal of accumulated interstitial proteins, by binding and causing phago-proteolysis by macrophages. However, there are some concerns regarding coumarin hepatoxicity, and there is a lack of evidence on the use of this medication in cirrhosis.

Low fat diets are currently recommended for the treatment of intestinal lymphangiectasia, as intestinal lymph flow is highly affected by oral fat intake[77]. For fat nutrition, medium-chain triglycerides supplementation should be used as they are directly absorbed through the portal venous system, without involvement of intestinal lacteal. Additionally, octreotide has been found helpful in patients with intestinal lymphangiectasia, by reducing splanchnic blood flow and the leakage of intestinal lymph[78]. Moreover, tranexamic acid has been found to cause significant reduction in protein loss in patients with intestinal lymphangiectasia, possibly due to the inhibition of tissue fibrinolytic activity that decreases the capillary permeability to protein[79]. Finally, transjugular intrahepatic porto-systemic shunt and liver transplantation have

been found to be effective therapy of PHT-induced protein-losing enteropathy, possibly caused by intestinal lymphangiectasia[80,81]. Regarding CA, a number of treatment options have been identified, including low-fat diet, medium-chain triglyceride, octreotide, total parenteral nutrition, embolization of leaking lymph vessel by radiological intervention, and surgical peritoneovenous shunt[39,82]. Nevertheless, there are no research reports comparing either of these treatment modalities. Initially, these patients should be managed with conservative approaches, and when they fail, repeated paracentesis should be used for symptomatic relief, and further invasive therapies may be considered.

It has been found that splenectomy effectively decreases portal pressure and corrects hypersplenism in patients with cirrhosis[83,84]. Since the progression of cirrhosis may result in a parallel increase in portal pressure, it would be worth investigating whether a reduction in portal pressure, after splenectomy, contributes to decreased lymph formation and decreased overload of the lymphatic system. However, in patients with advanced decompensated cirrhosis, where lymphatic dysfunction is maximal, splenectomy may not always be feasible[84]. Furthermore, caution is needed while contemplating albumin therapy in cirrhotic patients with lymphatic dysfunction. Henriksen *et al*[85] have recently found that in patients suffering from advanced cirrhosis, with diuretic-resistant ascites, the transport rate of albumin from plasma into the peritoneal cavity is highly elevated and exceeds the back transport rate of albumin into the plasma. Patients with advanced cirrhosis have accelerated trans-capillary escape rate of albumin, due to greater hydrostatic pressure and capillary permeability[86]. Hence, the molecules of albumin are more likely to extravasate rapidly into the interstitium. To recirculate the escaped albumin back to plasma, proper lymphatic functions are needed. However, in patients with advanced cirrhosis, the escaped albumin is less likely to be recirculated back into the plasma, due to deficient lymphatic function. This would not only fail to correct circulating hypovolemia, the reason for which it is given, but accumulation of albumin in the interstitium would facilitate development of reversed oncotic pressure gradient and extravascular movement of fluid, leading to worsening of edema and ascites[87]. Albumin, however, also has anti-inflammatory, immunomodulatory, and anti-oxidant properties[88]. It would be interesting to investigate these non-oncotic properties of albumin on lymphatic functions, as chronic inflammation and neurohormonal alterations play a significant role in lymphatic dysfunction of cirrhosis.

## CONCLUSION

In conclusion, a greater understanding of the lymphatic vascular system has emerged over the last two decades, following the discovery of specific lymphatic endothelial markers and technical advances in lymphatic imaging. However, the role of lymphatic dysfunctions in the pathophysiology of advanced cirrhosis is still poorly understood. Given the major role of the lymphatic system in body fluid homeostasis, immunity, and metabolism, it is plausible to understand that in patients with cirrhosis, a defective lymphatic system may have several clinical consequences. This field is, therefore, largely open to new research. A better understanding of lymphatic pathophysiology in cirrhosis will significantly enhance our ability to manage such patients and design targeted therapy.

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ORIGINAL ARTICLE

Basic Study

# Papaya improves non-alcoholic fatty liver disease in obese rats by attenuating oxidative stress, inflammation and lipogenic gene expression

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**Author contributions:** Deenin W performed the experiments, analysed and interpreted the data and wrote the manuscript; Malakul W, Boonsong T and Phoungpetchara I conceptually designed the study, interpreted the data and proofed the manuscript; Tunsophon S designed and supervised the study, interpreted the data, discussed the results and wrote and revised the manuscript; all authors read and approved the final manuscript.

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## Abstract

***BACKGROUND***

Non-alcoholic fatty liver disease (NAFLD) is a global health issue that is correlated with obesity and oxidative stress.

***AIM***

To evaluate the anti-NAFLD effect of papaya in high fat diet induced obesity in rats.

***METHODS***

Four-week-old male Sprague-Dawley rats were divided into four groups after 1 wk of acclimatization: Group 1 was the rats fed a normal diet (C); group 2 was the rats fed a high fat diet (HFD); group 3 was the rats fed a HFD with 0.5 mL of papaya juice/100 g body weight (HFL), and group 4 was the rats fed a HFD with 1 mL of papaya juice/100 g body weight (HFH) for 12 wk. At the end of the treatment, blood and tissue samples were collected for biochemical analyses and histological assessment.

***RESULTS***

The results of the HFH group showed significantly reduced body weight (HFH *vs*

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HFD, $P < 0.01$), decreased NAFLD score (HFH *vs* HFD, $P < 0.05$), and reduced hepatic total cholesterol (HFL *vs* HFD, $P < 0.01$; HFH *vs* HFD, $P < 0.001$), hepatic triglyceride (HFH *vs* HFD, $P < 0.05$), malondialdehyde (HFL, HFH *vs* HFD, $P < 0.001$), tumour necrosis factor-α (HFH *vs* HFD, $P < 0.05$) and interleukin-6 (HFH *vs* HFD, $P < 0.05$) when compared to the HFD group. However, the liver weight showed no significant difference among the groups. The activities of catalase and superoxide dismutase significantly increased in HFH when compared with the HFD group ($P < 0.05$ and $P < 0.001$, respectively). The suppression of transcriptional factors of hepatic lipogenesis, including sterol regulatory element-binding protein 1c and fatty acid synthase, were observed in the papaya treated group (HFH *vs* HFD, $P < 0.05$). These beneficial effects of papaya against HFD-induced NAFLD are through lowering hepatic lipid accumulation, suppressing the lipogenic pathway, improving the balance of antioxidant status, and lowering systemic inflammation.

*CONCLUSION*

These current results provide experimental-based evidence suggesting papaya is an efficacious medicinal fruit for use in the prevention or treatment of NAFLD.

**Key Words:** High fat diet; Lipogenic gene expression; Non-alcoholic fatty liver disease; Obesity; Oxidative stress; Papaya



**Core Tip:** High fat diet consumption causes non-alcoholic fatty liver disease (NAFLD). This is one of the major liver diseases found worldwide. Liver fat accumulation leads to dysfunction of liver due to oxidative stress and inflammation. Papaya is an important export fruit from Asian and Latin America. It is a nutrient rich fruit with many medicinal properties. Our present study clearly demonstrated that the hepatoprotective mechanism of papaya against NAFLD was a result of the association of the hypolipidemic, anti-inflammatory, and antioxidant activities. This study provides evidence for the beneficial effects of papaya to reverse the progression of NAFLD in obese rats.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is of growing concern since its prevalence is increasing worldwide[1]. NAFLD is characterised by an accumulation of triglycerides and fatty acids in hepatocytes. The circulating pool of free fatty acids (FFAs) is increased in obese individuals and accounts for the majority of lipid accumulation in NAFLD. Excessive consumption of diets rich in fat is related to oxidative stress in various tissues including vessels, adipose tissues and liver and consequent to disease development[2]. Normally, oxidative stress such as reactive oxygen species (ROS) and reactive nitrogen species are continuously generated from inside the cells (*e.g.*, electron transfer, cellular metabolism), but there is the counterbalance by the antioxidant system to defend the body from cellular or tissue damage[3]. In NAFLD, an imbalance of oxidant synthesis and antioxidants is the major contributor to the pathogenesis of the disease, leading to liver injury and hepatocyte deterioration[4]. Antioxidants have been suggested to be beneficial for health promotion and disease prevention. Therefore, we hypothesised that fruit rich in antioxidants may have potential benefit against NAFLD.

*Carica papaya* known as pawpaw or papaya is in the family of Caricaceae[5]. It is widely cultivated in many regions of the world, including Central and South America,

Asia, and Africa, and its principal markets for consumption are the United States and Europe[6]. Papaya is a nutraceutical plant with many medicinal properties. Some studies have reported its health benefits including the treatment of gastrointestinal related disorders, diabetes, hypertension, hypercholesterolemia and hepatotoxicity, and its anti-microbial, anti-parasitic, and anti-viral properties[7,8]. Almost all parts of papaya can be used, especially the fruit of *C. papaya*. It is a nutritional source that is high in fibre, minerals and strong antioxidants including vitamin A, C and E. However, its health benefits in NAFLD are still the subject of research.

The purpose of this study was to evaluate the effect of papaya juice in the treatment of NAFLD. The doses of papaya juice used in this study can be practically applied to human use. Since papaya is low cost, easily available and widely marketed worldwide, the results from this study could be implemented in nutritional intervention that may be used in the prevention and treatment of NAFLD.

## MATERIALS AND METHODS

### Plant material and preparation of papaya

The Holland variety of papaya fruit (*Carica papaya* L.) was derived from a supermarket in Phitsanulok, Thailand. The fruit was harvested at a ripe stage, when papaya presents yellow areas on 50%-75% of the skin[9]. The juice was freshly prepared by extraction from the homogenised flesh of the Holland cultivar and separated from the pulp by squeezing it several times. The juice was then centrifuged at 1500 × *g* for 20 min. The papaya composition as shown in Table 1 was analysed by Food and Nutrition Laboratory, Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand.

### Animals and experimental design

The NAFLD animal model was developed as described previously[10]. Four-week-old male Sprague-Dawley rats weighing between 100 and 120 g were purchased from the National Laboratory Animal Centre at Salaya campus, Mahidol University (Nakon Pratom, Thailand). All animal experiments were carried out after getting approval from the Animal Ethics Committee at the Centre for Animal Research at Naresuan University, Phitsanulok, Thailand (Approval number NU-AE 580714). All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals (8$^{th}$ edition, National Academies Press)[11]. The animals were acclimatised for 1 wk and then randomised into four groups ($n$ = 6-7). Group 1 was the control rats fed a commercial normal diet for 8 wk (C), while the three remaining groups (2-4) were fed a high fat diet (HFD) for 8 wk and oral gavage for 1 mo as follows; Group 1 was fed a normal diet for 8 wk and then treated with distilled water for an additional 4 wk, animals were maintained on a normal diet. After the first 8 wk period on HFD, animals in group 2 were fed a HFD for 4 wk, while those of groups 3 and 4 were kept on HFD and received 0.5 mL and 1 mL/100 g body weight/day of papaya juice, respectively.

The doses of papaya used in 0.5 mL of papaya juice/100 g body weight (HFL) and 1 mL of papaya juice/100 g body weight (HFH) were the equivalent of approximately 125 and 250 g of papaya consumed by a person, respectively. Diet composition of control and high fat diets were formulated according to AIN-93G as previously described with a slight modification[12]. Briefly, the high fat diets were composed of 1.5% cholesterol, 20% palm oil and 0.25% cholic acid. Body weights of rats were recorded weekly. At the end of the 12$^{th}$ week, the animals were euthanised by pentobarbital injection. The blood was drawn through cardiac puncture. Blood and tissue samples were collected and kept at -80 °C for further analysis.

### Biochemical analyses

The serum was used to measure aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) by Bio Lab Medical Centre (Phitsanulok, Thailand).

### Analysis of hepatic TAG and cholesterol content

Hepatic lipid was extracted according to a modified Folch method, as previously described[13]. Briefly, lipids were extracted from 0.5 g of liver with a mixture of chloroform/methanol (2:1, v/v) and dried under $N_2$. The pellets were dissolved and used for the analysis of hepatic lipid contents. The hepatic contents of triglyceride and

**Table 1 Composition of papaya**

| Nutrients | Value |
|---|---|
| Energy (kcal) | 26.62 ± 0.26 |
| Moisture (g) | 92.86 ± 0.06 |
| Protein (g) | 0.65 ± 0.02 |
| Total fat (g) | 0.00 ± 0.00 |
| Total carbohydrate (g) | 6.01 ± 0.09 |
| Soluble dietary fibre (g) | 0.87 ± 0.03 |
| Ash (g) | 0.49 ± 0.01 |
| Total sugar (g) | 3.97 ± 0.02 |
| Calcium (mg) | 25.95 ± 0.98 |
| Potassium (mg) | 14.50 ± 0.28 |
| Iron (mg) | 0.56 ± 0.01 |
| Total phenolic compounds (mg gallic acid/g papaya) | 0.56 ± 0.01 |
| Carotenoid profile: | |
| Beta-cryptoxanthin (μg) | 596.04 ± 15.27 |
| Lycopene (μg) | 1166.88 ± 11.24 |
| Beta-carotene (μg) | 78.96 ± 1.45 |

total cholesterol were determined using a colorimetric assay kit according to the instructions of the manufacturer (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany).

### Histopathological analyses

To analyse the histopathology of the liver, the tissue was fixed immediately after removal in 10% formalin. The liver tissue was then embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The histopathological features were scored for the liver lesions using NAFLD activity score (NAS) according to Xu *et al*[14]. NAS component represents the sum of score ranging from 0-8 for three histological features: Hepatocyte ballooning (0-2), lobular inflammation (0-3) and steatosis (0-3). The total NAS score of 0-3 was defined as not nonalcoholic steatohepatitis (NASH). The score greater than 5-8 was considered as NASH. The hepatic lipid accumulation assessment was modified from Malakul *et al*[12]. In brief, the frozen liver samples with optimal cutting temperature-embedded were cryosectioned at 5 μm with a cryostat, fixed in 4% v/v formalin for 10 min and then stained with Oil Red O working solution for triglycerides and free fatty acid staining.

### Hepatic lipid peroxidation

The isolated rat livers were homogenised in phosphate buffered saline (PBS), and the total protein content of liver tissues was measured using a Bradford assay kit (Sigma-Aldrich, St. Louis, MO, United States). The lipid peroxidation of the hepatic tissue homogenate was determined by a thiobarbituric acid assay. The solutions were prepared according to Liu *et al*[15]. Briefly, the mixture of 15% trichloroacetic acid, 0.25 N HCl (Sigma-Aldrich) and 0.37% 2-thiobarbitulic acid (POCH, Sowinskiego, Poland) with 1:1:1 ratio was prepared. Then 200 μL of these reagents were added in each eppendorf tube and incubated in heat block at 95 °C for 15 min. The solutions were centrifuged at 3500 × *g* for 25 min and the supernatant in each tube was pipetted to 96 well plates. The samples were then measured at absorbance 535 nm with malondialdehyde as a standard, and the unit was expressed as μmoL/mg protein.

### Catalase and superoxide dismutase activities

The livers were homogenised in ice cold PBS. The homogenate was centrifuged, and the supernatant were taken to measure the activities of catalase (CAT) and superoxide dismutase (SOD) by using commercial assay kits (Cayman Chemical Company, Ann Arbor, MI, United States). The final units for enzyme activities were normalised with

protein concentration.

### Determination of biomarkers of inflammation

The liver homogenates were used to determine the levels of tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) by using commercial assay kits (Sigma-Aldrich). The final units for TNF-α and IL-6 were normalised with protein concentration.

### Analysis of gene expression

Total ribonucleic acid (RNA) of the liver was isolated using RiboZol (Amresco, Dallas, TX, United States) according to the protocol provided by the manufacturer. The complementary deoxyribonucleic acid synthesis was performed in a reaction mixture containing 4 μL of reaction buffer, 2 μL of deoxyribonucleotide triphosphate, 1 μL of random primer, 1 μL of RNAse inhibitor, 1 μL of reverse transcriptase and 500 ng of total RNA. Polymerase chain reaction (PCR) was performed with PCR thermocycling. The PCR products were measured by agarose gel electrophoresis technique with 2% agarose gel and 1 × TBE running buffer (1M Tris, 0.9M boric acid and 1 mmoL/L EDTA). Deoxyribonucleic acid was stained with a fluorescent colour (Biotechnology, Daejeon, Korea). Each sample was assayed in triplicate, and β-actin was amplified in parallel to serve as an internal control for reverse transcription-PCR quantification. All mRNA gene expression data were normalised to the expression level of β-actin.

The sequences of the primers for genes used in this study were indicated as follows; *SREBP-1c*: forward 5′-TGGATTGCACATTTGAAGACAT-3′, reverse 5′-GCTCCTCTTTGATTCCAGGC-3′; *ACC:* forward 5′-GCCTCTTCCTGACAAACGAG-3′, reverse 5′-TCCATACGCCTGAAACATGA-3′; *FAS*: forward 5′-GGACAT GGTCACAGACGATGAC-3′, reverse 5′-GTCGAACTTGGACAGATCCTTCA-3′. *ACTB*: forward5′-TGTCCACCTTCCAGCAGATGT-3′, reverse 5′-AGCTCAGTAACAGTCGA -3′.

### Statistical analysis

Results are presented as the mean ± SE of the mean. Statistical analyses were performed using IBM SPSS version 23 (Armonk, NY, United States). Group difference was assessed by a one-way analysis of variance, followed by Tukey's test for multiple comparisons. A $P$ value < 0.05 was considered statistically significant.

## RESULTS

### Effects of papaya on liver weight, lipid contents and serum components in rats

The initial body weight and body weight at week 8 of all the experimental groups were not significantly different. However, at the end of treatment the HFD group showed significantly increased body weight when compared with the C group, while those parameters decreased in the HFH group. The result also showed that papaya improved hepatic lipid contents in HFD-fed rats. The HFD group showed significantly increased hepatic triglycerides (TG) and cholesterol levels when compared with the C. The TG levels were significantly decreased in the HFH ($P$ < 0.05), while total cholesterol (TC) was significantly decreased in both the HFL ($P$ < 0.01) and HFH ($P$ < 0.001) when compared with the HFD group. This result indicated that papaya markedly reduced the hepatic TG and TC contents. The serum levels of AST, ALT and ALP were significantly increased in rats fed a HFD. Higher levels of those enzymes suggest that a HFD can induce liver inflammation or liver damage. Moreover, the liver damage indices also significantly decreased in the papaya treated group when compared to the HFD group (Table 2). This result suggests that papaya administration may improve liver injury found in NAFLD.

### Effects of papaya on lipid accumulation

Oil Red O staining showed that hepatic lipid accumulation of HFD was significantly higher than that in the C group. The oral administration of papaya to HFD rats reduced steatosis and lipid droplet size as shown in Figure 1A. In addition, it showed that the liver samples from the HFD group showed significant fat deposition with the highest scores in steatosis, lobular inflammation and hepatocyte ballooning. The HFD group scores were significantly higher than those of the control group ($P$ < 0.001), which strongly indicated the development of NAFLD. Interestingly, the significant reduction of steatosis, lobular inflammation and hepatocyte ballooning was observed after 4 wk of treatment with papaya (Figure 1B).

**Table 2 Effects of papaya on body weight, liver weight, hepatic lipid contents and liver damage indices in high fat diet induced obesity in rats**

| | C | HFD | HFL | HFH |
|---|---|---|---|---|
| Initial weight (g) | 218.0 ± 12.18 | 236.5 ± 13.58 | 215.5 ± 14.01 | 221.8 ± 14.29 |
| Body weight at week 8 (g) | 398.6 ± 9.462 | 457.6 ± 18.93[a] | 462.9 ± 17.11[a] | 454.9 ± 7.584[a] |
| Body weight at week 12 (g) | 465.83 ± 11.13 | 536 ± 33.24[c] | 509.33 ± 33.57[a] | 471.33 ± 15.04[e] |
| Liver weight (% of body weight) | 2.66 ± 0.13 | 4.46 ± 0.3[c] | 4.49 ± 0.51[c] | 4.33 ± 0.26[c] |
| Hepatic triglycerides (mg/dL) | 140.60 ± 13.95 | 211.00 ± 26.25[a] | 172.50 ± 7.89 | 152.20 ± 12.68[d] |
| Hepatic cholesterol (mg/dL) | 74.30 ± 5.58 | 152.60 ± 9.44[c] | 112.40 ± 7.96[b,e] | 92.38 ± 6.66[f] |
| Serum ALT (U/mL) | 38.80 ± 2.29 | 214.00 ± 48.95[c] | 109.30 ± 20.85[d] | 86.00 ± 7.57[d] |
| Serum AST (U/mL) | 126.40 ± 4.72 | 302.70 ± 51.72[b] | 211.30 ± 7.88 | 137.70 ± 21.42[e] |
| Serum ALP (U/mL) | 60.60 ± 1.80 | 86.20 ± 4.60[b] | 91.00 ± 4.16[b] | 88.00 ± 6.08[b] |

Data are expressed as the mean ± SE of the mean ($n$ = 6-7).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$ *vs* C.
[d]$P < 0.05$.
[e]$P < 0.01$.
[f]$P < 0.001$ *vs* high fat diet group. ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; C: Control; HFD: High fat diet; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

### *Effects of papaya on the oxidative status and antioxidant activities*

Papaya improved lipid peroxidation in HFD-fed rats. The HFD group showed significantly increased lipid peroxidation when compared with the C ($P < 0.001$). Furthermore, lipid peroxidation was significantly decreased in the HFD treated with papaya 0.5 and 1 mL/100 g body weight ($P < 0.001$) when compared with the HFD group (Figure 2A). In contrast, the CAT and SOD activities were found to decrease in the HFD group, whereas those significantly increased in HFH group (Figure 2B and C).

### *Effects of papaya on proinflammatory cytokines in liver tissue*

The results showed that HFD in rats significantly increased the serum levels of TNF-α (Figure 3A) and IL-6 (Figure 3B), while these two cytokine levels significantly decreased in the HFD treated with papaya 1 mL/100 g body weight ($P < 0.05$). Taken together, papaya administration can counterbalance lipid peroxidation and inflammation, which is normally found in NAFLD. Improvements in antioxidant activity were also observed.

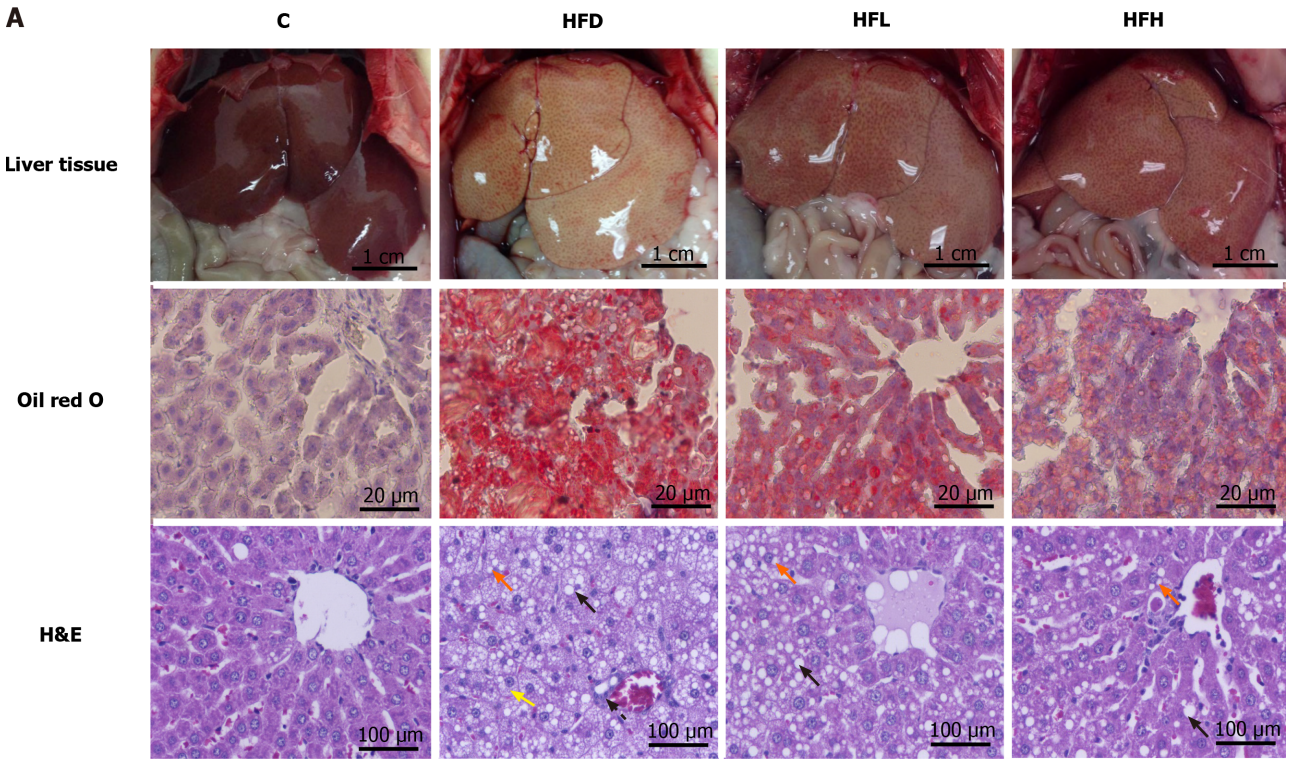
### *Effects of papaya on the de novo lipogenic gene in liver tissue*

The mRNA expression of *SREBP-1c* and *FAS* had a tendency to increase in HFD rats as compared with the control. A significantly decreased expression of those genes were observed in HFH rats ($P < 0.05$) as shown in Figure 4A, B and D, respectively. In contrast, the expression of *ACC* was not different among the groups (Figure 4C). The data indicated that a possible involvement of lipogenesis in the papaya treated group is partially mediated through *SREBP-1c*, which down-regulates the expression of *FAS*. This event may account for the decreased fatty acid metabolism in the liver of rats treated with high doses of papaya juice.

## DISCUSSION

Oxidative stress and inflammation are the main components that contributed to the pathogenesis of NAFLD. Many natural products rich in polyphenols, and strong antioxidant activity have been studied for their positive benefits in the treatment of NAFLD[16]. The presence of these bioactive compounds as well as the significant antioxidant activity *in vitro* has been observed in the pulp and fruit peel of papaya[9].

Our present study demonstrated that papaya attenuated lipid accumulation in




B

| | | | | |
|---|---|---|---|---|
| Steatosis | 0.00 ± 0.00 | 2.17 ± 0.75[c] | 1.67 ± 1.21[b] | 1.17 ± 0.75 |
| Lobular inflammation | 0.00 ± 0.00 | 2.00 ± 0.63[c] | 1.33 ± 1.03[a] | 1.00 ± 0.89 |
| Hepatocyte ballooning | 0.00 ± 0.00 | 1.83 ± 0.41[c] | 1.33 ± 0.52[c] | 1.50 ± 0.55[c] |
| NAS | 0.00 ± 0.00 | 6 ± 1.79[c] | 4.33 ± 2.76[c] | 3.67 ± 2.19[c,d] |

**Figure 1 Effect of papaya on non-alcoholic fatty liver disease.** A: Macroscopic and microscopic appearance in rat hepatocytes. Macrovesicular steatosis (black arrow) are large lipid droplets that are present in the hepatocytes. Microvesicular steatosis (red arrow) are small lipid droplets that are present in the hepatocytes. Hepatocyte ballooning is recognised as cell swelling and enlargement within the cytoplasm (yellow arrow). Lobular inflammation in non-alcoholic steatohepatitis foci (dotted line arrow) are scattered in the hepatic lobule; B: Comparative analysis of non-alcoholic fatty liver disease activity score for all treatment groups. Data are expressed as mean ± SE of the mean ($n$ = 6-7). [a]$P$ < 0.05, [b]$P$ < 0.01, [c]$P$ < 0.001 *vs* C, and [d]$P$ < 0.05 *vs* high fat diet group. C: Control; H&E: Hematoxylin and eosin; HFD: High fat diet; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight; NAS: Non-alcoholic fatty liver disease activity score.

HFD-induced obesity in rats. In this study the *in vivo* model of NAFLD was successfully established and developed to lipid accumulation in liver after feeding the rats an HFD. Those rats fed a HFD exhibited an increase in the weight of the liver and lipid contents, which is a feature of NAFLD[14]. The reverse alterations in hepatic lipid accumulation can be explained by the effects of papaya on lipid metabolism. The mechanism may be, in part, by the inhibition of pancreatic lipase by papaya[17]. Pancreatic lipase is an enzyme secreted from the pancreas and works in the small intestine to hydrolyse TG from diet to glycerol and free fatty acids. In this case, papaya juice hinders the digestion of TG, resulting in the reduction of lipid absorption and then promotion of the excretion of lipids outside the body. From previous studies, it has been shown that the excessive hepatic accumulation of TG and FFA induced hepatic steatosis[18]. From our study, it was demonstrated that the treatment with papaya ameliorates lipid accumulation in liver in HFD rats *via* the modulation of lipid metabolism-related molecules.

In NAFLD pathogenesis, imbalanced lipid metabolism leads to simple steatosis, oxidative damage and secretion of proinflammatory mediators. The liver serves as the major regulator for lipid metabolism that involves in several steps[19]. Hepatic lipid content is regulated by the cellular molecules that control the input and the output. The regulation depends on the metabolic status, the facilitation of hepatic fatty acid uptake, synthesis and storage in the liver, or the rapid metabolism to hepatic fatty acid oxidation as a source of energy may occur[20].

*SREBP-1c* exerts a significant control over the *de novo* synthesis of *FAS*[20]. It was further found that papaya eliminated hepatic steatosis in HFD rats. The latter effect

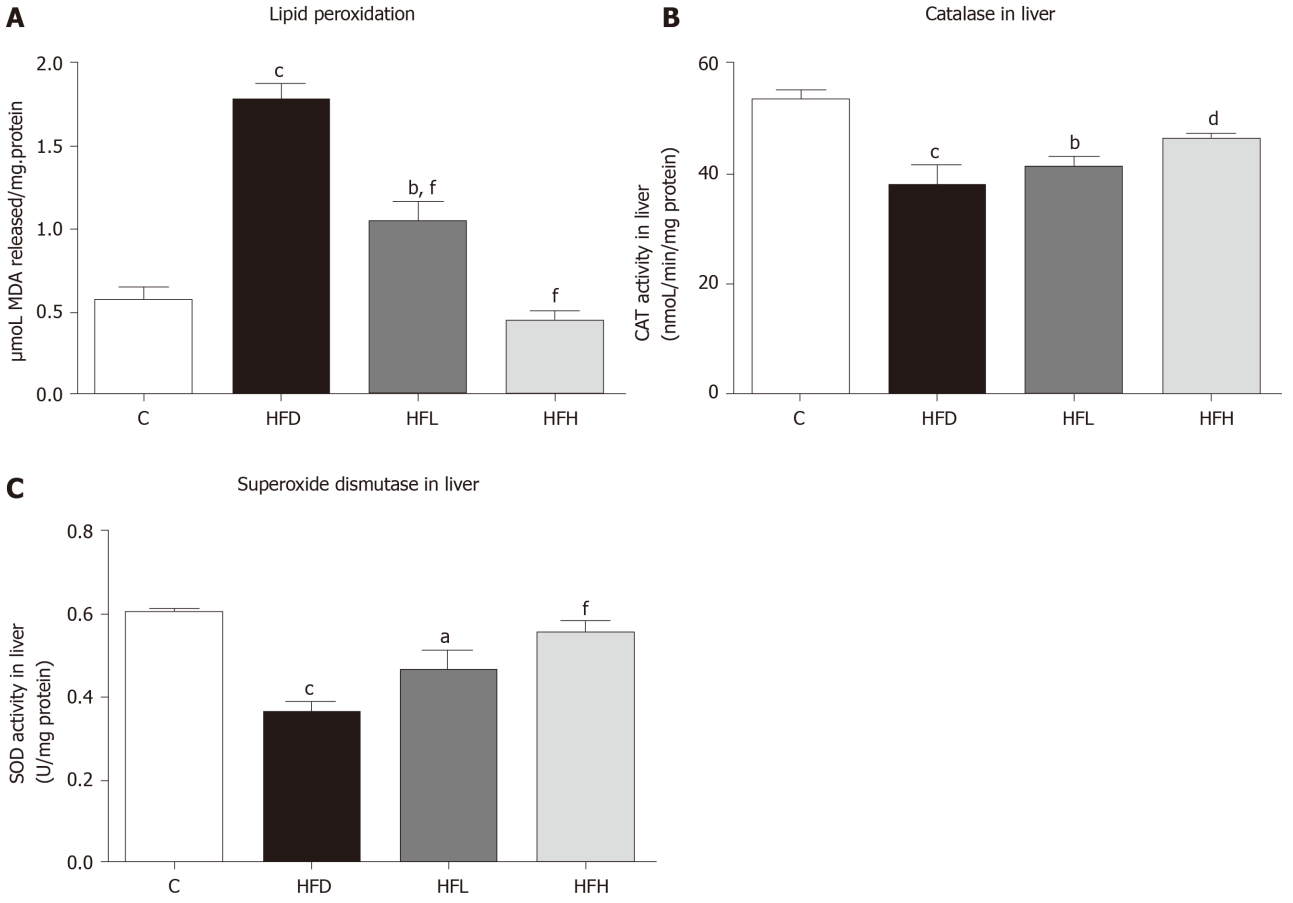



**Figure 2 Effects of papaya on antioxidant activities in liver tissue.** A: Lipid peroxidation in the liver; B: Activity of catalase (CAT) in the liver; C: Activity of superoxide dismutase (SOD) in the liver. Data are expressed as mean ± SE of the mean ($n$ = 6-7). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control (C), and $^{d}P < 0.05$, $^{f}P < 0.001$ *vs* high fat diet (HFD) group. HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight; MDA: Malondialdehyde.

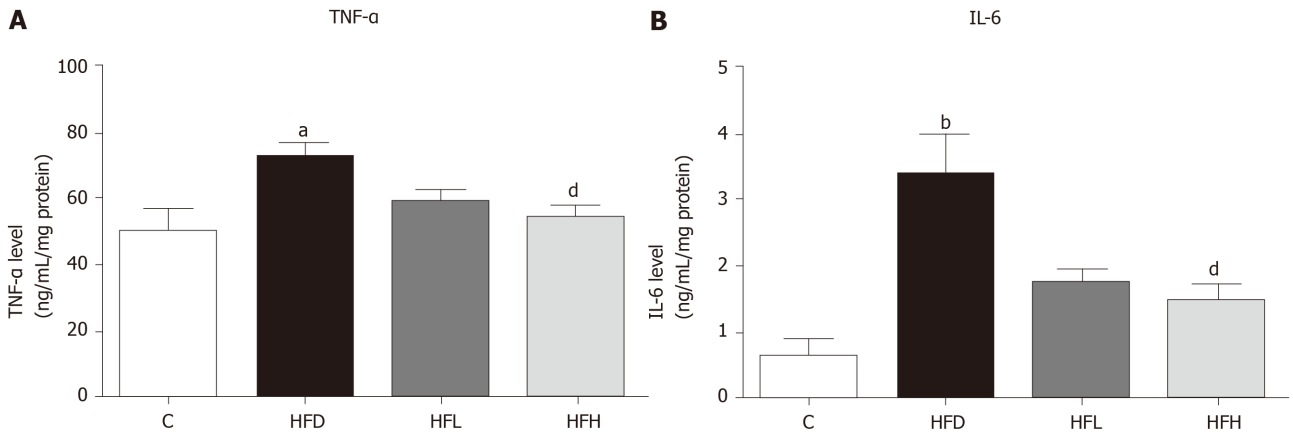



**Figure 3 Effects of papaya on proinflammatory cytokines in liver tissue.** A: Tumour necrosis factor-α (TNF-α) in the liver; B: Interleukin 6 (IL-6) in the liver. Data are expressed as mean ± SE of the mean ($n$ = 6-7). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control (C), and $^{d}P < 0.05$ *vs* high fat diet (HFD) group. HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

might be partially mediated by the regulation of *SREBP-1c*. *SREBP-1c* is an important transcription factor of *de novo* lipogenesis in the liver, while its downstream gene-*FAS* is responsible for fatty acid catabolism[21]. In the livers of obese rats treated with papaya, *SREBP-1c* and *FAS* were remarkably decreased. This implies that papaya exerts its anti-lipogenic effect in consequence of the suppressed regulation of *SREBP-1c* and *FAS*, leading to decreased hepatic lipid accumulation.

Several studies have also demonstrated the antioxidant capacity of β-carotene and

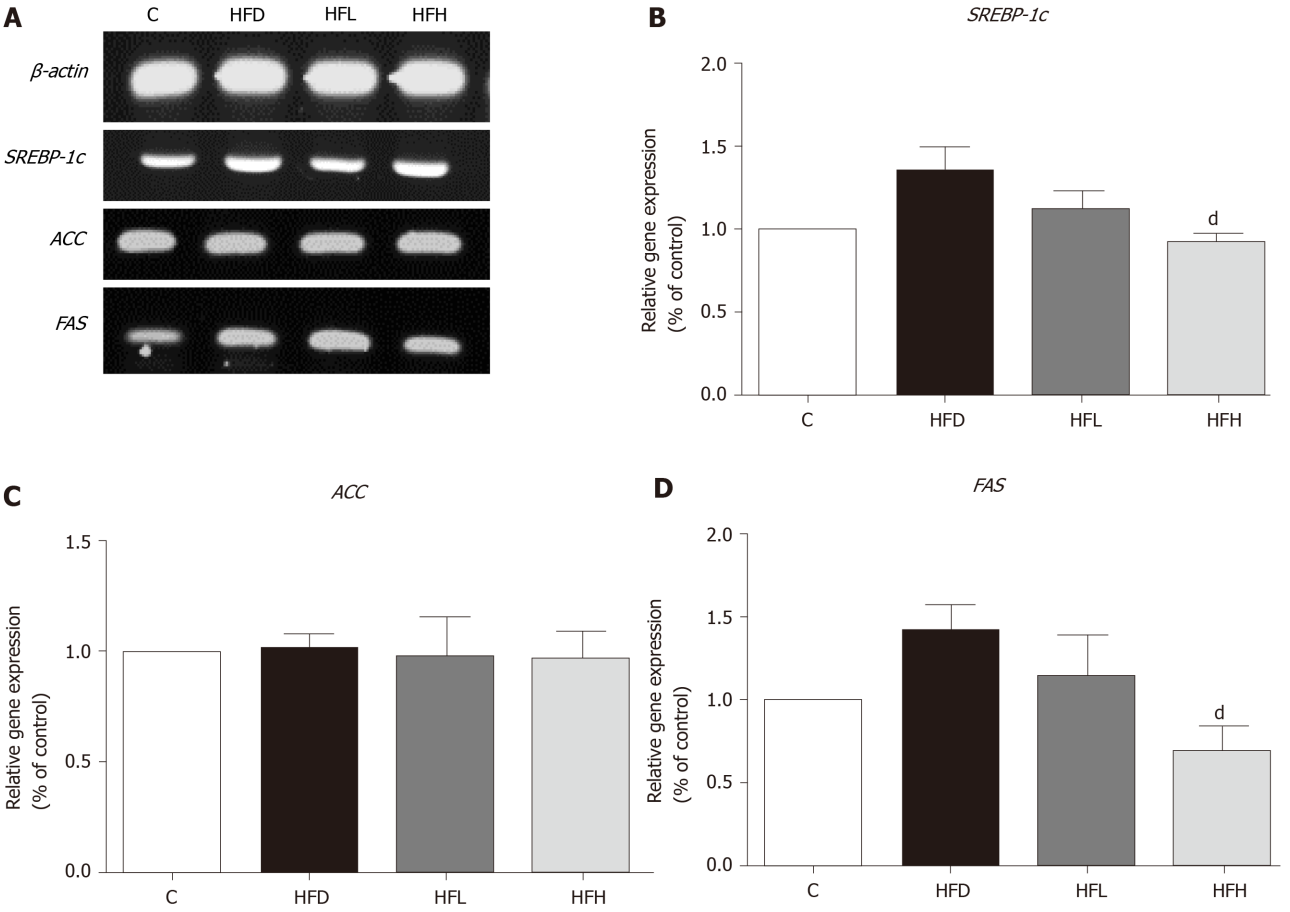



**Figure 4 Effects of papaya on *de novo* lipogenic gene expression in liver tissue.** A: Immunoblotting analysis of *SREBP-1c*, *ACC*, *FAS* and *ACTB*. *ACTB* was used as a normalization gene; B: Relative gene expression of *SREBP-1*; C: Relative gene expression of *ACC*; D: Relative gene expression of *FAS*. Data are expressed as mean ± SE of the mean ($n$ = 5). $^{d}P < 0.05$ *vs* high fat diet (HFD) group. C: Control; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

its act against oxidative stress in different models[22,23]. The significantly elevated hepatic content of TG, TC and malondialdehyde in NAFLD rats is a strong indicator of liver damage and oxidative stress[24]. The pathogenesis of NAFLD is widely accepted by the two-hit hypothesis; the first hit presents increasing levels of FAs and is a key part in the development of hepatic steatosis. Prolonging of hepatocellular damage and sensitised liver leads to the presence of oxidative stress and the release of cytokine or adipokine mediators, this situation is called a second hit[25]. More specifically, high fat consumption leads to increased FAs in liver and either enter β-oxidation or are stored as TG. The mitochondrial β-oxidation serves as energy sources and can generate numerous free radicals including ROS and lipid peroxidation from the electron transport chain though the mitochondrial respiration pathway[26]. Normally, the antioxidant defensive systems help to protect the organs against the deleterious substances[27]. Among these, SOD is a key antioxidant enzyme for the first defence reaction with the ROS-mediated cellular damage. SOD participates in the conversion of superoxide anions into less harmful $H_2O_2$ and oxygen. CAT is another antioxidant enzyme that can catalyse $H_2O_2$ into water and oxygen[28]. From our results, it clearly shows that SOD and CAT activities in the liver were significantly increased after papaya treatment. The mechanism is still unknown, but it might be because of the carotenoid compounds in papaya. Papaya is one of the important dietary sources for carotenoids including β-carotene and lycopene[29]. The liver is the main place for storage carotenoids, the powerful antioxidants from food, and this compound may help scavenge the results of oxidative stress produced in the liver[16].

High fat accumulation in the liver causes impairment of cellular homeostasis. ROS and lipid peroxidation generated in NAFLD are potent inducers of cytokine production and trigger the release of cytokine proinflammatory mediators such as TNF-α and IL-6[30]. TNF-α plays a crucial role in exert in a variety of biological effects including systemic inflammation and takes part in many stages of liver disease[31]. In contrast, IL-6 is secreted from various kinds of cells and is necessary to leukocyte

recruitment and tissue homeostasis[32]. Recent studies have been reported that IL-6 enhances liver inflammation and related to insulin resistance in NAFLD[29]. Proinflammatory cytokine overproduction causes hepatocyte dysfunction and develops fibrosis later on.

We demonstrated from our results that papaya can reduce liver inflammation by the inhibiting the overproduction and activity of proinflammatory cytokines generated in high fat induced hepatic inflammation tissue. The mechanism may be from indirect action of papaya to reduce ROS and can modulate the overwhelming production of cytokines. In addition, papaya itself may play a direct role in the inflammation processes. As reported earlier, papaya possesses anti-inflammatory and immuno-modulatory properties as stated both *in vitro* and i*n vivo* studies[33]. Liver inflammation can aggravate liver damage, resulting in the progression of fibrosis, cirrhosis or liver failure. Reduced inflammatory secretion from cytokines may prevent steatosis and alleviate the progression of the disease[34].

## CONCLUSION

This study demonstrates for the first time the hepatoprotective capacity of the papaya fruit on the damage caused by HFD induced hepatic steatosis. From the obtained results, it can be suggested that the mechanism of action of the hepatoprotective effect of the papaya against the hepatic lipid accumulation in NAFLD was the combined result of the association of the anti-lipogenic, anti-inflammatory and antioxidant activities of papaya (Figure 5).

Moreover, the doses of papaya used in this study can be of practical use in human medicine. The results of this study provide experimental-based evidence suggesting papaya is an efficacious nutritional strategy for use in the prevention or treatment of NAFLD. However, future research should be performed using human trials to elucidate the intervention of papaya in clinical and public health implications.

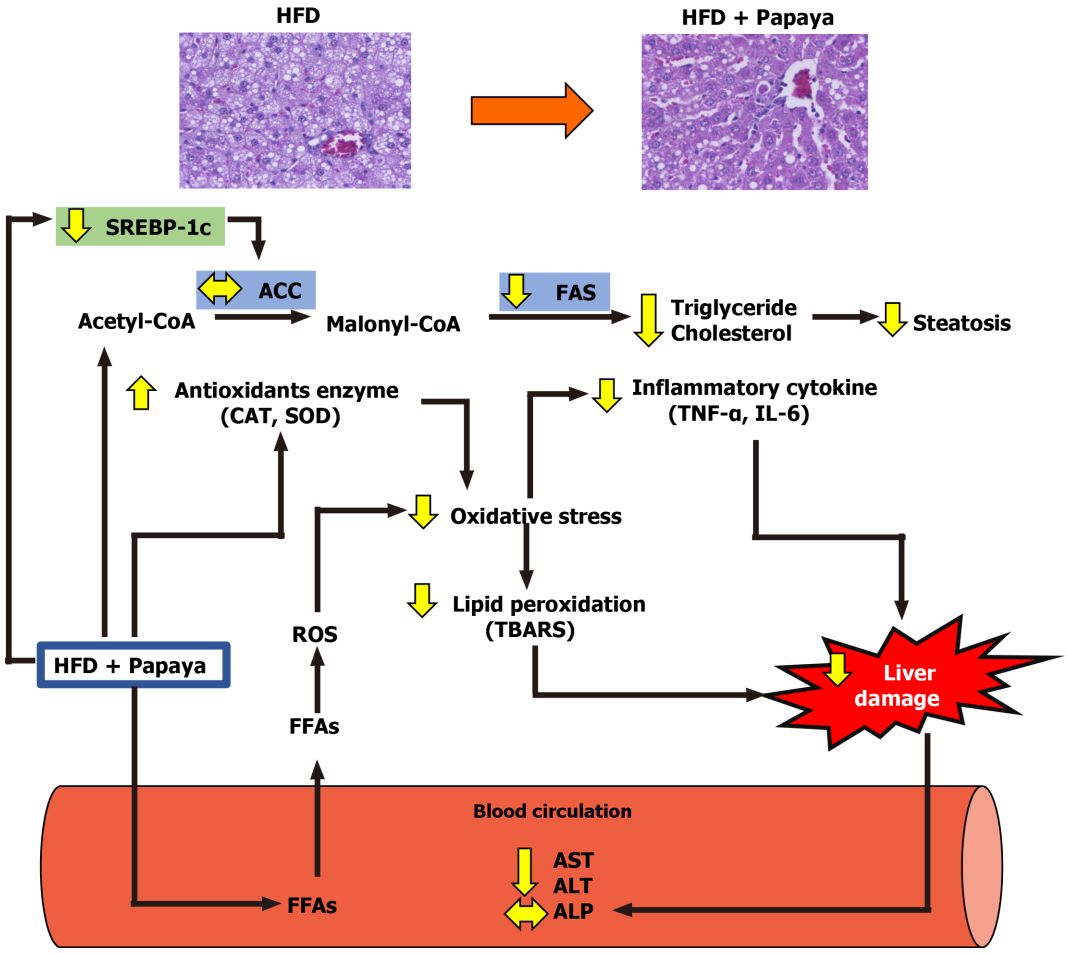



**Figure 5 Schematic diagram of possible mechanism of papaya juice on non-alcoholic fatty liver disease.** The beneficial effect of papaya against hepatic steatosis in obese rats may occur through the inhibition of lipogenic pathways by reducing *SREBP-1c* and *FAS* gene expression, causing the reduction of hepatic fat accumulation. Papaya can improve enzymatic antioxidants [catalase (CAT) and superoxide dismutase (SOD)] and decrease lipid peroxidation in the liver. The administration of papaya significantly decreased proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) to modulate liver damage. Papaya is therefore able to reduce the activities of aspartate transaminase (AST) and alanine transaminase (ALT) in serum. Overall, this study provides evidence for the beneficial effects of papaya to reverse the progression of non-alcoholic fatty liver disease in obese rats. ALP: Alkaline phosphatase; FFAs: Free fatty acids; HFD: High fat diet; ROS: Reactive oxygen species; TBARS: 2-Thiobarbituric acid reactive substances.

## ARTICLE HIGHLIGHTS

### *Research background*

High fat diet consumption causes fat accumulation in liver [nonalcoholic fatty liver disease (NAFLD)], which leads to liver dysfunction due to oxidative stress and inflammation

### *Research motivation*

Papaya is a nutritional, healthy and affordable fruit. It is available in all regions of the world and can be found year-round. Additional scientific evidence on the health and nutritional benefits of papaya are needed to promote health and papaya consumption.

### *Research objectives*

To evaluate papaya's health benefit against NAFLD in obese rats.

### *Research methods*

Rats were fed with a high fat diet for 12 wk to induce obesity. Papaya juice at the implement doses were administered to the rats. Hepatic lipid contents, oxidative stress, inflammatory cytokines, lipogenic genes and liver pathology were assessed.

### *Research results*

The hepatoprotective action of papaya against the accumulation of hepatic fat was a

result of the association of the hypolipidemic effect partially through a suppression of *SREBP-1c* and *FAS*, anti-inflammatory and antioxidant activities.

### Research conclusions

The results of this study provide experimental-based evidence that can contribute to the implement of papaya in the prevention and treatment of obesity and associated metabolic disorders.

### Research perspectives

Our study offers an optimistic view of an anti-NAFLD effect of papaya; however, further evidence from human clinical studies is necessary.

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ORIGINAL ARTICLE

Basic Study

# Promotive action of 2-acetylaminofluorene on hepatic precancerous lesions initiated by diethylnitrosamine in rats: Molecular study

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**Author contributions:** Hasanin AH and El Gayar N conceived the idea and designed the animal model and statistical analysis; Habib EK performed histochemical examinations; Matboli M conducted bioinformatics analysis, biochemical and molecular assay; all authors drafted the manuscript and critically reviewed the manuscript and approved the final version of the manuscript for publication.

**Institutional animal care and use committee statement:** All animal procedures were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine (approval No. 17585).

**Conflict-of-interest statement:** The authors declare no competing interest.

**Data sharing statement:** No

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## Abstract

***BACKGROUND***

Diethylnitrosamine (DEN) induces hepatic neoplastic lesions over a prolonged period.

***AIM***

To investigate the promotive action of 2-acetylaminofluorene (2-AAF) when combined with DEN in order to develop a rat model for induction of pre-cancerous lesion and investigate the molecular mechanism underlying the activity of 2-AAF.

***METHODS***

The pre-precancerous lesions were initiated by intraperitoneal injection of DEN for three weeks consecutively, followed by one intraperitoneal injection of 2-AAF at three different doses (100, 200 and 300 mg/kg). Rats were separated into naïve, DEN, DEN + 100 mg 2-AAF, DEN + 200 mg 2-AAF, and DEN + 300 mg 2-AAF groups. Rats were sacrificed after 10 wk and 16 wk. Liver functions, level of alpha-fetoprotein, glutathione S-transferase-P and proliferating cell nuclear antigen staining of liver tissues were performed. The mRNA level of RAB11A, BAX, p53, and Cyclin E and epigenetic regulation by long-noncoding RNA (lncRNA) RP11-513I15.6, miR-1262 (microRNA), and miR-1298 were assessed in the sera and liver tissues of the rats.

***RESULTS***

2-AAF administration significantly increased the percent area of the precancerous

additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.



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foci and cell proliferation along with a significant decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA was associated with a marked decrease in lncRNA RP11-513I15.6 expression with a significant increase in both miR-1262 and miR-1298.

*CONCLUSION*

2-AFF promoted hepatic precancerous lesions initiated through DEN by decreasing autophagy, apoptosis, and tumor suppression genes, along with increased cell proliferation, in a time- and dose-dependent manner. These actions were mediated under the epigenetic regulation of lncRNA RP11-513I15.6/miR-1262/miR-1298.

**Key Words:** Acetylaminofluorene; Hepatic precancerous lesion; Diethylnitrosamine; Autophagy; Apoptosis; MicroRNA



**Core Tip:** 2-Acetylaminofluorene epigenetically regulated the expression of long-noncoding RNA RP11-513I15.6/miRNA-1262/miR-1298 (microRNA, miRNA) resulted in decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA leading to increased hepatocyte proliferation and decreased apoptosis promoting hepatocellular promoted precancerous lesion in rat models.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th common cancer and the 2nd leading cause of cancer mortality all over the world[1]. Its incidence is elevated, which is attributed to the rising proportion of individuals infected with hepatitis C virus[2]. The molecular pathogenesis of cancer and the underlying tumor biology has been progressing. Spontaneous animal models, induced models, transplantable models, transgenic models, and viral models were used to investigate the biological mechanism of HCC with respect to the liver-targeted key pathways[3]. Rodent has a short life span due to which the cellular transformation is observed from initiation to malignancy, thereby rendering it as a preferred model system[4]. However, modeling a malignant liver disease is challenging due to the urgent need for optimal models for preclinical studies.

Several hepatotoxic agents, such as carcinogen diethylnitrosamine (DEN), have been repeatedly administered to induce general liver disease and HCC over a prolonged period. DEN produces small foci of dysplastic hepatocytes *via* ethylation of various nucleophilic sites in deoxyribonucleic acid[5], resulting in cirrhosis and liver cancer within 18 wk as presented by mutations in β-catenin[6] and p53[7]. HCC induced by DEN activates the H-ras proto-oncogene[8]. Interestingly, variable time intervals, tumor promoters, DEN doses, and application routes were applied by various groups to induce hepatic precancerous lesions in a dose- and time-dependent manner. A two-stage model was established using DEN as a genotoxic compound and phenobarbital to induce HCC[9]. Another two-step HCC model was established according to the Solt-Farber protocol; herein, the initiation by DEN was followed by partial hepatectomy, leading to an elevated number of initiated cells[10].

2-Acetylaminofluorene (2-AAF) serves as a model carcinogen with genotoxic and epigenetic properties[11]. The present study proposed that genotoxic 2-AAF metabolites produce G to T transversion-initiated cells along with cirrhotic alteration due to chronic toxic effect on mitochondrial respiration[12]. Also, electron drainage by 2-AAF causes an uncoupling effect on oxidative phosphorylation[13].

Malik *et al*[14] reported a protocol for HCC induction in the liver without hepatectomy, wherein male Wistar rats were injected with DEN intraperitoneally, and then, 2-AAF repeatedly. This model showed oxidative stress, cell damage, and advanced HCC.

The present study aimed to investigate the development of precancerous lesions by DEN injection intraperitoneally (100 mg/kg body weight), followed by a single intraperitoneal (i.p.) injection of promoter 2-AAF at three different doses (100, 200 and 300 mg/kg) at two intervals of 10 wk and 16 wk, respectively.

## MATERIALS AND METHODS

### Chemicals

DEN with ≥ 99% purity (CAT number 55-18-5) and 2-AAF with ≥ 98% purity (CAT number 53-96-3) were purchased from (Sigma-Aldrich, St. Louis, United States).

### Experimental protocol

A total of 60 adult male Wistar rats (200-250 g) were used. The animals were maintained at 22-24 °C and twelve hours light/dark cycles and received standard rat chow and tap water. All animal experiments were carried out according to the National Institute of Health guide for dealing with laboratory animals (National Research Council (US) Institute for Laboratory Animal Research. No. 85-23, revised 1996). The study was approved by Ain Shams University, Faculty of Medicine Institutional Animal Ethics Committee (approval No. 17585). The animals were acclimatized for 1 wk and weighed before each injection for accurate determination of the drug dosage.

Wister rats were randomly and equally divided into naïve, DEN, DEN + 100 mg 2-AAF, DEN + 200 mg 2-AAF, and DEN + 300 mg 2-AAF groups. The four DEN groups were injected i.p. with 100 mg/kg per week for 3 wk, followed by 1 wk interval. Then, 2-AAF was injected once intraperitoneally at 3 different doses for the 2-AAF three groups (100, 200 and 300 mg/kg). The naïve group was injected with 0.9% NaCl as described above. In each group, half of the animals were sacrificed at the end of week 10 and the remaining at week 16 (Figure 1).

### Specimen collection

Rats were anesthetized before withdrawing the retro-orbital blood samples; sera were collected by centrifugation at 1200 × *g* for 10 min. Subsequently, the rats were sacrificed, and liver samples collected. All the samples were maintained at -80 °C for further tests of liver function, and the level of alpha-fetoprotein (AFP) and RNA extraction in the liver samples were examined.

### Tissue preparation for histological and immunohistochemical examinations

The liver specimens were collected from all animals in each group, with fixation in 10% neutral formaldehyde for 24 h, followed by dehydration, then embedded in paraffin blocks. Then, 5 μm sections were subjected to hematoxylin-eosin (HE) staining to detect any histopathological changes. Images were captured using an Olympus BX50 Light microscope (Olympus, Japan).

### Glutathione S transferase-placental immunohistochemistry

The sections were dewaxed using xylene, followed by hydration using ethanol gradient. The endogenous peroxidase activity was inhibited by hydrogen peroxide. Subsequently, the sections were washed with water and rinsed with phosphate-buffered saline (PBS) before probing with glutathione S-transferase-P (GST-P) primary antibody (1:250; Abcam, cat.# AB106268, San Francesco, CA, United States) at 4 °C overnight. The GST-P-positive area stained brown. The morphometric analysis was carried out using Leica Q win V.3 software after capturing the images using a Leica DM2500 microscope (Leica, Wetzlar, Germany).

### Proliferating cell nuclear antigen immunohistochemistry staining

The sections were prepared for proliferating cell nuclear antigen (PCNA) staining (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, United States) for 2 h as described above. Irrespective of the location within the hepatic lobule of the staining intensity, the nuclei were scored as positive or negative. The PCNA labeling indices are represented as the expression of positively stained nuclei (10 fields/slide at × 400).

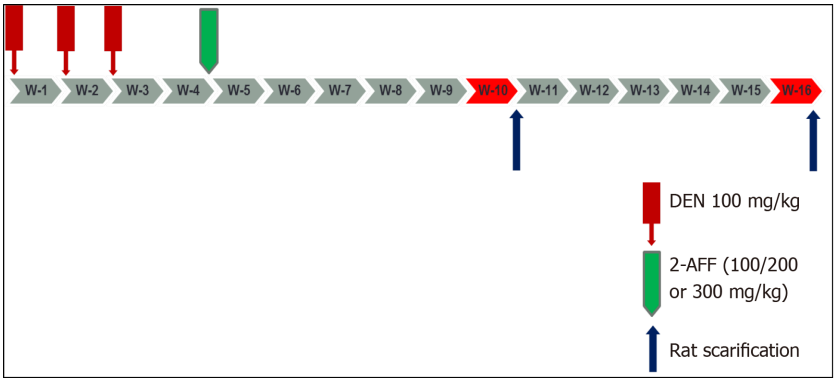



**Figure 1 Schematic of the study design.** DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene.

### *AFP and liver function*

The levels of alanine aminotransferase (ALT), AFP, total bilirubin, and direct bilirubin were analyzed quantitatively using a commercial ELISA kit on sera samples.

### *Molecular assay*

**Bioinformatics-based selection of molecular parameters to investigate the oncogenic mechanism of the chemicals used in the HCC model:** The molecular biomarker panel was obtained in two steps: (1) A panel of key genes, such as Ras-related in brain11gene (RAB11A), p53, BAX and cell cycle-related gene Cyclin E1 according to Gene Atlas Data Base (https://www.ebi.ac.uk/gxa/home) and protein Atlas Data Base ( https://www.proteinatlas.org/) that play a major role in hepatic carcinogenesis, including autophagy, apoptotic genes, and cell cycle; and (2) lncRNA-RP11-513I15.6 was selected using a database of long-noncoding RNA (lncRNA) that act as competitive endogenous RNA (ceRNAs) (http://gyanxet-beta.com/Lncedb/index.php). This lncRNA acts as a master regulator of the target mRNAs by competing with miR (microRNA, miRNA)-1262 and miR-1298 binding with the genes mentioned above. The selected lncRNA and miRNA were based on the specificity to HCC, competing endogenous RNA score, and the number of target sites of mRNA. Finally, the pathway enrichment analysis by Diana database (http://www.microrna.gr/miRPathv2) for both miR-1262 and miR-1298 revealed that these were linked to autophagy, cell cycle regulation, cell adhesion, and other pathways associated to carcinogenesis.

### *Total RNA extraction*

Total RNA was extracted from sera samples by miRNEasy® RNA isolation kit (Qiagen, Düsseldorf, Germany). The RNA integrity and concentration were determined on an Ultraspec 1000 UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, United Kingdom). The RNA purity was 1.8-2. Subsequently, the total RNA was reverse transcribed into complementary DNA by miScript II RT Kit (Qiagen, Düsseldorf, Germany) on a Hybaid thermal cycler (Thermo Electron, Waltham, MA, United States).

### *Real-time quantitative Polymerase Chain Reaction of the RNA panel*

The expression of mRNA and lncRNA in the rat sera and liver tissues was measured by RT² SYBR Green ROX real-time quantitative polymerase chain reaction (qPCR) Mastermix and Quantitect SYBR Green Mastermix Kit (Qiagen, Düsseldorf, Germany), respectively. The specific primers were provided (Qiagen, Düsseldorf, Germany), using Step One Plus™ System (Applied Biosystems Inc., Foster City, CA, United States). B-actin (accession NM_001101) served as the endogenous control.

The miRNA expression in the sera and liver tissue was investigated according to the protocol of miScript SYBR Green kit Qiagen (Düsseldorf, Germany). *RNU-6* served as the endogenous control. The specific PCR primers were synthesized by Qiagen (Düsseldorf, Germay).

The PCR program was according to the following cycles: Denaturation at 95 °C for 15 min followed by forty cycles of denaturation for 10 s at 94 °C, then annealing for 30 s at 55 °C, and finally extension for 34 s at 70 °C. Each reaction was done in duplicate.

The threshold cycle (Ct) value of each sample was calculated using the StepOnePlus™ software v2.2.2 (Applied Biosystems). *Ct* value > 36 was considered

negative. The specificities of the amplicons were confirmed using the melting curve analysis software of Applied Biosystems. The expression of the target molecules was measured using the $2^{-\Delta\Delta Ct}$ method[15]. The expression of the target gene was normalized against that of the housekeeping gene for the samples and compared to the reference sample.

### Statistical analysis

The values are expressed as means ± SD. The statistical differences among all groups were assessed using one-way ANOVA, and Tukey's test. $P < 0.05$ was considered to be statistically significant. The statistical analyses were done using Graphpad Prism, version 5.0. (2007: San Diego, United States).

## RESULTS

The naïve groups at weeks 10 and 16 that did not show significant differences were pooled as a single group.

### Histological and immunohistochemical examination

The liver sections of the naïve control group stained with HE revealed normal architecture of hepatic lobules, central veins, and portal triads. Neither localized lesion nor alternating pre precancerous foci or dysplastic nodules were observed throughout the experimental period (Figure 2A-D).

The histopathology of the liver sections of different groups with DEN either alone or when combined with 2-AAF showed the development of multistage hepatocellular pre precancerous lesions. An apparent increase in the incidence, number, and size of the lesions was observed as a result of increased dose and duration of the usage of DEN and 2-AAF. The liver specimens of rats sacrificed at week 10 showed small early and well-differentiated foci of cellular alteration after injection of DEN solely (Figure 2E), while varying numbers of multiple aggregations of small nodules were present after administration of both DEN + 2-AAF (Figure 2F-H). The simultaneous occurrence of multiple nodules reflected either the dissemination of hepatocytes with cellular atypia from a single primary lesion to form satellite nodules or the synchronous development of several other independent lesions. The localized lesions of foci of cellular alteration did not compress the surrounding hepatic parenchyma but merged with it imperceptibly. However, lack of or minimal disruption of hepatic lobular architecture was observed.

The histological analysis of these pre- precancerous lesions varied greatly from week 10-16 with respect to different stages of differentiation and growth patterns. The lesions observed by the end of week 16 were large and less differentiated (Figure 2I-P). Multiple dysplastic nodules were scattered, compressing the surrounding liver parenchyma and occupying most of the examined fields. These dysplastic nodules were uniform lesions and discriminated from the surrounding liver tissue based on their morphology, cytoplasmic staining, size of the nucleus, and presence of cellular atypia. The nodular cells did not show sinusoidal spaces and were large with clear cytoplasm.

The immunohistochemically-stained liver sections with the GST-P antibody revealed the presence of multiple GST-P-positive areas in all groups after administration of DEN + 2-AAF. Moreover, small positive areas of cellular foci were noted in the group treated with DEN and sacrificed at week 10 (Figure 3A). Multiple GST-P-positive areas, variable in size, were scattered in-between negatively stained hepatocytes among groups treated with DEN + 2-AFF and sacrificed at week 10 (Figure 3B-D). The number and size of the GST-P-positive areas were markedly increased in groups that received DEN + 2-AFF and sacrificed at week 16, especially those that received high doses showed large positive hyperplastic nodules occupying most of the examined fields (Figure 3E-H). The % surface area of GST-P-positive hepatic lesions was measured among different groups and statistically analyzed (Figure 3I).

The immunohistochemical analysis showed an elevated expression of PCNA in groups that received DEN + 2-AAF as compared to those treated with DEN alone. The higher the dose of 2-AAF combined with DEN and longer the duration, higher the expression rate. Strikingly, significant differences were detected between DEN/2-AAF 200 and 300 as compared to DEN/2-AAF 100 at weeks 10 and 16, respectively (Figure 4 and Table 1).

**Table 1 Expression rate of hepatocytes positive for proliferating cell nuclear antigen was calculated as number of positive field expression in 10 fields per rat liver tissue**

| Group | 10 wk duration | 16 wk duration |
|---|---|---|
| DEN | + | ++ |
| DEN + 100 AAF | + | ++ |
| DEN + 200 AAF | ++ | +++ |
| DEN + 300 AAF | ++ | +++ |

+: Positive expression found in 1-3 fields; ++: Positive expression found in 4-6 fields; +++: Positive expression found in 7-10 field. DEN: Diethylnitrosamine; AAF: Acetylaminofluorene.

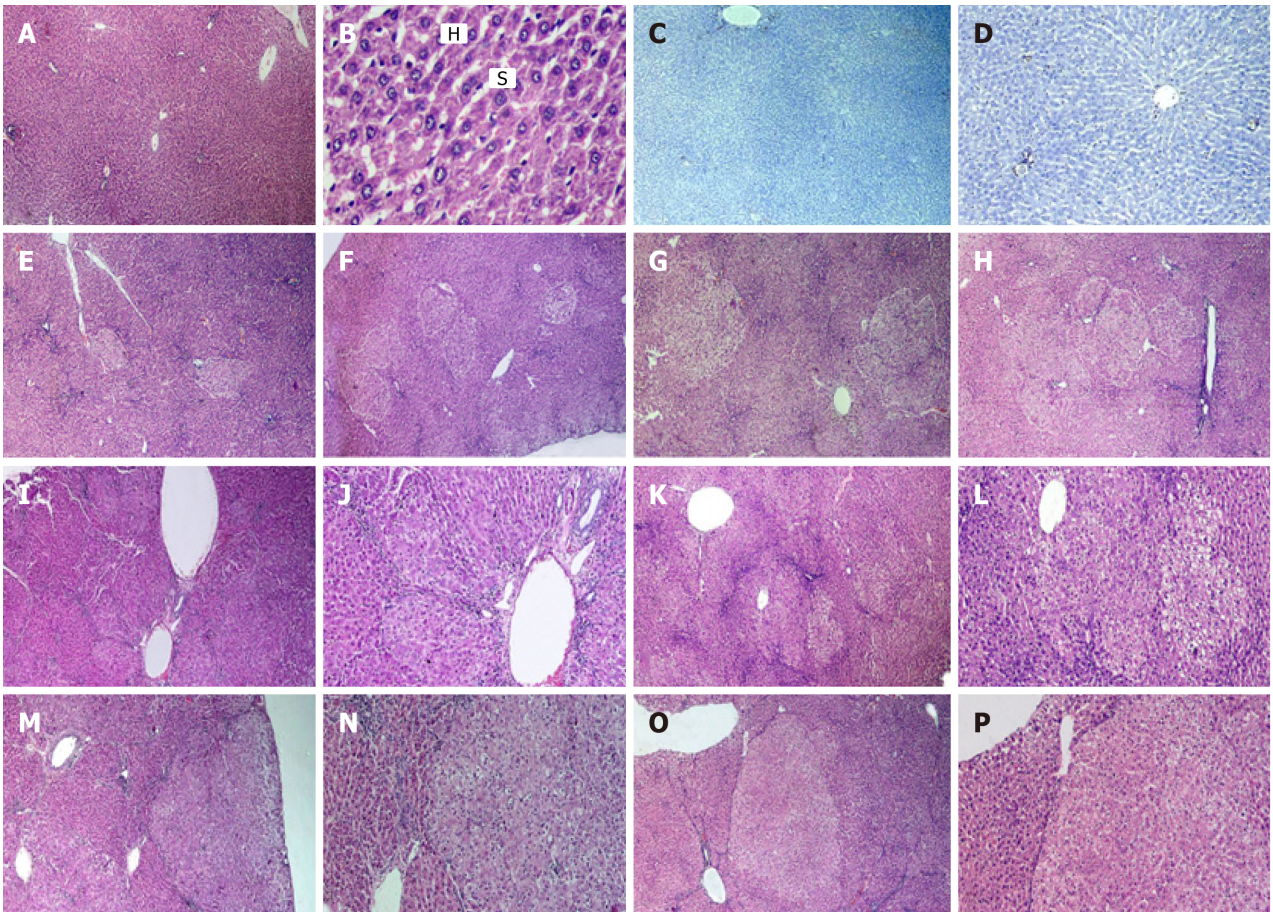



**Figure 2 Histological and immunohistochemical examination.** A-D: Images of liver sections of naive group. Hematoxylin-eosin (HE) stained sections show normal hepatic architecture, portal triad, central vein and radiating cords of hepatocytes (H) with blood sinusoids (S) present in between (A and B). Immunohistochemically-stained section with anti-glutathione S transferase-P demonstrating negative reaction (C). Immunohistochemically-stained section with proliferating cell nuclear antigen antibodies (D); E-H: HE images of liver sections of rats that received diethylnitrosamine (DEN) and different doses of 2-acetylaminofluorene (2-AAF) and were sacrificed at week 10. Show multiple foci of cellular alteration of different sizes (dotted shapes), not compressing the surrounding hepatic parenchyma. DEN group (E), DEN+ 2-AAF 100 mg group (F), DEN + 2-AAF 200 mg group (G) and DEN + 2-AAF 300 mg group (H); I-P: HE liver sections of rats that received DEN and different doses of 2-AAF sacrificed at week 16, show larger, well discriminated, less differentiated dysplastic nodules compressing the surrounding liver tissue with disruption of hepatic lobular architecture were observed. DEN group (I and J), DEN + 100 mg 2AAF group (K and L), DEN + 200 mg 2AAF group (M and N), DEN + 300 mg 2AAF group (O and P). A, C, E-H × 40; D, J, L, N and P × 100; I, K, M and O × 40, B × 400.

### *Effect on liver function and AFP*

Table 2 showed that by the end of weeks 10 and 16, liver function tests (ALT, albumin, T-bilirubin, D-bilirubin) and AFP had a significant decline after DEN and 2-AAF were administered at three doses as compared to the naïve group. 2-AAF addition to DEN significantly increased the level of AFP as compared to DEN alone with significant differences between 2-AAF doses at the two time points in a dose-dependent manner.

**Table 2 Effect of diethylnitrosamine and 2-acetylaminofluorene on alpha-fetoprotein and liver function**

| | AFP | ALT | Total bilirubin | Direct bilirubin | Albumin |
|---|---|---|---|---|---|
| Naïve | 22.8 ± 1.13 | 33.3 ± 6.83 | 0.30 ± 0.18 | 0.27 ± 0.14 | 3.77 ± 0.23 |
| Week 10 | | | | | |
| DEN | 89.2 ± 28.8[d] | 63.0 ± 27.5[d] | 1.44 ± 0.45[d] | 1.03 ± 0.14[d] | 2.49 ± 0.15[d] |
| DEN + 100 AAF | 116 ± 52.1[d] | 78.3 ± 17.8[d] | 2.07 ± 0.44[d,e] | 1.40 ± 0.39[d] | 2.83 ± 0.19[d] |
| DEN + 200 AAF | 223 ± 124[b,d] | 82.7 ± 12.7[d] | 2.73 ± 0.23[a,d,e] | 1.67 ± 0.19[a,d,e] | 2.73 ± 0.14[d] |
| DEN + 300 AAF | 305 ± 126[d,e] | 98.0 ± 10.7[d,e] | 3.13 ± 0.36[b,d,e] | 2.13 ± 0.61[d,e] | 3.15 ± 0.38[c,d,e] |
| Week 16 | | | | | |
| DEN | 159 ± 32.2[d] | 94.1 ± 6.4[d] | 2.13 ± 0.55[d] | 1.60 ± 0.39[d] | 2.0 ± 0.62[d] |
| DEN + 100 AAF | 290 ± 241[d] | 104 ± 31.9[d] | 2.23 ± 0.36[d] | 2.25 ± 0.63[d] | 2.57 ± 0.37[d] |
| DEN + 200 AAF | 815 ± 143[a,d,f] | 128 ± 36.9[d] | 4.10 ± 0.39[a,d,f] | 2.53 ± 0.63[a,d,f] | 2.17 ± 0.29[d] |
| DEN + 300 AAF | 1059 ± 360[b,d,f] | 210 ± 63.2[b,c,d,f] | 4.47 ± 0.99[b,d,f] | 3.10 ± 0.39[d,f] | 2.13 ± 0.67[b,c,d,f] |

Values are mean ± SD; number of animals = 6 rats/each group.
[a]$P < 0.05$ when DEN + 200 acetylaminofluorene (AAF) is compared to the DEN + 100 AAF.
[b]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 100 AAF.
[c]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 200 AAF.
[d]$P < 0.05$ compared to the naïve group.
[e]$P < 0.05$ compared to the diethylnitrosamine (DEN) group at week 10 group.
[f]$P < 0.05$ compared to the DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase.

### *Effect of DEN/2-AAF on the expression of RAB11A, BAX, p53, Cyclin E mRNA among the rat groups*

The fold-changes in the relative quantification (RQ) of RAB11A mRNA in rats' liver tissues and sera showed a significant decrease as compared to naïve rats in all groups at both weeks 10 and 16. Compared to DEN alone, a significant decrease was noted in the RQ of RAB11A mRNA in DEN/2-AAF 200 and 300 in sera and tissues at weeks 10 and 16 as compared to the significant change in DEN/2-AAF 100 in tissue at week 10. Moreover, only a significant decrease was detected in DEN/2-AAF 300 as compared to DEN/2-AAF 100 in serum at week 10 (Figure 5A).

Compared to the naïve group, rats that received DEN solely or when combined to 2-AAF for 10 wk or 16 wk showed a significant decrease in the level of *BAX* mRNA in both liver tissues and sera. 2-AAF addition to DEN significantly decreased the expression of BAX mRNA as compared to DEN alone, except for 2-AAF at a dose of 100mg, in the serum at week 10. Only DEN/2-AAF 300 showed a significant decrease as compared to DEN/2-AAF 100 at week 10 in the liver tissues. The serum BAX mRNA level exhibited insignificant differences among the three DEN/2-AAF groups at both weeks 10 and 16 (Figure 5B).

Furthermore, compared to the naïve group, all groups that received DEN alone or combined with 2-AAF, a significant decrease was detected in the rat liver tissue and sera p53 mRNA. All 2-AAF groups showed a significant decrease over DEN alone except for 2-AAF 100 in the liver tissues at week 10. However, insignificant differences were noted among the three groups DEN/2-AAF 100, 200 and 300 at both weeks 10 and 16 in both liver tissues and sera (Figure 5C).

The Cyclin E mRNA in the rat liver tissues showed a significant increase between DEN/2-AAF 200 and 300 as compared to DEN/2-AAF 100 at week 10. In addition, a significant increase was noted between DEN/2-AAF 300 and DEN/2-AAF100, 200. Furthermore, rats that received DEN either alone or combined with 2-AAF showed a significant increase in the serum Cyclin E mRNA level as compared to the naïve group. All rats that received 2-AAF exhibited a significant increase in Cyclin E mRNA over DEN alone, except for 2-AAF 100, in the rat sera at week 10. Also, a significant increase was observed in DEN/2-AAF 200 and 300 over DEN/2-AAF 100 in the liver tissues at week 10. In addition, a significant increase occurred in 2-AAF 300 over 2-AAF 100 and 200 in the tissues at week 16. A significant increase was noted in 2-AAF 300 over both 2-AAF 100 and 200 at week 10 and in 2-AAF 300 over 2-AAF 100 at week 16 in rat sera (Figure 5D).

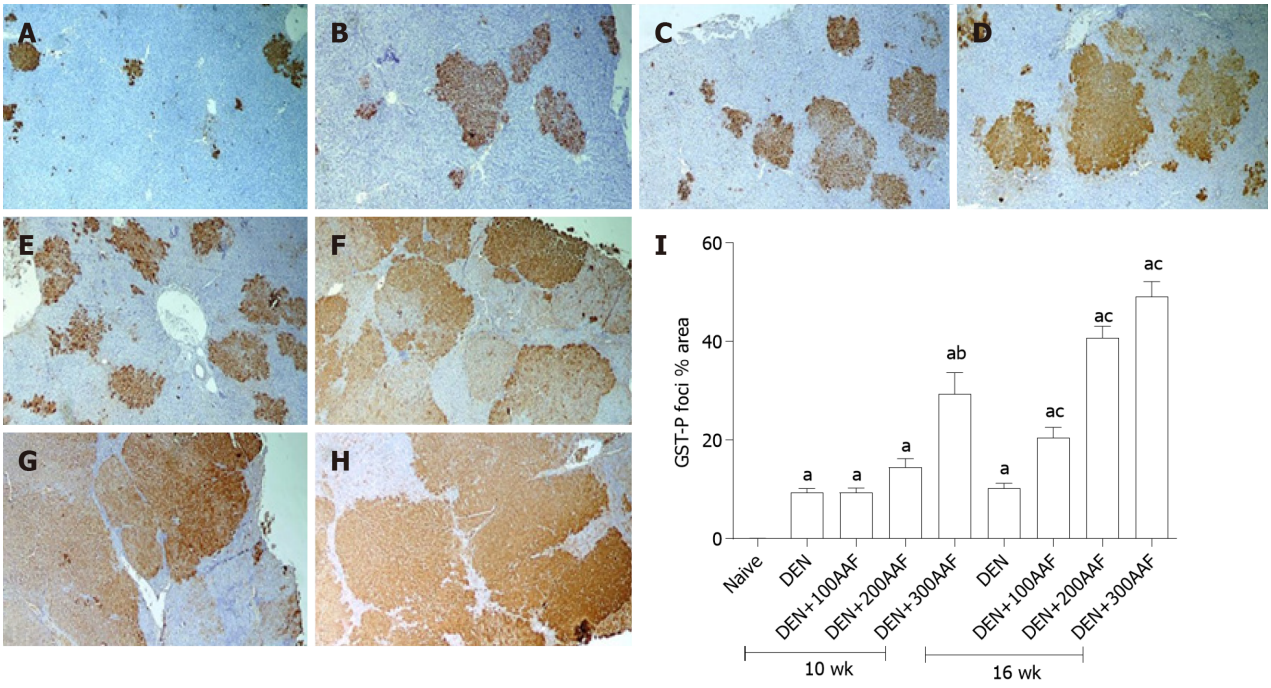



**Figure 3 Histological and immunohistochemical examination.** A-H: Images of rats' liver sections immunohistochemically-stained with glutathione S transferase-P (GST-P) antibody, show multiple GST-P-positive hepatic foci and nodules (brown stained collection of cells) of different sizes scatter in-between negatively stained hepatic parenchyma. Rats sacrificed at week 10 (A-D), rats sacrificed at week 16 (E-H) [A and E: diethylnitrosamine (DEN) group; B and F: DEN + 100mg 2-acetylaminofluorene (2-AAF) group; C and G: DEN + 200 mg 2-AAF group; D and H: DEN + 300 mg 2-AAF (× 40)]; I: shows the effect of DEN and 2-AAF at different doses on GSTP foci % area in the liver. Values are mean ± SE; number of animals = 6 rats/each group. $^{a}P < 0.05$ compared to naïve group; $^{b}P < 0.05$ compared to DEN group at week 10, $^{c}P < 0.05$ compared to DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; GST-P: Glutathione S transferase-P.

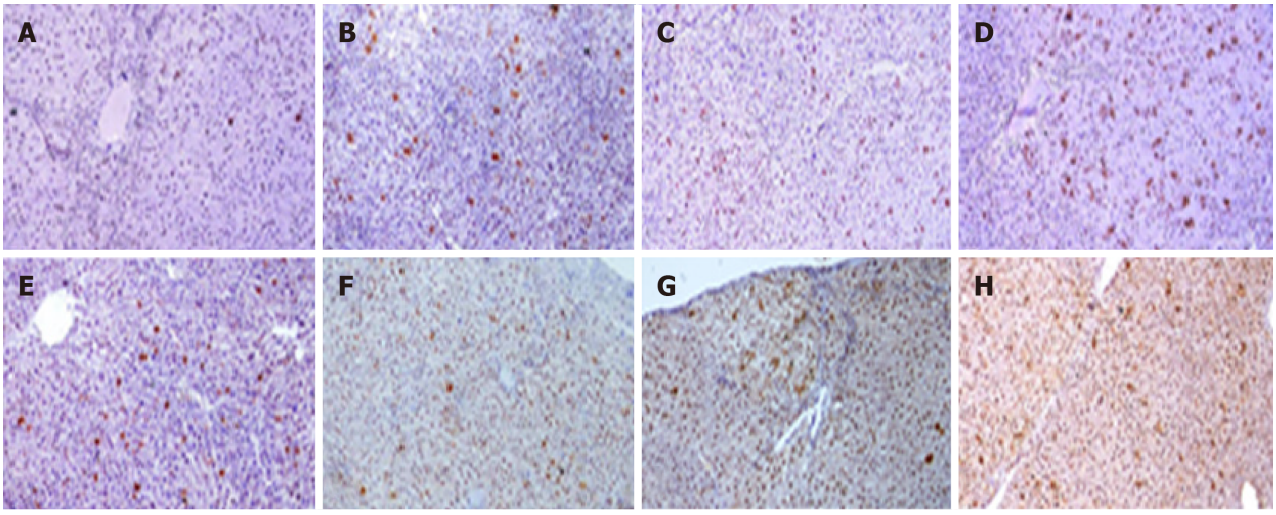
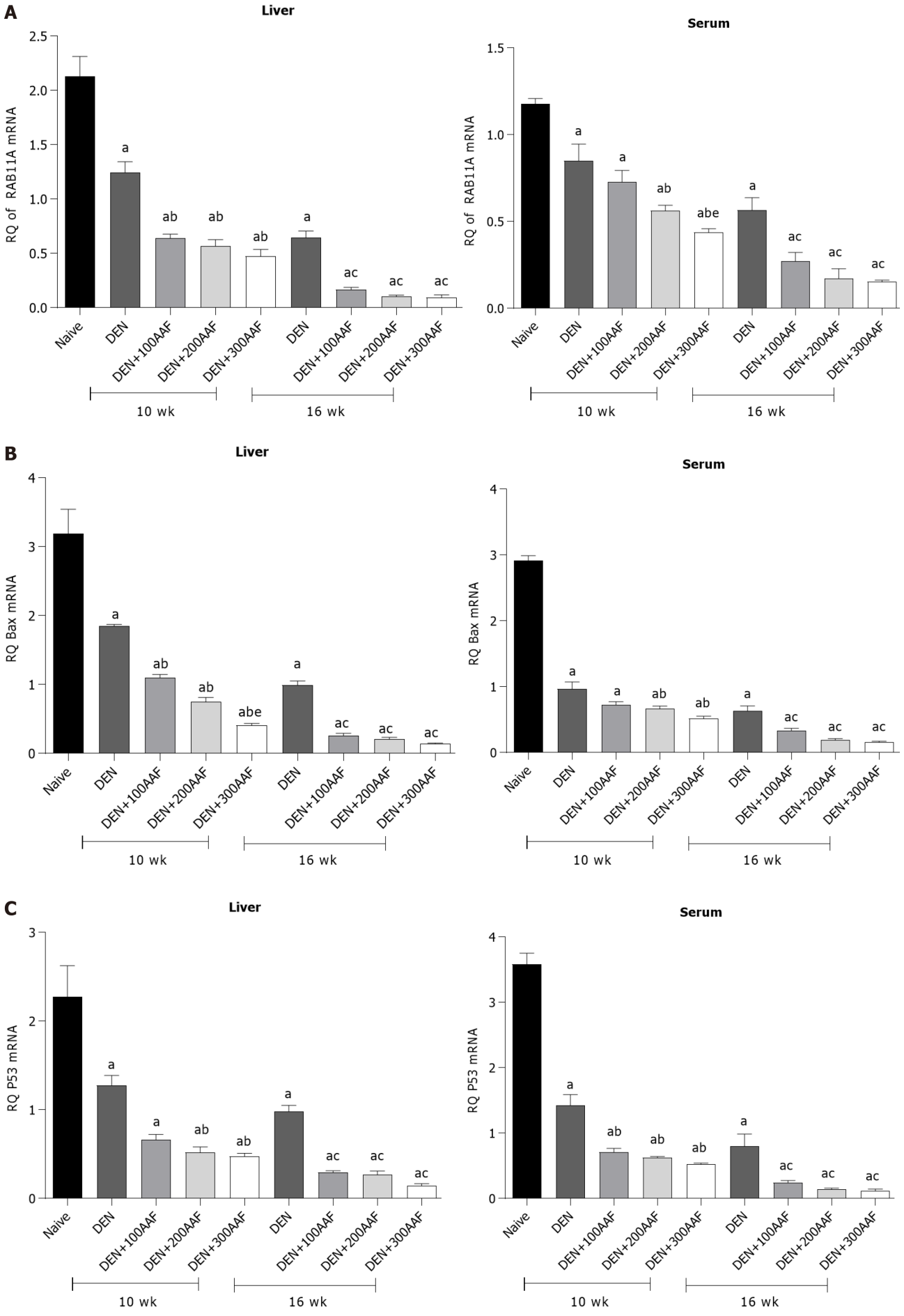



**Figure 4 Images of rats' liver sections stained immunohistochemically with proliferating cell nuclear antigen.** Positive immune-reactive nucleus (brown dots) scatter in-between negatively stained liver tissue of rats who received diethylnitrosamine (DEN) and different doses of 2-acetylaminofluorene (2-AAF). A-D: Rats sacrificed at week 10; E-H: Rats sacrificed at week 16 (A and E: DEN group; B and F: DEN + 100 mg 2AAF group; C and G: DEN + 200 mg 2AAF group; D and H: DEN + 300 mg 2AAF; magnification × 100).

Finally, 2-AAF administration resulted in a significant increase in the level of Cyclin E mRNA with a concomitant decrease in RAB11A, p53, and BAX mRNA expression in the liver tissues and sera as compared to DEN alone. Also, significant differences were reported for 2-AAF 300 as compared to the other 2 doses, especially in the level of Cyclin E mRNA.


A
Liver
RQ of RAB11A mRNA
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
ab
ab
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
10 wk
16 wk
Serum
RQ of RAB11A mRNA
1.5
1.0
0.5
0.0
a
a
ab
abe
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
10 wk
16 wk
B
Liver
RQ Bax mRNA
4
3
2
1
0
a
ab
ab
abe
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
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10 wk
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Serum
RQ Bax mRNA
4
3
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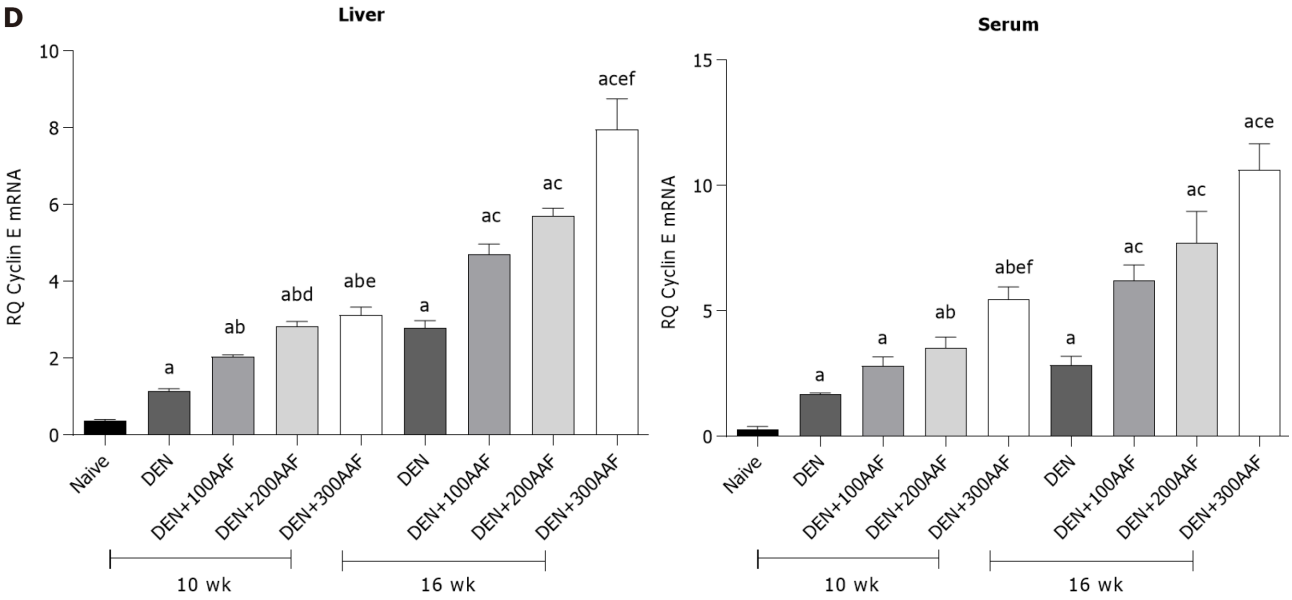


**Figure 5 Effect of diethylnitrosamine and 2-acetylaminofluorene at different doses.** A: Relative quantification (RQ) of RAB11A mRNA; B: RQ of BAX mRNA; C: RQ of p53 mRNA; D: RQ of Cyclin E mRNA in the liver and serum in rats. Values are mean ± SE; number of animals = 6 rats/each group. [a]*P* < 0.05 compared to the naïve group; [b]*P* < 0.05 compared to the diethylnitrosamine (DEN) group at week 10 group; [c]*P* < 0.05 compared to the DEN at week 16 group; [d]*P* < 0.05 when DEN 200 + acetylaminofluorene (AAF) is compared to the DEN 100 + AAF; [e]*P* < 0.05 when DEN 300 + AAF is compared to the DEN 100 + AAF; [f]*P* < 0.05 when DEN 300 + AAF is compared to the DEN 200 + AAF. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene.

### *Effect of DEN/2-AAF on the expression of lncRNA-RP11-513I15.6, miR-1262, and miR-1298 among the rat groups*

The levels of lncRNA-RP11-513I15.6, miRNA-1262, and miR-1298 were assessed in the liver tissues and sera of all groups at the end of weeks 10 and 16. One-way ANOVA and Tukey's multiple comparison test showed significant differential expression in RQ among the studied groups.

Compared to the naïve group, the RQ of lncRNA-RP11-513I15.6 in rat liver tissues and sera in DEN and DEN/2-AAF groups showed a significant decrease at both weeks 10 and 16. A significant decrease was noted in the 2-AAF groups as compared to DEN alone, except for 2-AAF 100 mg, in the liver in week 10 and 2-AAF 100 mg in the sera at week 16. At week 10, a significant difference was observed between DEN/2-AAF 200 and DEN/2-AAF 300 than DEN/2-AAF100 mg in liver tissues. At week 16, a significant difference was noted in DEN/2-AAF 300 over DEN/2-AAF 100 mg in liver tissues, while the differences between the three groups either on week 10 or 16 were insignificant (Table 3).

miR-1262 exhibited a significant increase in the rats who received either DEN alone or in combination with 2-AFF as compared to the naïve group. Compared to DEN alone, all 2-AAF groups showed a significant increase except for 2-AAF 100 at week 10 in both liver tissues and sera. At week 10, 2-AAF 300 mg showed a significant difference over 2-AAF 100 mg and 200 mg in liver tissues. Moreover, at week 16, a considerable difference was observed between 2-AAF 200 mg and 300 mg over DEN/2-AAF 100 mg. At the serum level, significant differences were detected in DEN/2-AAF 300 mg over DEN/2-AAF 100 mg in both weeks 10 or 16 (Table 3).

Compared to the naïve group, all groups that received DEN or DEN in combination with 2-AAF showed a remarkable increase in the level of miR-1298. Compared to DEN alone, all groups that received 2-AFF showed a significant increase in the level of miR-1298, except for 2-AFF 100 mg, at week 10 in both liver tissues and sera. At week 10 in liver tissues, DEN/2-AAF 200 mg and 300 mg showed a significant increase over DEN/2-AAF 100 mg, while at week 16, a significant difference was detected in DEN/2-AAF 300 mg over both DEN/2-AAF 100 mg and 200 mg. At week 10, significant differences were noted in DEN/2-AAF 300 over DEN/2-AAF 100 mg and 200 mg in the sera, and at week 16, a significant increase was observed in DEN/2-AAF 300 mg over DEN/2-AAF 100 mg (Table 3).

Finally, 2-AAF administration exhibited a significant increase in miR-1298 and miR-1262 with a concomitant decrease in lncRNA-RP11-513I15.6 expression in the liver tissues and sera over DEN alone; also, significant differences were observed in 2-AFF

**Table 3 Effect of diethylnitrosamine and 2-acetylaminofluorene on relative quantification of lncRNA-RP11-513I15.6 (long-noncoding RNA), relative quantification of miR-1262 and relative quantification of miR-1298 (microRNA)**

| | RQ of lncRNA-RP11-513I15.6 | | RQ of miR-1262 | | RQ of miR-1298 | |
|---|---|---|---|---|---|---|
| | Liver | Serum | Liver | Serum | Liver | Serum |
| Naïve | 2.33 ± 0.31 | 1.86 ± 0.41 | 0.38 ± 0.09 | 0.26 ± 0.34 | 0.77 ± 0.26 | 0.1 ± 0.04 |
| Week 10 | | | | | | |
| DEN | 1.31 ± 0.36[d] | 0.99 ± 0.15[d] | 1.63 ± 0.28[d] | 1.40 ± 0.13[d] | 1.55 ± 0.37[d] | 1.08 ± 0.11[d] |
| DEN + 100 AAF | 1.03 ± 0.2[d] | 0.63 ± 0.13[d,e] | 2.26 ± 0.54[d] | 2.23 ± 0.19[d] | 1.85 ± 0.12[d] | 1.48 ± 0.56[d] |
| DEN + 200 AAF | 0.58 ± 0.12[a,d,e] | 0.44 ± 0.05[d,e] | 2.52 ± 0.44[d,e] | 2.81 ± 0.49[d,e] | 2.46 ± 0.37[d,e] | 1.82 ± 0.47[b,c,d,e] |
| DEN + 300 AAF | 0.47 ± 0.037[b,d,e] | 0.39 ± 0.005[d,e] | 3.9 ± 0.36[d,e] | 3.59 ± 1.10[b,d,e] | 2.88 ± 0.11[d,e] | 3.30 ± 0.18[d,e] |
| Week 16 | | | | | | |
| DEN | 0.76 ± 0.1[d] | 0.52 ± 0.13[d] | 3.12 ± 0.62[d] | 2.15 ± 0.08[d] | 2.52 ± 0.56[d] | 2.23 ± 0.26[d] |
| DEN + 100 AAF | 0.46 ± 0.04[d,f] | 0.22 ± 0.04[d] | 4.48 ± 0.63[d,f] | 4.08 ± 0.32[d] | 3.92 ± 0.61[d,f] | 4.56 ± 0.61[d,f] |
| DEN + 200 AAF | 0.26 ± 0.07[d,f] | 0.14 ± 0.07[d,f] | 5.71 ± 0.76[a,d,f] | 7.38 ± 2.24[d,f] | 4.58 ± 0.56[d,f] | 5.78 ± 1.72[d,f] |
| DEN + 300 AAF | 0.14 ± 0.04[b,d,f] | 0.12 ± 0.06[d,f] | 6.45 ± 1.04[b,d,f] | 9.78 ± 4.32[b,d,f] | 5.89 ± 1.27[d,f] | 7.38 ± 2.05[b,d,f] |

Values are mean ± SD; number of animals = 6 rats/each group.
[a]$P < 0.05$ when DEN + 200 acetylaminofluorene (AAF) is compared to the DEN + 100 AAF.
[b]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 100 AAF.
[c]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 200 AAF.
[d]$P < 0.05$ compared to the naïve group.
[e]$P < 0.05$ compared to the diethylnitrosamine (DEN) group at week 10 group.
[f]$P < 0.05$ compared to the DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; RQ: Relative quantification; lncRNA: Long-noncoding RNA; miR: MicroRNA.

300 mg over the other two doses.

## DISCUSSION

The nodules and cancer progression has been analyzed using animal models of carcinogenesis[16]. The present study aimed to develop a model of chemically-induced pre precancerous nodules in rat liver using DEN + 2-AAF and explore the putative molecular mechanism at the genetic and epigenetic levels. The conformation of premalignant epithelial tissues was disrupted by pre- and neoplastic liver nodules in experimental animals before the onset of cancer[17]. DEN is used to induce precancerous and cancerous lesions. It is metabolically activated by the liver cytochrome cytochrome P450 (CYP450) system, followed by induced DNA damage and oxidative stress in hepatocytes during cancer initiation[18]. The drawback of this model is the duration required for appropriate tumor development[19]. The initiated cells can be stimulated to proliferate and form hepatocyte foci and nodules by the administration of promotor agent, such as 2-AAF that causes toxicity, cell death, and carcinogenesis[20]. Carcinogens exert their carcinogenicity through either epigenetic effects without direct interaction with DNA or genotoxic effects[21].

GST-P immunohistochemistry served as an optimal marker of hepatic pre precancerous in rats[22]. In addition, PCNA is an essential cell cycle regulator; its expression serves as a tool for studying cell proliferation and identifying the replicating cells[23]. The nuclei of hepatocytes with positive PCNA immunostaining indicate hepatic regeneration. Also, a large number of cells circulating in GST-P-positive areas were observed. Furthermore, liver regeneration induced by massive hepatic necrosis was associated with the proliferation of hepatocytes.

Accumulating evidence suggested that oncogenic transformation is associated with resistance or impeded apoptotic pathway. The cancer therapy targets such autophagic imbalance[24]. RAB proteins are members of the Ras superfamily consisting of small monomeric GTPases that regulate the intracellular trafficking of several cell types. RAB11 GTPases are involved in the recycling of endosomes as well as controlling trafficking and autophagy process[25]. Previous studies demonstrated a significant role

of RAB11A in pancreatic cancer[26] and non-small cell lung cancer[27].

A majority of the tumors present defects in the cell cycle, especially the loss of tumor suppressor p53, which prevents cell proliferation in response to DNA damage or dysregulation of oncogenes, inducing apoptosis or cellular senescence. p53 heterozygous mutant is susceptible to the occurrence of HCC[28,29]. Cyclin was overexpressed in many human cancers, including ovarian and breast cancers. AKT acts as a cytoplasmic central regulator of cell cycle signaling (Cyclin D1 and E) and cell survival ($Mdm^2$/p53)[30,31]. Cyclin E1 is a regulatory subunit of Cyclin-dependent kinase 2 (CDK2). Cyclin E1 is upregulated in human HCCs and associated with poor prognosis[32,33]. Notably, the dysfunction of apoptosis with dysregulation of BCL-2 and BAX has been reported in many cancers, including bladder cancer[34]. BAX is a central regulator of cell death, leading to mitochondrial dysfunction. Also, it is one of the proapoptotic Bcl-2 family proteins that regulate apoptosis in normal and cancer cells[35].

Interestingly, previous studies reported the role of tumor suppressor miR-1262 in cancers. The expression of miR-1262 was dysregulated in the lung[36] and colon cancers[37]. On the other hand, hsa-miR-1298 is a microRNA gene, correlated to undefined RNA class and localized on the X chromosome (Xq23), (114715233-114715344 bp), 112 bases in length. Calvisi *et al*[6] demonstrated the secretion of circulating miR-21, miR-221a, miR-519d and miR-1228 in HCC patients. The high mobility group "A" family consisted of lncRNA RP11-513I15.6, which encoded the small nuclear proteins. Moreover, it play a significant role as an oncogene and is frequently overexpressed in different malignancies, such as HCC out[38], breast cancer[39], and ovarian cancer[40].

## CONCLUSION

Administration of DEN to rats produced changes in hepatocytes with increased GST-P and PCNA expression and development of precancerous hepatic foci. The transformed cells proliferated when challenged with another carcinogen (2-AAF) as a promoter. These changes increased with the elevated dose of 2-AFF and duration of the experiment. DEN and 2-AAF affected the mRNA-biomarkers, including RAB11A, BAX, p53, and Cyclin E. Thus, the oncogenic properties of DEN and 2-AAF were observed in induced HCC model, which might be attributed to the suppression of p53, autophagy, and apoptosis along with the activation of the cell cycle. Moreover, it significantly increased the level of miR-1262 and miR-1298 with a concomitant decrease in the expression of lncRNA-RP11-513I15.6. This phenomenon led to the hypothesis that lncRNA-RP11-513I15.6 is a part of competing endogenous RNA, decreasing the level of miR-1262 and miR-1298, which, in turn, regulates the selected target mRNAs.

## ARTICLE HIGHLIGHTS

### *Research background*

2-Acetylaminofluorene (2-AAF) dose dependently promoted hepatic precancerous lesion. Over diethylnitrosamine (DEN), 2-AAF decreased autophagy. Over DEN, 2-AAF decreased apoptosis and tumor suppression gene. Over DEN, 2-AAF increased hepatic cell proliferation. 2-AAF epigenetically regulated long-noncoding RNA (lncRNA) RP11-513I15.6/miRNA-1262/miR-1298 (microRNA = miRNA = miR).

### *Research motivation*

Urgent need for hepatocellular carcinoma (HCC) rat model for preclinical trials.

### *Research objectives*

The present study aimed to develop a model of chemically-induced pre precancerous nodules in rat liver using DEN + 2-AAF and explore the putative molecular mechanism at the genetic and epigenetic levels.

### *Research methods*

Bioinformatics-based selection of molecular parameters to investigate the oncogenic mechanism of the chemicals used in the HCC model followed by induction of animal

model by. intraperitoneal injection of DEN for three weeks consecutively, followed by one intraperitoneal injection of 2-AAF at three different doses (100, 200 and 300 mg/kg. Rats were sacrificed after 10 wk and 16 wk. Liver functions, level of alpha-fetoprotein, glutathione S-transferase-P and proliferating cell nuclear antigen staining of liver tissues were performed. The mRNA level of RAB11A, BAX, p53, and Cyclin E and epigenetic regulation by lncRNA RP11-513I15.6, miR-1262, and miR-1298 were assessed in the sera and liver tissues of the rats.

### Research results

2-AAF administration significantly increased the percent area of the precancerous foci and cell proliferation along with a significant decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA was associated with a marked decrease in lncRNA RP11-513I15.6 expression with a significant increase in both miR-1262 and miR-1298.

### Research conclusions

Administration of DEN to rats produced changes in hepatocytes with increased glutathione S-transferase-P and proliferating cell nuclear antigen expression and development of precancerous hepatic foci. The transformed cells proliferated when challenged with another carcinogen (2-AAF) as a promoter. These changes increased with the elevated dose of 2-AFF and duration of the experiment. DEN and 2-AAF affected the mRNA-biomarkers, including RAB11A, BAX, p53, and Cyclin E. Thus, the oncogenic properties of DEN and 2-AAF were observed in induced HCC model, which might be attributed to the suppression of p53, autophagy, and apoptosis along with the activation of the cell cycle. Moreover, it significantly increased the level of miR-1262 and miR-1298 with a concomitant decrease in the expression of lncRNA-RP11-513I15.6. This phenomenon led to the hypothesis that lncRNA-RP11-513I15.6 is a part of competing endogenous RNA, decreasing the level of miR-1262 and miR-1298, which, in turn, regulates the selected target mRNAs.

### Research perspectives

More *in vitro* functional studies are urgently need to explore the competing endogenur role of lncRNA in HCC pathogenesis.

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ORIGINAL ARTICLE

Basic Study

# BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) regulation of β-catenin signaling in the progression of drug-induced hepatic fibrosis and carcinogenesis

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## Abstract

### *BACKGROUND*

BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) is a liver tumor suppressor, which is downregulated in a large number of patients with liver diseases. BRUCE facilitates DNA damage repair to protect the mouse liver against the hepatocarcinogen diethylnitrosamine (DEN)-dependent acute liver injury and carcinogenesis. While there exists an established pathologic connection between fibrosis and hepatocellular carcinoma (HCC), DEN exposure alone does not induce robust hepatic fibrosis. Further studies are warranted to identify new suppressive mechanisms contributing to DEN-induced fibrosis and HCC.

### *AIM*

To investigate the suppressive mechanisms of BRUCE in hepatic fibrosis and HCC development.

### *METHODS*

mentorship to CLV; Du CY performed critical revision of the manuscript, obtained funding, study supervision and manuscript revisions.

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Male C57/BL6/J control mice [loxp/Loxp; albumin-cre (Alb-cre)$^-$] and BRUCE Alb-Cre KO mice (loxp/Loxp; Alb-Cre$^+$) were injected with a single dose of DEN at postnatal day 15 and sacrificed at different time points to examine liver disease progression.

*RESULTS*

By using a liver-specific BRUCE knockout (LKO) mouse model, we found that BRUCE deficiency, in conjunction with DEN exposure, induced hepatic fibrosis in both premalignant as well as malignant stages, thus recapitulating the chronic fibrosis background often observed in HCC patients. Activated in fibrosis and HCC, β-catenin activity depends on its stabilization and subsequent translocation to the nucleus. Interestingly, we observed that livers from BRUCE KO mice demonstrated an increased nuclear accumulation and elevated activity of β-catenin in the three stages of carcinogenesis: Pre-malignancy, tumor initiation, and HCC. This suggests that BRUCE negatively regulates β-catenin activity during liver disease progression. β-catenin can be activated by phosphorylation by protein kinases, such as protein kinase A (PKA), which phosphorylates it at Ser-675 (pSer-675-β-catenin). Mechanistically, BRUCE and PKA were colocalized in the cytoplasm of hepatocytes where PKA activity is maintained at the basal level. However, in BRUCE deficient mouse livers or a human liver cancer cell line, both PKA activity and pSer-675-β-catenin levels were observed to be elevated.

*CONCLUSION*

Our data support a "BRUCE-PKA-β-catenin" signaling axis in the mouse liver. The BRUCE interaction with PKA in hepatocytes suppresses PKA-dependent phosphorylation and activation of β-catenin. This study implicates BRUCE as a novel negative regulator of both PKA and β-catenin in chronic liver disease progression. Furthermore, BRUCE-liver specific KO mice serve as a promising model for understanding hepatic fibrosis and HCC in patients with aberrant activation of PKA and β-catenin.

**Key Words:** BIR repeat-containing ubiquitin conjugating enzyme; Diethylnitrosamine; Mouse model; Liver fibrosis; Liver cancer; Hepatocellular carcinoma



**Core Tip:** Upon diethylnitrosamine (DEN) exposure, BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) liver-deficiency accelerates chronic liver diseases such as fibrosis and hepatocellular carcinoma (HCC) in mice. Our previous study established the role of BRUCE in the protection of the liver against DEN-induced liver injury and subsequent disease progression. Here we report a chronic fibrosis background induced by hepatic BRUCE knockout in mice that recapitulates the fibrosis background in HCC patients. We also report a BRUCE-dependent suppression of β-catenin activity through the suppression of protein kinase A (PKA) activity. This study provides a therapeutic potential involving the inhibition of PKA and β-catenin activities in patients with liver disease that carry BRUCE inactivating mutations.

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## INTRODUCTION

The liver is constantly exposed to a variety of viral and bacterial products, environmental toxins, as well as alcohol intake and food antigens. The liver can

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rapidly detect damaging agents and protects itself against the damage without generating widespread inflammation and fibrosis, which are leading causes for liver cancers[1]. Diethylnitrosamine (DEN) is one of the most potent hepatocarcinogens that induces carcinogenic liver injury and development of hepatocellular carcinoma (HCC) in rodents. The application of DEN in rodents has become an attractive experimental model to study the pathogenetic alterations underlying hepato-genotoxic injury and the formation of HCC[2,3]. HCC represents the primary form of liver malignancy and the fourth most common cause of cancer-related deaths worldwide[4]. It has been well documented that the time and incidence of HCCs initiated by DEN differ greatly among mouse strains. DEN-induced HCC development is delayed in the tumor-resistant C57/BL6/J strain as compared to the more sensitive C3H/HE strain[5]. In addition, to induce robust hepatic fibrosis, a hallmark of human HCC development[6,7], administration of a single agent of DEN is insufficient in the C57/BL6/J strain, but when coupled with additional chemicals such as carbon tetrachloride, a fibrogenic agent, hepatic fibrosis is accelerated[8].

BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a hybrid ubiquitin conjugase and ligase[9]. BRUCE has two major pro-survival functions *in vitro*: Promotion of DNA damage repair and suppression of apoptosis. In the cell nucleus, BRUCE promotes DNA damage repair by homologous recombination (HR) to preserve genomic stability[10-12]. To achieve this function, BRUCE is recruited to damaged chromatin adjacent to DNA breaks, where it facilitates chromatin relaxation and accessibility, allowing for HR factors to be loaded onto DNA breaks to facilitate HR repair[11,12]. In the cytoplasm, BRUCE acts as a member of the inhibitor of apoptosis protein (IAP) family of proteins[13-15]. It inhibits the intrinsic mitochondrial pathway of apoptosis by post-translational ubiquitination of pro-apoptotic proteins to promote their degradation by the ubiquitin-proteasome system (UPS)[13,15-18]. In addition to these *in vitro* functions, we and others have demonstrated an *in vivo* anti-apoptosis function of BRUCE in mice, where it suppresses the mitochondrial pathway of apoptosis[16,17]. Furthermore, we have reported a DNA repair function of BRUCE in the protection of the mouse liver[3]. Utilizing an albumin-cre (Alb-cre) mediated liver-specific BRUCE knockout (LKO) mouse model, we have demonstrated for the first time that the BRUCE-ATR (Ataxia Telangiectasia and Rad3-related) signaling axis protects against DEN-induced liver injury and DEN-initiated HCC. BRUCE LKO mice had increased hepatocellular DNA damage accumulation induced by DEN, downregulated ATR-mediated DNA damage response, and an exacerbated HCC development with a fibrotic background[3]. However, the mechanisms underlying the liver fibrosis and HCC have not yet been characterized in this model.

Liver fibrosis is characterized by an excessive accumulation of the extracellular matrix (ECM) resulting in scar tissue formation[19]. Hepatocyte damage and death is an initial consequence of liver injury that initiates several events leading to the recruitment of inflammatory cells and the activation of hepatic stellate cells (HSCs)[20]. Upon liver damage, apoptotic hepatocytes release damage associated molecular patterns (DAMPs) and proinflammatory factors to activate neighboring Kupffer cells and HSCs, thereby inducing persistent inflammatory responses and fibrosis, respectively[19-21]. Activated HSCs are the principal source for deposition of ECM as activated HSCs are responsible for producing an excessive amount of ECM components, mainly collagens[20]. Although liver fibrosis occurs as a wound healing response to chronic toxin-mediated liver injury, chronic liver fibrosis can eventually lead to cirrhosis and HCC[2,22,23]. It is highly likely that the prognosis of liver fibrosis and HCC depend on genetic variations among multiple genes and the interactions of these genes with environmental factors and each other[24].

β-catenin is expressed throughout the adult liver. It is well documented that Wnt/β-catenin signaling regulates liver homeostasis, injury and tumorigenesis[25]. The nuclear expression and accumulation of β-catenin is an indication of its activation. As a leading contributor to chronic liver disease progression, aberrant β-catenin activation is detected during the early stages of chronic inflammation, fibrosis, steatosis, steatohepatitis, and hepatoblastoma as well as late stage of HCC. Aberrant activation of the Wnt/β-catenin signaling pathway (overexpression, mutations, increased nuclear expression of β-catenin) is found in up to 50% of human HCCs and correlated with tumor progression and poor prognosis[26,27].

While a hepatic fibrosis background is a hallmark of human HCC, the regulators of this pathology remain largely unclear. The HCC developed in our BRUCE LKO mouse model is associated with fibrosis[3], suggesting that BRUCE is a regulator of this pathology. Therefore, the BRUCE liver-KO mouse model allows us to examine how BRUCE regulates fibrosis and HCC. In this study, we used our mouse model to examine the pro-fibrotic and pro-tumorigenic signaling pathways. Furthermore, we

also investigated the stages of chronic liver disease to determine the point of fibrosis induction, as well as tumor initiation in the BRUCE liver-KO mice.

## MATERIALS AND METHODS

### *Generation of genetically modified conditional LKO mice*

The BRUCE Alb-Cre KO mice (C57/BL6 mice) were previously described[3]. Genotypes were confirmed by PCR and ablation of BRUCE protein expression in mouse liver tissues confirmed by Western blot.

### *DEN induction*

To initiate chronic liver disease pathogenesis, DEN (Sigma, #N0756) was delivered intraperitoneally (i.p.) into control and BRUCE liver KO mice of 14-day old male mice at 25 mg/kg of body weight. Control and KO mice were sacrificed at the following time points: 3-, 6-, 8-, and 14-mo post exposure to DEN and livers were collected for further studies.

### *Hematoxylin and eosin staining*

Slides were first dewaxed by three xylene washes for 6 min each. Slides were placed into two washes of 100% ethanol for 15 s each followed by a single 95% and 70% ethanol wash, for 15 s each. Slides were then washed with tap water for 1 min then dipped into filtered hematoxylin for 10-12 min. Slides were then rinsed in several washes of tap water until the water was clear. Slides were then dipped twice into a 0.3% Acid Solution (made with ethanol and HCl) then rinsed with tap water for 2 min. Slides were placed into 0.3% ammonia water (made with ammonium hydroxide and distilled water) until the tissue acquired a blue jean color. Slides were rinsed for 2 min in tap water then incubated in 95% ethanol for 20 s. Slides were then placed into Eosin-Y solution for 30 s-1 min, then dehydrated. The dehydration process included: 95% ethanol incubation for 20 s, three 100% ethanol incubations for 20 s, and three xylene incubators for 15 s each. Finally, slides were mounted.

### *Sirius red staining*

Dewaxed slides were hydrated in an ethanol series: 100% for 5 min, 100% for 5 min, 95% for 3 min, and 70% for 3 min. Slides were then incubated in pico-sirius red for one hour followed by two times of washes in acidified water (made with glacial acetic acid). Slides were dried using filter paper then dehydrated in 3 changes of 100% ethanol for 3 min each. Finally, slides were incubated in xylene for 3 min each then mounted.

### *Sirius red image analysis*

To quantify Sirius red images, the scale bar was first measured using the straight-line tool, creating a line along the length of the scale bar. Following this, the scale bar was measured by selecting Analyze > Set Scale to set the scale to micrometers. The scale bar length in micrometers was entered into the "known distance" space and the "um" was entered into "unit of length." To split the image into three channels, we selected Image > Type > RGB stack then select Image > Stacks > Make Montage to view all three channels at once. The green channel (middle) was selected using the square tool then we selected Image > Adjust > Threshold. The slider was moved lower until the collagen is highlighted in red, then "Set" was selected. The square tool was then used to delete the scale bar area, which was then painted white to prevent its inclusion from the calculated area. Finally, to set measurements, we selected Analyze > Set Measurements and selected "area", "area fraction", "limit to threshold", and "display label". Finally, to measure we selected Analyze > Measure. The average of the measurements was taken as well as the standard deviation and graphed to represent the quantification analysis.

### *Liver RNA isolation and RNA sequencing*

Liver samples were placed in RNAlater® Solution (Ambion, #AM7020) and kept at 4 °C. Liver RNA isolation was performed using the mirVana™ miRNA Isolation Kit (Ambion, #AM1560) according to the manufacturer's protocol. RNA was sent to University of Cincinnati Genomics, Epigenomics and Sequencing Core for sequencing analysis.

### *Immunohistochemistry protocol*

Paraffin-embedded (formalin-fixed) liver tissue was sectioned to 5-8 μm thickness. Slides were deparaffinized in a series of xylene treatments (5 min). Slides were then rehydrated in an ethanol series (100% for 5 min, 100% for 5 min, 95% for 3 min, and 70% for 3 min). Slides were rinsed with 1 × PBS for 5 min. Antigen retrieval was performed using a solution of 0.1 M Citric Acid and 0.1 M Sodium Citrate. Antigen retrieval solution was boiled for 10 min, then slides were placed in the solution in Coplin jars and boiled in the microwave, 5 min at 100% power, then 5 min at 60% power twice. During each boil, top off the antigen retrieval solution with distilled water. Endogenous peroxidase was blocked by incubating the slides in 30% $H_2O_2$ in Methanol. Slides were washed twice in PBS for 5 min. Slides are blocked in 5% normal Goat Serum (Vector Labs, #S-1000) in PBST (made with 0.1% Triton X-100) for one hour at room-temperature. Primary antibody incubation was done overnight at 4 °C. Slides are washed twice with PBS for 5 min. Then slides were incubated with a secondary antibody for one hour at room-temperature. Slides were then washed twice in PBS for 10 min and incubated for 30 min with a Vectastain Elite ABC solution according to the manufacturer's instructions (Vector Labs, #PK-6100). Slides were then washed twice in PBST for 5 min. Slides were developed by DAB (Sigma, #D3939). Slides were rinsed in tap water followed by a counterstain with hematoxylin. Slides were rinsed with tap water until water is clear then incubated in an acid rinse for 1 min. Slides were rinsed again and incubated with a bluing solution for 1 min. Slides were rinsed then dehydrated in an ethanol series, followed by xylene washes. Slides were mounted and analyzed. Primary Antibodies used in this study include alpha-smooth muscle actin (α-SMA) (CST 19245T), β-catenin (CST 9582), and Ki67 (CST 12202). The secondary antibody used was a biotinylated goat anti-rabbit immunoglobulin G antibody (Vector Labs, #BA-1000).

### *Image analysis α-SMA data*

Images were analyzed using the "Fiji" version of ImageJ software. Image was opened. Color Deconvolution was selected for images stained specifically in the nuclei. To decrease the interference of cytoplasmic staining, images that had nuclear and cytoplasmic staining, under the image pull down, RGB stack was selected under type. To decrease cytoplasmic signal, go to Image > Type > RGB stack. Once the RGB window appears, select Image > Stacks > Make Montagne then perform color deconvolution. For both nuclear-specific and other images, select the Vectors pulldown > "HDAB". The "Colour_2" image window was selected and measured. The units of intensity derived in the results window were transferred to an excel spreadsheet. The optimal density (O.D.) was calculated using the formula, O.D. = log (max intensity/mean intensity), where the max intensity should be 255. The average optimal density and standard deviations were calculated and graphed.

### *Ki67 scoring*

Slides were examined and percent nuclear positive hepatocytes *per* field (under 20 × magnification) were counted *per* 100 cells.

### *β-catenin scoring*

Slides were examined under 20 × magnification and percent nuclear positive cells were calculated per field using the cell counter feature in Fiji.

### *Preparation of mouse liver subcellular fractions*

Control and LKO livers of mice 3- and 8-mo post-DEN exposure were harvested. Cytoplasmic and nuclear fractions were prepared from these livers using the Thermo Scientific Subcellular Protein Fractionation Kit for Tissues (Cat. No. 87790), according to the manufacturer's recommendations.

### *Immunoblotting*

Protein extracts (40-100 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter. The filter was blocked with 5% dry milk in PBST for 1 h at room temperature, followed by incubation with primary antibody overnight at 4 °C or 3 h at room temperature. The filter was then washed in PBST 3 times for 5 min each, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing with PBST, the filter was developed with ECL for 1 min and exposed to X-ray film. Quantification of the polypeptide bands was performed with the Fiji software.

### Liver cDNA preparation and RT-PCR analysis

Liver cDNA was isolated from RNA templates as described previously[3]. The PCR mix was made using the 1/10 cDNA solution as the template, primers, and the DreamTaq PCR master mix (2 ×) (Thermo Fischer Scientific, #K1071). The PCR products were separated by electrophoresis on a 2% agarose gel. RT-PCR was setup according to the iQ™ Sybr® Green Supermix (BioRad, #170-8882) with the 1/10 cDNA as the template. PCR conditions for the semi-quantitative and RT-PCR are as follows: Initial denaturation-95 °C for 1 cycle; for 40 cycles: Denaturing-95 °C, annealing-Tm as indicated below, extension-72 °C; and an optional hold at 4 °C. Gene primers were obtained from Integrated DNA Technologies.

### RNA sequencing

RNA from Control and LKO livers of mice exposed to DEN for 8 mo was isolated as described previously[3]. RNA samples were submitted to the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati.

### Cell culture and transfection

HepG2 and THLE2 cells were purchased from ATCC. HepG2 cells were cultured in DMEM high glucose medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a $CO_2$(5%) incubator. THLE2 cells were cultured in special medium as suggested by ATCC. The siRNA transfection of cells was mediated by lipofectamine RNAiMAX (Thermo, Cat. No. 13778030) following manufacturer's instruction.

### Immunofluorescence analysis

THLE2 cells were fixed and stained with primary antibodies against BRUCE and protein kinase A (PKA). After washes, cells were incubated with secondary antibodies coupled with Alexa Fluor 594 and Alexa Fluor 488, respectively. Samples were analyzed and photos acquired under Zeiss Fluorescence Microscope.

### Preparation of whole cell lysates of human cancer cells

Cell pellets were lysed and sonicated to elute whole cell lysates in RIPA buffer with protease inhibitor tablets (Roche) and phosphatase inhibitors of 10 mmol/L NaF and 50 mmol/L β-glycerophosphate. The lysates were centrifuged at 15000 g for 20 min and the supernatant was collected.

**Antibodies:** The antibodies used in this study were: BRUCE from Novus (NB300-264); α-SMA (CST 19245T); Total β-catenin (CST 9582); Ki67 (CST 12202); phospho-β-catenin Ser-675 (CST 4176)**;** Lamin A/C (CST 4777); Actin (CST 3700); Glyceraldehyde-3-phosphate dehydrogenase (CST 2118); phospho-PKA substrate (RRXS*/T*) (100G7E) (CST 9624)

**Reagents and siRNAs:** DEN (#N0756) from Sigma; BRUCE siRNA and control siRNA were synthesized by Dharmacon[16]. Control siRNA sequence is UUCUCCGAACG-UGUCACGUdTdT. The BRUCE siRNA sequence is GGCACAGCAGCTCTTATCA.

**Data analysis:** The results are expressed as the means ± SD of the determinations. The statistical significance of the difference was determined by a two-tailed Student's *t*-test.

## RESULTS

### Liver-specific KO of BRUCE promotes early tumor onset and an exacerbated HCC mimicking patient-like histological features in DEN-exposed mice

DEN administration in mice promotes chronic liver injury and HCC development[5]. Control (loxp/Loxp; Alb-Cre$^-$) and LKO (loxp/Loxp; Alb-cre$^+$) mice were exposed to DEN at postnatal day 15 to induce liver injury and malignant transformation to HCC. Mice with and without BRUCE expression in the liver were sacrificed at various time points for studies of liver disease progression (Figure 1A). Fifty percent of the LKO mice developed tumors 8 mo after DEN administration, while the control littermates did not begin to develop tumors until 10 mo post DEN administration (Figure 1B and C). By 14 mo, 100% of the LKO mice developed HCC ($n$ = 17) whereas 80% of the control developed HCC ($n$ = 10) (Figure 1B). More of the LKO mice developed an exacerbated HCC phenotype (Figure 1B and D). Histology of the HCC tumors revealed a trabecular architecture identical to the histologic patterns of HCC patients

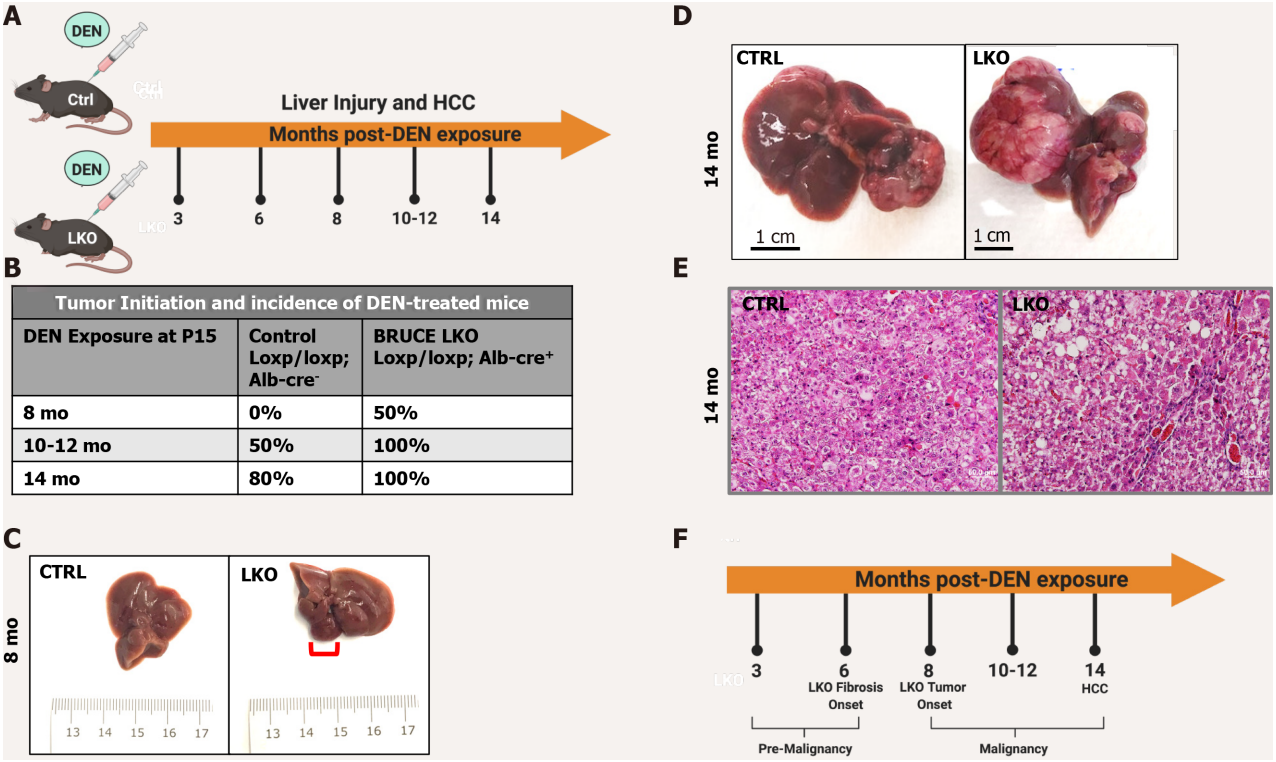



**Figure 1 Diethylnitrosamine-induced hepatic malignancy leads to earlier tumor initiation and an exacerbated patient hepatocellular carcinoma-like phenotype in BRUCE LKO mice.** A: Diethylnitrosamine (DEN) model. Control and BRUCE LKO mice were treated with DEN over a time course schedule as indicated; B: Tumor onset in LKO mice happened after 8 mo post-DEN exposure, while tumor onset did not begin in control mice until 10-12 mo; C: Tumor in LKO mouse traced in red; D: After 14 mo of DEN exposure, control and LKO mice develop hepatocellular carcinoma; however, the LKO mice have a more exacerbated phenotype; E: Hematoxylin and Eosin staining reveals a trabecular histologic feature in LKO but not control; F: Timeline of key events of DEN-induced hepatic malignancy model. DEN: Diethylnitrosamine; LKO: Liver-specific knockout; HCC: Hepatocellular carcinoma; CTRL: Control; BRUCE: BIR repeat-containing ubiquitin conjugating enzyme.

with poor prognosis (Figure 1E). The pathogenic highlights of this study were summarized into a pre-malignant and a malignant stage (Figure 1F). Together the data suggests that hepatic BRUCE deficiency accelerates and exacerbates DEN-induced HCC development in C57/BL6/J mice.

### *Hepatic BRUCE deficiency promotes hepatocyte damage and compensatory proliferation*

We have reported that DEN administration to BRUCE LKO mice induces more DNA damage accumulation in hepatocytes than that in control mice. We have also demonstrated that the repair of DEN induced hepatocellular DNA damage requires the BRUCE-ATR DNA repair axis[3]. Recently it has been reported that excessive hepatocyte apoptosis plays a tumor-promoting role in nonalcoholic steatohepatitis (NASH)-associated liver cancer in mice[28]. We and others have reported on the importance of BRUCE in the regulation of DNA damage response as well as inhibiting apoptosis[11,12,16], yet the connection of the aforementioned roles of BRUCE have not yet been examined for whether sustained DNA damage correlates with or triggers apoptosis in hepatocytes[29,30]. In addition, DEN exposure induces hepatocellular DNA damage and gene mutations in mice which is a carcinogenic mechanism underlying DEN-initiated liver injury and development of HCC[31]. However, the apoptotic regulators that are critical in the regulation of hepatic apoptosis induced by exposure to DEN have not been well established. We reasoned that BRUCE LKO mice have lost the IAP function of BRUCE in the liver, thus DEN exposure of LKO mice could induce more prominent hepatocyte apoptosis than in the control. Indeed, our RNA-seq analysis found that DEN administration results in a higher level of apoptotic gene expressions (Figure 2A), suggesting that hepatic BRUCE protects against DEN-induced hepatic apoptosis.

One of the pathological outcomes of hepatocyte apoptosis is the facilitation of hepatic inflammation and fibrosis through two major mechanisms: (1) Replenishment of lost hepatocytes *via* compensatory proliferation of quiescent hepatocytes through

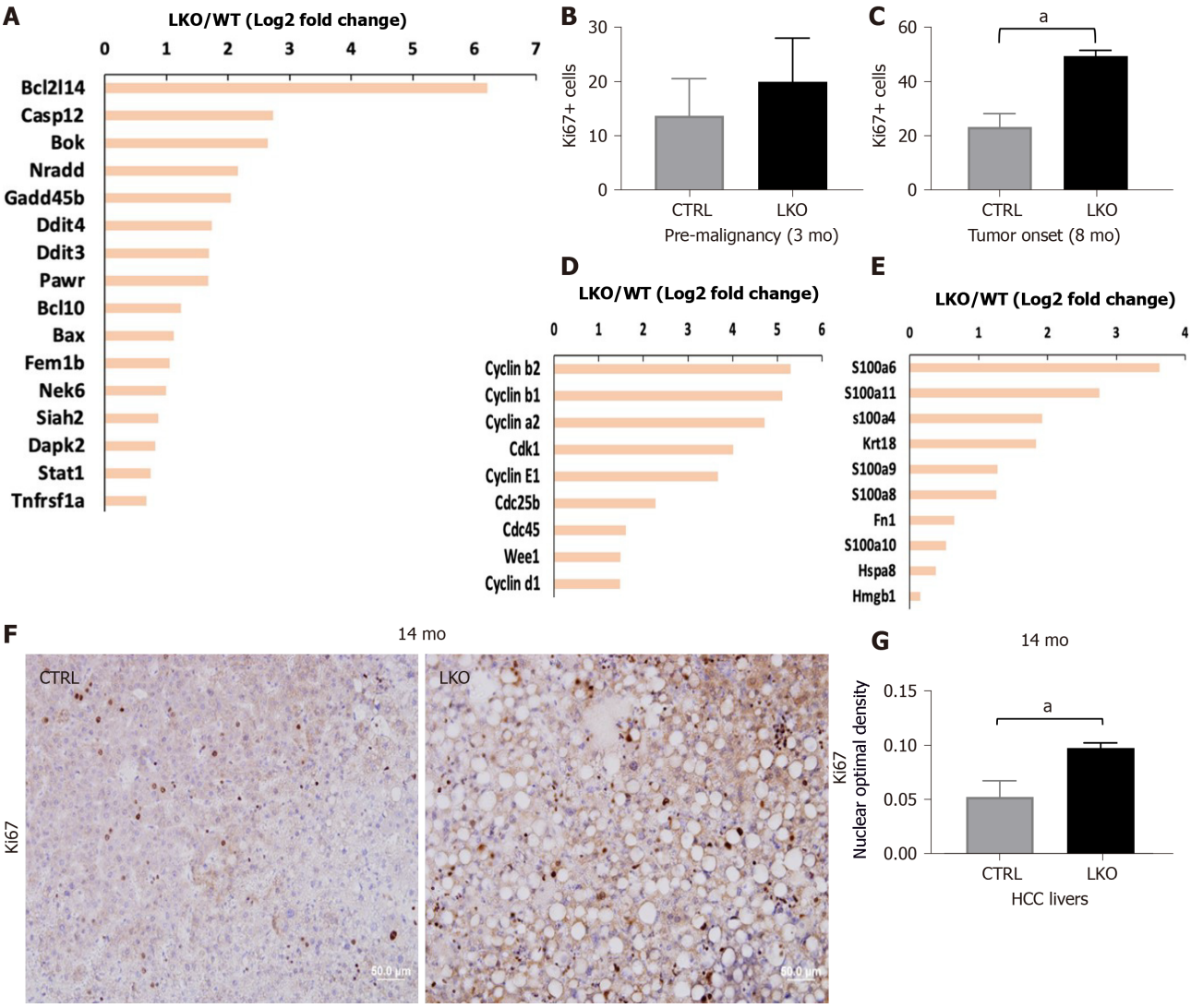



**Figure 2 BRUCE deficiency increases diethylnitrosamine-induced liver injury and hepatic proliferation.** A: Apoptotic gene expression is increased in liver-specific BRUCE KO livers at the time of tumor onset; B: Hepatic proliferation was measured by immunohistochemistry staining of the livers against Ki67. Livers exposed to diethylnitrosamine for 3 mo have an increase of Ki67+ cells; C: At the time of tumor onset in LKO livers there is an increase of Ki67+ positive cells; D: At the time of tumor onset, RNA-seq analysis reveals an increase of known cell cycle markers; E: Damage associated molecular patterns in the LKO livers; F and G: Ki67 staining by immunohistochemistry in 14 mo hepatocellular carcinoma livers, as well as quantification, show an increase of proliferation in LKO livers. $^{a}P<0.05$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; CTRL: Control.

feed-forward apoptosis-proliferation circles[26,32]; and (2) Release of proinflammatory DAMPs which result in liver inflammation and fibrosis[33]. Our immunohistochemical staining of a proliferation marker, Ki67, showed enhanced hepatocyte compensatory proliferation in BRUCE-deficient mouse livers compared to the control, during the pre-malignant stage (3 mo post DEN administration) (Figure 2B). This proliferation continued to the time of tumor onset (8 mo post DEN exposure) (Figure 2C). This elevated hepatic proliferation is supported by increased expression of multiple critical cell-cycle regulatory genes in BRUCE-deficient livers by RNA-seq analysis (Figure 2D). In addition, gene expression of DAMP molecules including HMGB1 and S100 are also increased in KO livers (Figure 2E). Moreover, increased cellular proliferation in human HCC has been correlated with tumor progression and poor prognosis[34,35]. During the malignant stage of 14 mo post DEN exposure, the HCC tissues from LKO mice exhibited increased Ki67 expression compared to the HCCs from control mice (Figure 2F and G). Together our data demonstrate that hepatic BRUCE deficiency results in elevated hepatic cell apoptosis and proliferation. Moreover, the release of DAMPs would exacerbate liver inflammation.

### *Hepatic BRUCE deficiency accelerates fibrosis in mice exposed to DEN*

Hepatic fibrosis is characterized by an excessive accumulation of ECM in which collagen fibers are the major component produced by activated HSCs. A unique

feature of liver cancer is its close association to liver fibrosis. More than 80% of HCCs develop in fibrotic or cirrhotic livers, suggesting an important role of liver fibrosis in the pre-malignant environment of the liver[6]. Although fibrosis is a feature of human HCC, it is not necessarily recapitulated in various murine liver disease models, which makes these models inferior in modeling the progression of fibrosis to HCC. Interestingly, the LKO mice developed significant fibrosis in the pre-malignant stage at 6 mo following DEN administration, as shown by the Sirius red staining of collagen fibers (Figure 3A and B). Notably the collagen fibers in BRUCE KO livers showed signs of the advanced stage of "bridging fibrosis"[36], as evidenced by the fibrotic spreading that extends between portal and central vein areas (Figure 3A). This bridging fibrosis is in sharp contrast to the control mice in which fibrosis was limited to portal or venular areas (Figure 3A). Upon chronic liver damage, injured hepatocytes undergo cell death which releases DAMP molecules and activate the normally quiescent HSCs[33]. To directly examine HSC activation, we analyzed the expression of α-SMA, which is expressed by HSCs and reflects their activation into a myofibroblast-like phenotype. The results revealed an increased α-SMA expression in HSCs which suggests an elevated activation of HSCs in LKO mice as compared to the control (Figure 3C and D). To validate these pro-fibrotic events at the gene expression level, RNA-seq analysis was conducted and multiple pro-fibrotic or fibrotic genes were found to have higher levels of expression in the LKO liver than that of the control (Figure 3E). Furthermore, elevated inflammatory gene expression was evident in LKO mice compared to control (Figure 3F). As liver fibrosis is characterized by the deposition of fibrillar collagens, the elevated gene expression of multiple types of collagens (Figure 3E) support the elevated fibrosis phenotype. In addition, HSCs contribute to the accumulation of ECM by producing excessive amounts of pro-fibrotic factors such as tissue inhibitor of metalloproteinases (TIMPs)[37]. Indeed, we observed an elevated level of *TIMP1* gene expression in LKO mice (Figure 3F). Moreover, the turnover of ECM, controlled by matrix metalloproteinases (MMPs), also promotes fibrosis and therefore a number of MMPs are highly expressed in liver fibrosis[19]. We also found that the gene expression of a number of MMPs were increased in LKO mouse livers (Figure 3F). Altogether, the data indicate that DEN exposure of BRUCE LKO mice aggravates hepatic fibrosis at both the histological and gene expression levels.

To ascertain whether the hepatic fibrosis is sustained during the malignancy stage, HCC tissues from 14-mo exposed mice were analyzed for both Sirius red staining and α-SMA expression. The results from both analyses demonstrated an exacerbated fibrosis that concur with HCCs (Figure 3G-J), demonstrating that sustained and chronic fibrosis coexists with HCCs, which is a hallmark of human HCCs. Altogether, hepatic BRUCE deficiency in the DEN-induced HCC model is sufficient to drive fibrosis in both pre-malignant and malignant stages.

### *Hepatic BRUCE deficiency promotes β-catenin signaling in the premalignant stage*

Activated Wnt/β-catenin pathway is indicative of the stabilization of β-catenin in the cytoplasm and its subsequent translocation to the cell nucleus, where it achieves its gene transcription function by activating gene expressions for promotion of hepatic inflammation and fibrosis[38-41]. Remarkably, there was a pronounced increase of nuclear localization of β-catenin in the liver sections from BRUCE LKO mice in the pre-malignant stage of 3 mo (Figure 4A and B) as well as at tumor onset of 8 mo post DEN exposure (Figure 4C and D). The nuclear localization of β-catenin in liver tissue sections assessed by immunohistochemistry (IHC) was further validated by a biochemical approach. Specifically, liver protein extracts from mice at the pre-malignant and tumor-onset stages were further fractionated into cytoplasmic and nuclear fractions and immunoblotted for β-catenin. There was a much higher level of total β-catenin in the nuclear fraction than in the cytosol of both control and LKO samples (Figure 4E). Promoted by the increase of nuclear β-catenin in liver tissue sections, we postulated that there could be an increase in β-catenin activity in the nuclear fraction of LKO liver samples. To test this possibility, we compared the levels of β-catenin phosphorylation at Ser-675, an activated form of β-catenin phosphorylated by cAMP-dependent PKA[42,43] in the control and LKO samples. Indeed, there was a dramatic increase of phospho-β-catenin at Ser-675 in the nuclear fractions of the LKO livers in both stages of pre-malignancy and tumor onset (Figure 4E), suggesting that β-catenin plays an important role in the promotion of hepatic inflammation and fibrosis in the early, pre-malignant stage. The RNA-seq analysis confirmed an upregulation in the expression of multiple Wnt ligands and regulators (Figure 4F), and β-catenin target genes (Figure 4G). Together the data demonstrate an aberrant activation of the Wnt/β-catenin pathway, which plays a pro-

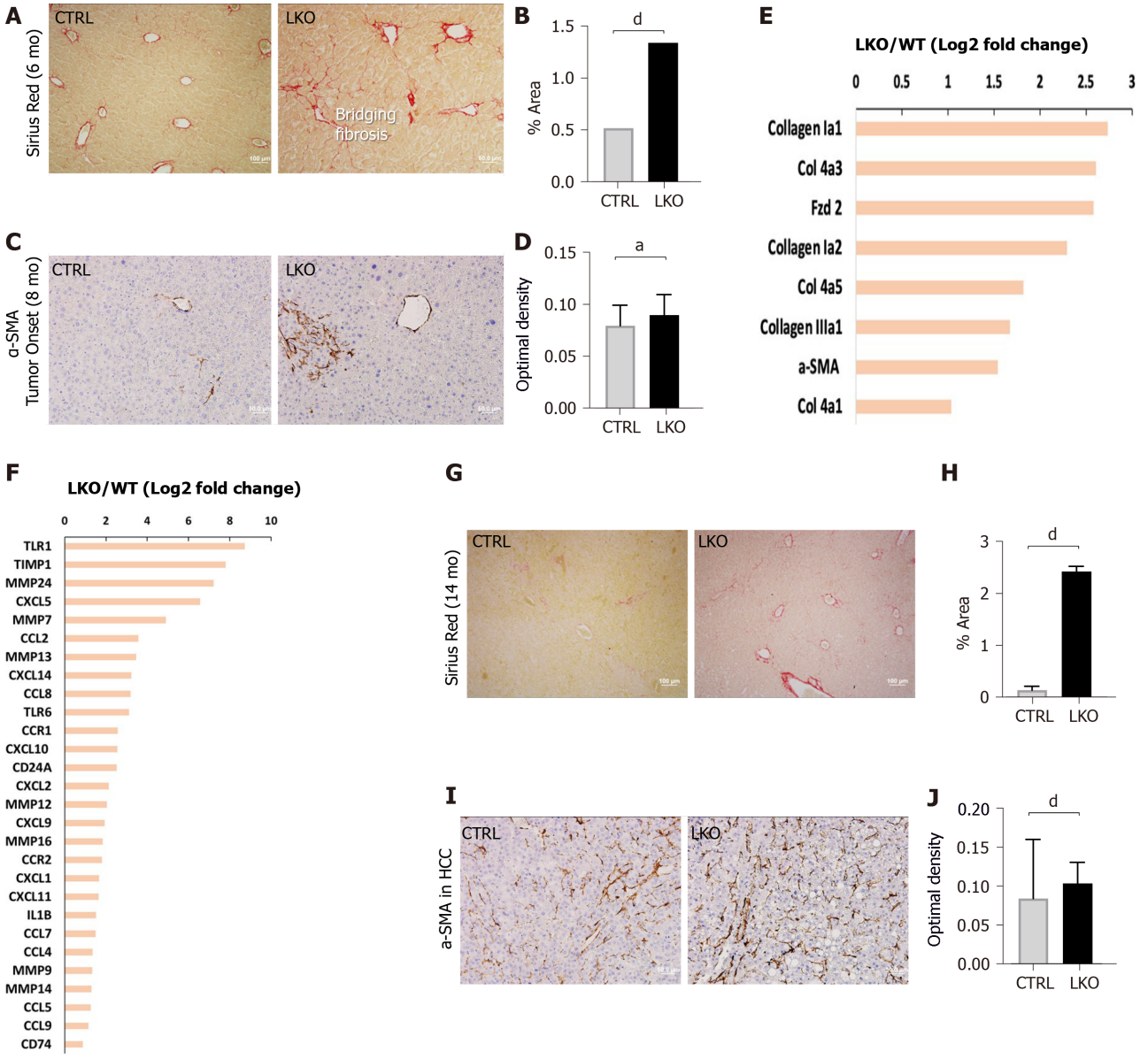



**Figure 3 BRUCE deficiency accelerates and increases diethylnitrosamine-induced fibrosis.** A and B: Sirius red staining of control and liver-specific BRUCE KO livers 6 mo post-diethylnitrosamine (DEN) exposure show an increase of Sirius red staining in the LKO livers, verified by quantification to the right; C and D: α-smooth muscle actin (SMA) immunohistochemistry at LKO tumor onset show an increase in α-SMA in LKO livers which is quantified in; E and F: RNA-seq analysis at the time of LKO tumor onset reveal that LKO livers demonstrate key patterns of human hepatic fibrosis, such as increased collagens and increased α-SMA as well as increased inflammation-related markers, such as CCL2; G and H: Sirius red staining of 14 mo post-DEN exposed HCC livers reveal an increase of collagen deposition, which was quantified; I and J: α-SMA immunohistochemistry of HCC livers, including quantification demonstrate an increase of activated hepatic stellate cells in 14 mo DEN-exposed LKO livers. $^{a}P < 0.05$; $^{d}P < 0.001$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; α-SMA: α-smooth muscle actin; CTRL: Control.

inflammatory and fibrotic role in LKO livers as shown in Figure 3. Collectively, these results indicate a new mechanism for an upregulated Wnt/β-catenin pathway resulting from hepatic BRUCE deficiency during the pre-malignant stage of hepatic inflammation and fibrosis in mice.

### *Hepatic BRUCE deficiency upregulates β-catenin signaling in malignant HCC livers*

At age 14 mo, BRUCE LKO mice had a more exacerbated DEN-induced HCC phenotype (Figure 1D) consistent with a human HCC-like trabecular histological feature (Figure 1E). Livers from LKO mice maintained the nuclear localization of β-catenin (Figure 5A and B). Additionally, mRNA levels of β-catenin were measured by qRT-PCR and found to be increased in the LKO livers (Figure 5C). To confirm the IHC analysis of increased β-catenin, we analyzed β-catenin protein levels by Western blot analysis (Figure 5D) and noticed a concomitant increase in protein expression in LKO HCC livers. Additionally, cyclin D1, a downstream target of β-catenin, was increased

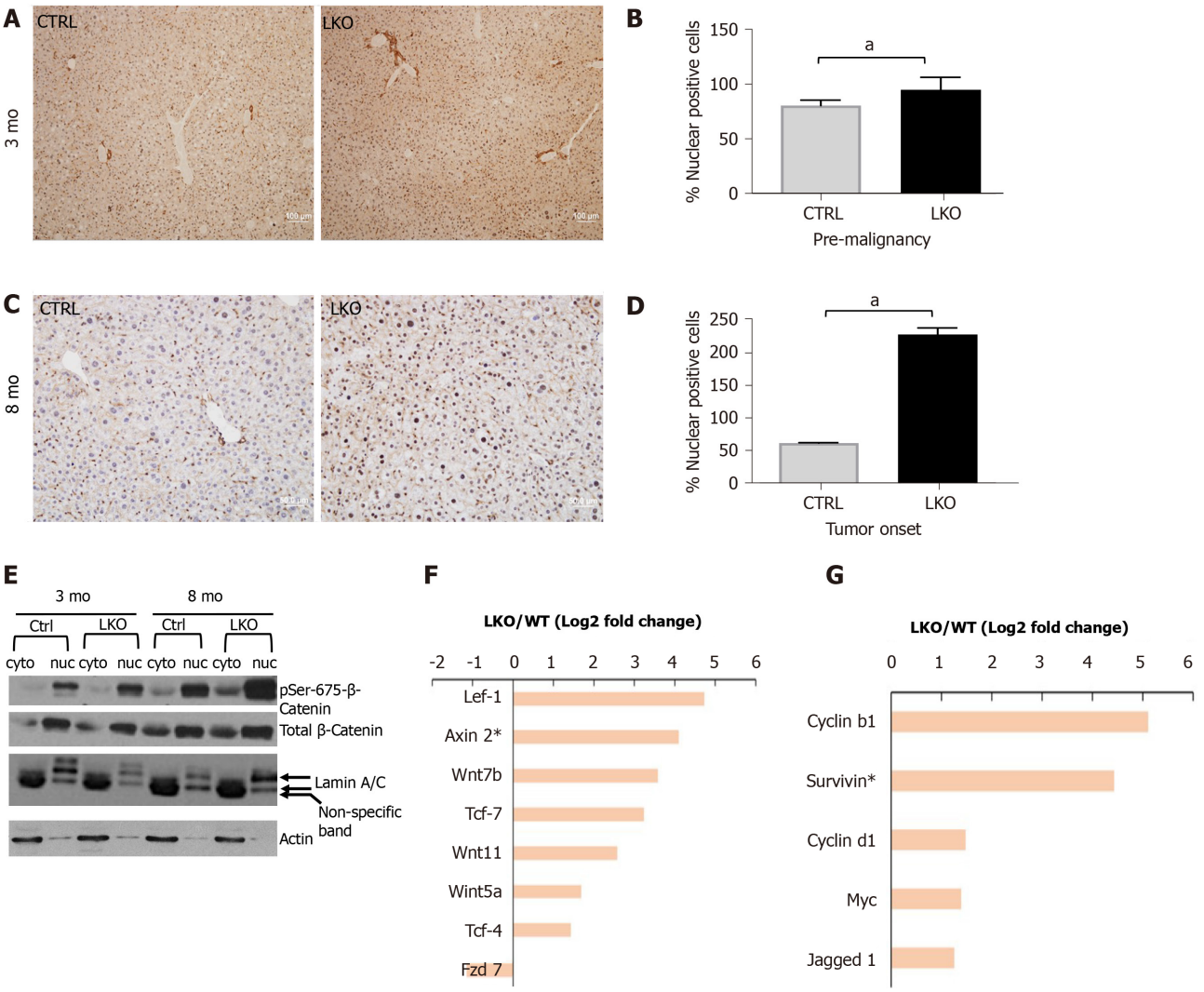



**Figure 4 BRUCE deficiency promotes β-catenin activation in mice.** A and B: After three months post-diethylnitrosamine (DEN) exposure, there is an increase in nuclear β-catenin staining by immunohistochemistry in the BRUCE KO livers. See quantification to the right; C and D: At tumor onset in LKO livers, nuclear β-catenin, shown by immunohistochemical staining, is increased in the LKO livers and quantified to the right; E: Phosphorylation of β-catenin at Ser-675 is increased in both the cytoplasmic and nuclear fractions of the LKO livers both pre-malignancy (3 mo post-DEN) and at tumor onset (8 mo post-DEN); F and G: At the time of tumor onset in LKO livers (8 mo post-DEN exposure), RNA sequencing analysis determined an increase in several canonical Wnt/β-catenin pathway members and target genes. [a]*P*< 0.05. LKO: Liver-specific knockout; CTRL: Control.

at the protein level in LKO HCC tissues (Figure 5E). Together the data demonstrate that BRUCE deficiency increases β-catenin nuclear accumulation in DEN-induced HCC. As β-catenin activity plays a critical oncogenic role in the development of HCC, these data suggest that upregulated β-catenin activity induced by BRUCE deficiency contributes to the accelerated HCC development in mice.

### *Loss of BRUCE stabilizes β-catenin through regulation of PKA activity in vitro and in vivo*

An increase of nuclear β-catenin in LKO mice at the stages of pre-malignant (3 mo), tumor onset (8 mo) and malignant (14 mo) (Figure 4) suggests that hepatic BRUCE regulates β-catenin activation. To investigate the underlying mechanisms, we utilized an *in vitro* cell culture system of the human liver cancer cell line HepG2 which allows for knockdown (KD) experiments. We first determined if an increase of phospho-β-catenin at Ser-675 can be induced by KD of BRUCE expression in HepG2 cells. HepG2 cells were transfected with either a control or a BRUCE siRNA followed by preparation of the whole cell protein lysates for Western blot analysis. Knockdown of BRUCE in HepG2 cells resulted in increased levels of both the total β-catenin protein and phospho-β-catenin at Ser-675 (Figure 6A), demonstrating that BRUCE negatively regulates β-catenin activation. Since phosphorylation of β-catenin at Ser-675 is PKA-dependent, we reasoned that loss of BRUCE expression might be linked to the

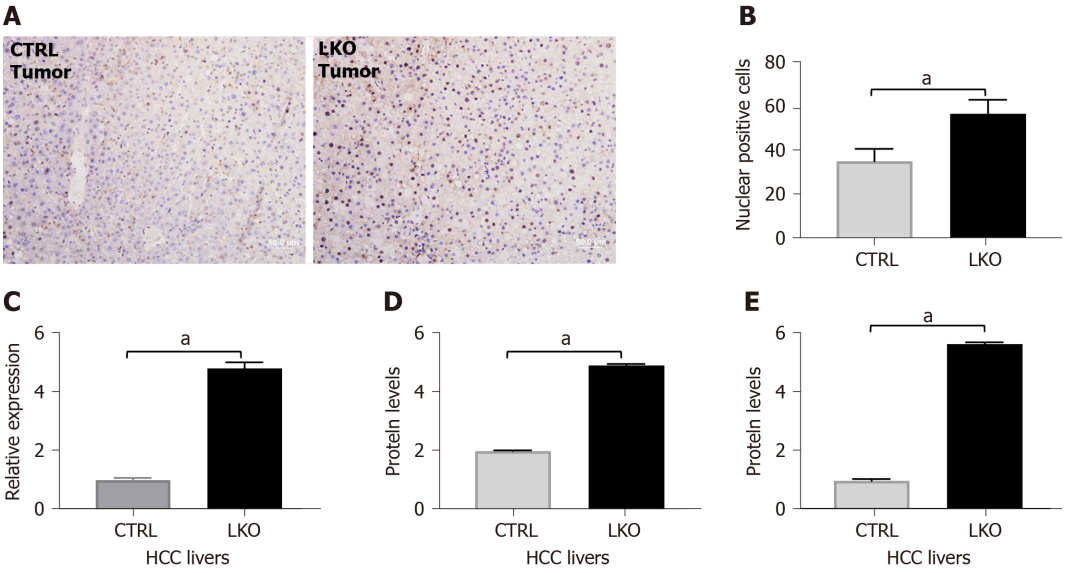


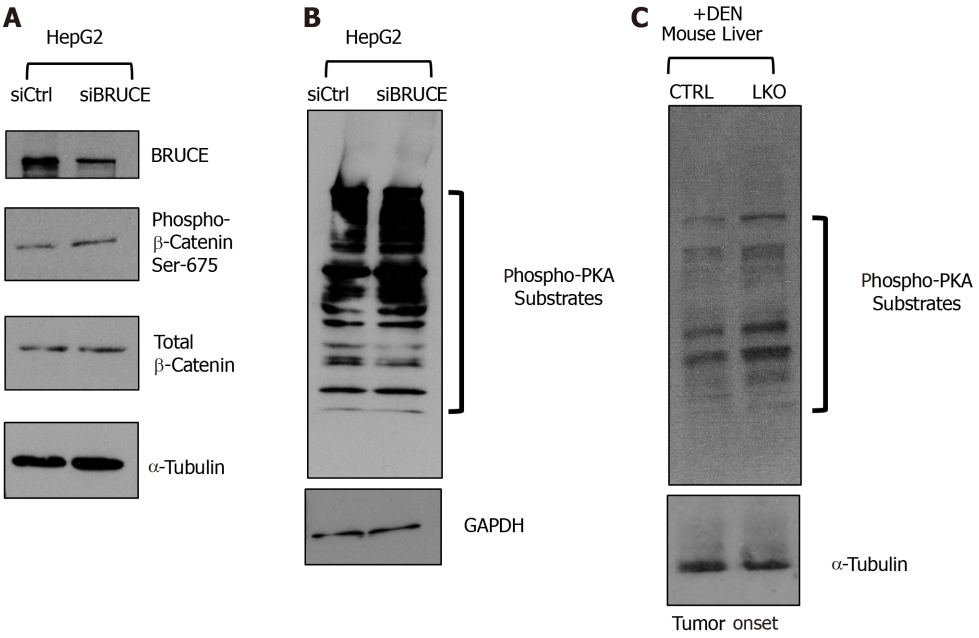

**Figure 5 BRUCE deficiency increases nuclear β-catenin and activity in hepatocellular carcinoma livers.** A and B: Immunohistochemistry of β-catenin in liver tumors after 14 mo of DEN-exposure which is quantified in; C: RT-PCR analysis of β-catenin in liver tumors after 14 mo post-DEN revealed an increase in mRNA levels in BRUCE knockout livers compared to control; D and E: Graphical representation of western blot analysis of β-catenin in liver tumors after 14 mo of DEN-exposure and cyclin D1, a downstream target of β-catenin. $^{a}P < 0.05$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; CTRL: Control.

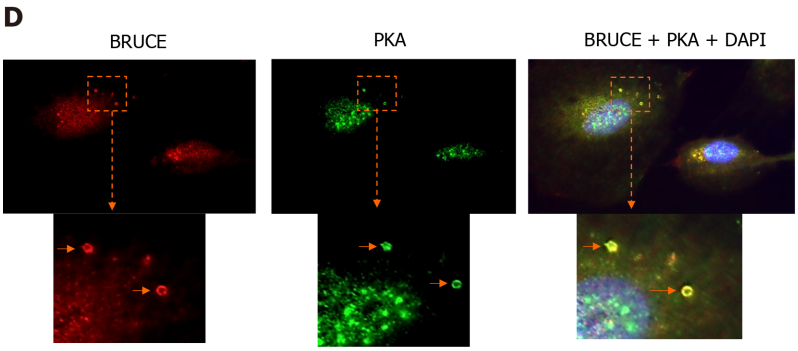
activation of PKA activity. To test this possibility, we compared PKA activity in cells with and without BRUCE knockdown by examination of the levels of PKA phosphorylated substrates using an antibody specific to PKA substrates. The Western blotting results showed an increase in phospho-PKA substrates in BRUCE KD cells (Figure 6B), demonstrating that the loss of BRUCE expression induces activation of PKA, thereby resulting in a higher PKA activity. This link of BRUCE loss to PKA activity elevation was also reproduced *in vivo* in DEN-exposed mice, demonstrated by the increase of phospho-PKA substrates by Western blot analysis of liver protein extracts at the time of tumor onset in the LKO mice (Figure 6C). To further delineate the correlation of BRUCE loss with the upregulation of PKA activity, we performed co-immunofluorescence analysis of both proteins with a human normal hepatocyte line, THLE2. We found that BRUCE and PKA were co-localized in endosomes (Figure 6D). BRUCE is reported to be on endosomes in non-hepatocytes[44]; however, this is the initial report of BRUCE and PKA colocalization on endosomes in human hepatocytes. This colocalization suggests that in endosomes of hepatocytes, BRUCE interacts with PKA to restrain hyperactivation of PKA, whereas loss of BRUCE releases the restriction of PKA activation and thus PKA activity is elevated.

With the observation of PKA-dependent phospho-β-catenin at Ser-675 upon liver injury with DEN, we propose a new signaling axis of BRUCE-PKA-β-catenin in the regulation of liver function. In this axis, hepatic BRUCE suppresses hyperactivation of PKA activity, thereby preventing aberrant phosphorylation and activation of β-catenin as well as its subsequent profibrogenic and oncogenic functions. In livers devoid of BRUCE, there is a loss of the BRUCE-dependent negative regulation of PKA activation; therefore, PKA phosphorylates and activates β-catenin to aggravate hepatic fibrosis and accelerate HCC (schematic, Figure 6E).

## DISCUSSION

We have previously reported the clinical relevance of BRUCE in liver diseases in which BRUCE downregulation is found in a large portion of liver disease patients, including fibrosis, hepatitis, NASH, and HCC[3]. Upon assessment of BRUCE protein expression levels in liver specimens (male and female patients), we found that BRUCE levels were reduced in 54.5% of hepatitis samples ($n$ = 22), 46.7% of cirrhosis samples ($n$ = 30), and 84% of HCC samples ($n$ = 25)[3]. These findings suggest a correlation between BRUCE expression levels and various liver disease stages. Additionally, we previously reported a 6% rate of deleterious BRUCE mutations in HCC patients, as deduced through the Cancer Genome Atlas. This rate was comparable to the mutation rate of other key DNA damage response (DDR) genes such as, *ATR*, *BRCA1* and



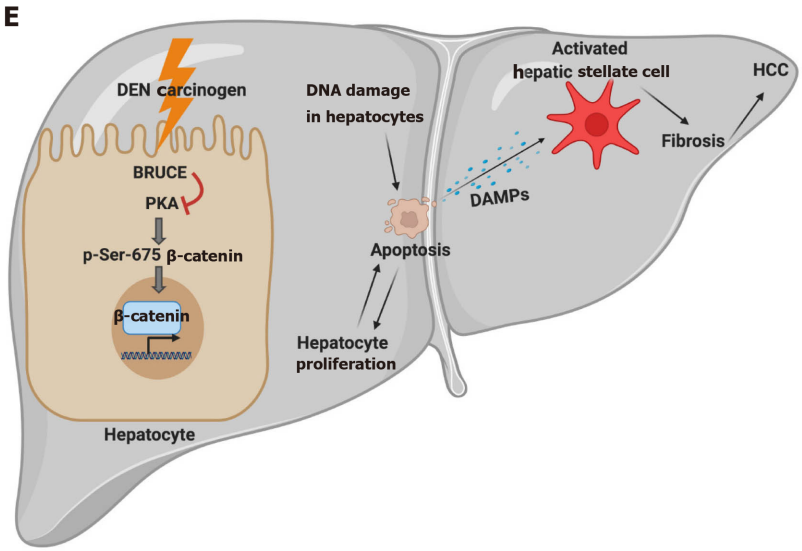




**Figure 6 BRUCE-dependent regulation of β-catenin links to protein kinase activity.** A: Whole cell lysates of HepG2 cells transfected with either an siCtrl or siBRUCE were blotted for BRUCE, phospho- and total-β-catenin, as well as a tubulin control; B: Lysates described in (A) were blotted for phospho-protein kinase A (PKA) substrates to measure PKA activity, as well as a glyceraldehyde-3-phosphate dehydrogenase control; C: Western blot analysis of mouse liver tissue lysates from control and liver-specific BRUCE knockout (LKO) exposed to diethylnitrosamine (DEN) for phospho-PKA substrates showing an increase in PKA activity in LKO livers at the time of tumor onset (8 mo); D: Immunofluorescence staining showing colocalization of BRUCE (red) and PKA (green) in endosomes (arrows) in normal human THLE2 hepatocyte line with cell nucleus counterstained with DAPI. The cellular areas outlined in dashed squares are enlarged and shown below; scale bar 20 μm; E: A working model showing a new BRUCE-PKA-β-catenin signaling axis involved in the regulation of fibrosis and HCC. BRUCE regulates β-catenin activation by inhibiting PKA-dependent phosphorylation-activation of β-catenin for hepatic proliferation and carcinogenesis. Mechanistically, BRUCE interacts with PKA in the hepatocyte cytoplasm to restrain PKA activity. When this interaction is disrupted by KO of BRUCE in the mouse liver, or by KD of BRUCE expression in

liver cancer cell line, the repression of PKA is derepressed and PKA-dependent phosphorylation-activation of β-catenin at Ser-675 occurs which results in hepatic proliferation. Meanwhile hepatocytes undergo apoptosis induced by DEN-DNA damage and these apoptotic hepatocytes release damage associated molecular patterns to activate hepatic stellate cells. The BRUCE-PKA-β-catenin signaling axis, together with DEN induced DNA damage, hepatic cell death, and oxidative stress, result in an early onset of fibrosis and accelerated HCC. DEN: Diethylnitrosamine; LKO: Liver-specific knockout; BRUCE: BIR repeat-containing ubiquitin conjugating enzyme; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PKA: Protein kinase A; DAPI: 4',6-diamidino-2-phenylindole; HCC: Hepatocellular carcinoma; DAMPs: Damage associated molecular patterns; CTRL: Control.

*BRCA2* in HCC patients. Furthermore, we delineated frameshift and nonsense mutations of BRUCE, particularly in BRUCE's ubiquitin conjugating (UBC) domain[3]. Our group has previously established that the UBC domain of BRUCE is necessary for its DDR function[11,12]. Therefore, deleterious BRUCE mutations would inactivate BRUCE's DDR function and could contribute to overall genomic instability leading to HCC development[3,12].

BRUCE has two major functions. It facilitates DDR to maintain genomic stability and as an IAP-family member, it suppresses apoptosis to maintain cell viability[15,16]. Liver KO of BRUCE abolishes both of these functions in the liver. While DDR inactivation and genomic instability promote HCC development in BRUCE-deficient settings[3], inflammation and fibrosis are well characterized risk factors in HCC pathogenesis[45,46]. Therefore, we focused on the progression of fibrosis in this study. The loss of hepatic BRUCE together with DEN administration contributes to an increase in DAMPs which lay the foundation for fibrosis as well as contribute to the progression of HCC. Loss of BRUCE's anti-apoptotic function results in elevated hepatocyte apoptosis and the release of DAMPs, which promote compensatory hepatocyte proliferation (Figure 2). Upon release of DAMPs, the quiescent HSCs will become activated which will progressively trigger the onset of fibrosis (Figure 3). As previously reported, increased hepatic fibrosis and compensatory proliferation are contributors to both HCC and poor prognosis[6,33-35,47].

Nonetheless, there are a number of risk factors that predominate the development of HCC in humans. Infection with hepatitis B or C viruses, alcohol consumption and metabolic syndrome are also major risk factors. Since DNA damage and apoptosis are likely common to liver fibrosis and HCC induced by these risk factors, BRUCE is anticipated to also protect against liver diseases induced by these risk factors, which is our future direction for this research. In addition to DNA repair and anti-apoptosis, BRUCE also regulates autophagy and cellular energy levels as we previously published[48]. As autophagy is involved in the regulation of liver homeostasis and liver injury and because of the robust autophagic activity found in liver tissue[49-51], it is likely that BRUCE also regulates liver injury, fibrosis and carcinogenesis through autophagy. BRUCE likely coordinates multiple signaling pathways including autophagy, DNA repair and apoptosis to preserve liver homeostasis.

Liver diseases present a huge health threat and are on the rise. However, the molecular pathways leading to fibrosis and HCC are not fully defined, which have hampered the development of mechanism-based therapeutic intervention. Aberrant β-catenin activation and its nuclear localization in the promotion of liver disease is found in up to 50% of human HCCs[22,27]. It is believed that aberrant β-catenin activation is a key contributor to chronic liver disease progression. Finding upstream regulators of β-catenin pathogenic activation is necessary for identification of the right sub-group of chronic liver disease patients for considering mechanism-based therapeutic targeting.

This study provides new insights into the molecular pathways that contribute to liver fibrosis and HCC. We revealed a previously unknown hepatocellular "BRUCE-PKA-β-catenin signaling axis" involved in the regulation of fibrosis and HCC (Figure 6E). In this signaling axis, we have identified a novel role of hepatocellular BRUCE in the suppression of aberrant activation of β-catenin through preventing PKA-mediated phosphorylation and activation of β-catenin both *in vivo* and *in vitro*. Mechanistically, we have revealed a novel interaction between BRUCE and PKA in the hepatocyte cytoplasm at endosomes, which provides the support for a functional interaction of these two proteins in the regulation of liver functions. This is further supported by our observations that upon disruption of BRUCE function either by liver KO (animal) or KD (HepG2 cell line), the repression of PKA is derepressed and PKA-dependent phosphorylation of β-catenin at Ser-675 occurs. This β-catenin phosphorylation is associated with the early onset of fibrosis and accelerated HCC in our mouse model (Figure 6E).

How might BRUCE regulate PKA protein levels and its activation? BRUCE itself is a hybrid protein harboring ubiquitin conjugase and ligase activities[15]. During the intrinsic mitochondrial pathway of apoptosis, BRUCE catalyzes ubiquitination of pro-

apoptotic proteins SMAC, Caspase-9 and others to reduce cellular apoptotic capacity to tip the balance of life and death towards cell death[17,51]. In addition, during DNA damage response induced by DNA double-strand and single-strand breaks, BRUCE ubiquitin ligase activity cooperates with the deubiquitinase USP8 to regulate ATM and ATR DNA damage responses to facilitate HR repair of DNA breaks[3,11,12]. Therefore, we propose that hepatic BRUCE-regulated protein ubiquitination signaling controls liver functions and conversely, lack of BRUCE expression results in dysregulation of ubiquitin signaling and accelerates liver disease development. BRUCE repression of PKA hyperactivation suggests a possible ubiquitination mechanism, in which BRUCE normally represses PKA activity through promotion of PKA ubiquitination and subsequent degradation through the UPS, thereby preventing β-catenin phosphorylation and activation. In the absence of BRUCE through genetic ablation of BRUCE in the mouse liver or gene knockdown in liver cancer cell lines, PKA becomes stabilized and β-catenin is activated. This possibility is currently under investigation in the lab.

This study has opened new avenues for focusing on BRUCE protection against liver injury, fibrosis and liver cancer. In addition to the "BRUCE-PKA-β-catenin signaling axis", other functions of BRUCE can also impact liver disease progression. In this regard, we have shown that BRUCE's function in the promotion of DNA damage repair is implicated in HCC development initiated by DEN[3]. Being an IAP in the suppression of mitochondrial pathway of apoptosis, BRUCE deficiency can make hepatocytes more susceptible to apoptosis under hepatic oxidative stress and detoxication, which are physiological processes inherent to livers. BRUCE also impacts autophagy and loss of BRUCE reduces cellular energy and increases autophagy flux[47]. Since autophagy regulates liver functions, the impact of BRUCE on liver autophagy and its connection with liver disease progression is under investigation in our lab. Future studies will focus on the interplay among these pathways in the maintenance of liver homeostasis and suppression of liver diseases in genetically modified murine models, including humanized murine models as it has shown promises to better understand human liver fibrosis.

The Wnt/β-catenin pathway has been regarded as a crucial mechanism involved in fibrosis and hepatocarcinogenesis. However, only a limited number of efficient targeted therapies are available for aberrant activation of this pathway in inhibiting chronic liver disease progression. Findings from this study provide the rationale to stratify the subset of liver disease patients with BRUCE mutant or deficiency and to test the therapeutic potential of targeting aberrant activation of the cAMP-PKA and Wnt/β-catenin pathways.

## CONCLUSION

We previously reported the clinical relevance of somatic deleterious mutations in BRUCE or its downregulation in a large patient population with hepatitis, fibrosis and HCC[3]. In conclusion, this study identifies BRUCE as a suppressor of liver fibrosis in the premalignant and malignant stages in a DEN-induced hepatocarcinogenic murine model. Mechanistically, this study elucidates a previously unrecognized "BRUCE-PKA-β-catenin" signaling pathway contributing to hepatic proliferation, fibrosis and malignancy. Specifically, by using *in vitro* and *in vivo* approaches, we showed that hepatic BRUCE-deficiency releases its suppression of PKA kinase activity, leading to PKA-dependent phosphorylation and activation of β-catenin. In contrast to DEN exposure alone, which does not induce robust fibrosis, DEN treatment in a BRUCE null background accelerates fibrosis, which likely drives the early HCC development in BRUCE LKO mice. Considering the significant clinical relevance of BRUCE in patients with liver diseases, this study has demonstrated that our BRUCE LKO mouse model is a promising model for recapitulating human liver disease progression for dissecting the complicated pathological mechanisms underlying liver disease progression.

## ARTICLE HIGHLIGHTS

### *Research background*

BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a known ubiquitin conjugase/Ligase hybrid that has been shown to inhibit apoptosis, regulate efficient

DNA repair, and most recently promote tumor suppression in the liver. Our group previously showed that upon liver injury with diethylnitrosamine (DEN), loss of hepatic BRUCE promoted fibrosis and exacerbated hepatocellular carcinoma (HCC) development in mice.

### Research motivation

About 80% of HCCs develop in fibrotic or cirrhotic livers, demonstrating the importance of understanding liver fibrosis as a factor contributing to hepatic malignancy. Identifying mechanisms that can regulate both fibrosis and HCC development simultaneously provides the possibility of opening therapeutic windows for treating fibrosis and HCC. Considering that over 50% of human HCCs have aberrant β-catenin mutations, targeting the Wnt/β-catenin has shown much promise. The key upstream regulators of this pathway that suppress fibrosis and HCC development remain elusive.

### Research objectives

The objective of this study was to evaluate the mechanisms of BRUCE in inhibiting hepatic fibrosis and HCC upon liver injury induction.

### Research methods

Male C57/BL6/J control mice [loxp/Loxp; albumin-cre (Alb-cre)$^-$] and BRUCE Alb-Cre KO mice (loxp/Loxp; Alb-Cre$^+$) were injected with a single dose of DEN at postnatal day 15. Mice were sacrificed at various time points to examine liver disease progression and liver biopsies were used in the analyses of the proposed mechanism.

### Research results

Based on the exacerbation of fibrosis and HCC phenotypes observed in the liver-specific BRUCE knockout (LKO) mice that we previously reported, we hypothesized that, "the onset of fibrosis and tumorigenesis are likely earlier events in LKO mice". In the present study, we found that upon DEN-induction, BRUCE LKO livers developed fibrosis as early as after 6 mo of exposure. Additionally, the LKO mice developed tumors as early as 8-months after exposure compared to the WT tumor onset after 10 mo of DEN exposure. Furthermore, we observed increased accumulation of β-catenin, including its activity in LKO liver samples. The phosphorylation of β-catenin was determined by measuring nuclear levels of total β-catenin, and Ser-675 phosphorylated β-catenin. Additionally, the activity of protein kinase A (PKA), one of the upstream kinases that phosphorylates β-catenin at Ser-675, was found to be increased in both BRUCE-deficient mouse livers and a human liver cancer cell line. More importantly, BRUCE and PKA were found to be colocalized in the cytoplasm of hepatocytes.

### Research conclusions

In conclusion, this study further demonstrated BRUCE's liver tumor suppressive function, by identifying the early onset of tumorigenesis in LKO mice. Furthermore, the current study elucidated a novel role of BRUCE in the negative regulation of PKA activity in order to negatively regulate β-catenin stabilization and activity. Together, BRUCE's regulation of β-catenin through PKA, is a likely mechanism used to suppress hepatic diseases, such as fibrosis and HCC.

### Research perspectives

While further investigation is warranted, this study revealed the novel role of BRUCE in hepatic regulation of β-catenin upon liver injury. Further establishing BRUCE's regulation of PKA activity can possibly provide more promising therapeutic approaches for treating liver disease patients with aberrant expression of BRUCE and β-catenin.

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ORIGINAL ARTICLE

Retrospective Study

# Early tacrolimus exposure does not impact long-term outcomes after liver transplantation

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**Author contributions:** Gastaca M designed and performed the research and wrote the paper; Ruiz P and Bustamante J designed the research and supervised the report; Martinez-Indart L provided the statistical analysis; Ventoso A, Palomares I, Prieto M, Salvador P, and Senosiain M acquired and analyzed the data; Testillano M, and Suárez MJ and Valdivieso A supervised the report; all authors approved the final version of the article.

**Institutional review board statement:** This study was reviewed and approved by the Research Ethics Committee of the Hospital Universitario Cruces, No.

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## Abstract

### *BACKGROUND*

Tacrolimus trough levels (TTL) during the first weeks after liver transplantation (LT) have been related with long-term renal function and hepatocellular carcinoma recurrence. Nevertheless, the significance of trough levels of tacrolimus during the early post-transplant period for the long-term outcome is under debate

### *AIM*

To evaluate the effect of TTL during the first month on the long-term outcomes after LT.

### *METHODS*

One hundred fifty-five LT recipients treated *de novo* with once-daily tacrolimus were retrospectively studied. Patients with repeated LT or combined transplantation were excluded as well as those who presented renal dysfunction prior to transplantation and/or those who needed induction therapy. Patients were classified into 2 groups according to their mean TTL within the first month after transplantation: ≤ 10 ($n$ = 98) and > 10 ng/mL ($n$ = 57). Multivariate analyses were performed to assess risk factors for patient mortality.

### *RESULTS*

CEIC E13/08.

**Informed consent statement:** Patients gave written consent to be included in the liver transplantation prospective data base. The requirement for specific informed consent for this study was waived because of the retrospective nature of the study.

**Conflict-of-interest statement:** MG is a member of advisory boards and has received honoraria from Astellas, Novartis and Chiesi. JB has received honoraria from Astellas and Novartis. AV has received honoraria from Astellas and Novartis. All other authors have no conflicts to declare.

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Mean levels within the first month post-transplant were 7.4 ± 1.7 and 12.6 ± 2.2 ng/mL in the ≤ 10 and > 10 groups, respectively. Donor age was higher in the high TTL group 62.9 ± 16.8 years *vs* 45.7 ± 17.5 years ($P$ = 0.002) whilst mycophenolate-mofetil was more frequently used in the low TTL group 32.7% *vs* 15.8% ($P$ = 0.02). Recipient features were generally similar across groups. After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in: Mean estimated glomerular filtration rate ($P$ = 0.69), hepatocellular carcinoma recurrence ($P$ = 0.44), *de novo* tumors ($P$ = 0.77), new-onset diabetes ($P$ = 0.13), or biopsy-proven acute rejection rate (12.2% and 8.8%, respectively; $P$ = 0.50). Eighteen patients died during the follow-up and were evenly distributed across groups ($P$ = 0.83). Five-year patient survival was 90.5% and 84.9%, respectively ($P$ = 0.44), while 5-year graft survival was 88.2% and 80.8%, respectively ($P$ = 0.42). Early TTL was not an independent factor for patient mortality in multivariate analyses.

*CONCLUSION*

Differences in tacrolimus levels restricted to the first month after transplant did not result in significant differences in long-term outcomes of LT recipients.

**Key Words:** Liver transplantation; Tacrolimus levels; Prolonged released tacrolimus; Once-daily tacrolimus; Renal function; Survival; Outcomes



**Core Tip:** This is a retrospective study to evaluate the effect of early tacrolimus trough levels (TTL) on the long-term outcomes after liver transplantation. Patients were classified into 2 groups according to mean TTL within the first month: ≤ 10 ($n$ = 98) and > 10 ng/mL ($n$ = 57). After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in: Mean estimated glomerular filtration rate, hepatocellular carcinoma recurrence, *de novo* tumors, biopsy-proven acute rejection rate and five-year patient and graft survival. Differences in tacrolimus levels within the first month after liver transplant did not result in significant differences in long-term outcomes.

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## INTRODUCTION

Tacrolimus represents the keystone of current immunosuppressive regimens after liver transplantation (LT)[1]. Monitoring of trough drug levels is required to maintain them within the therapeutic range[2]. In the case of LT, there is some debate regarding the significance of trough levels of tacrolimus in the early post-transplant period for the long-term outcome. Initial recommendations were extrapolated from kidney transplantation, but LT does not require the high doses needed to prevent acute cellular rejection (ACR) in other allografts[3]. In this regard, various studies have explored the idea of minimizing initial tacrolimus trough levels (TTL)[4-6].

Mean TTL < 10 ng/mL within the first month after LT was associated with less renal impairment within 1 year in a recent meta-analysis[7]. In this study, tacrolimus concentration between 6 and 10 ng/mL were recommended as more appropriate after LT. Mean TTL > 10 ng/mL within the first month after LT but not thereafter has been also associated with increased risk of hepatocellular carcinoma (HCC) recurrence[8]. High exposure to calcineurin inhibitors was an independent predictor of HCC recurrence by multivariate analysis in this study (RR: 2.82; $P$ = 0.005). Moreover, Rodríguez-Perálvarez *et al*[3] reported that mean TTL of 7-10 ng/mL during the first

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two weeks after LT was effective in preventing ACR, and was related with significantly superior results in graft survival than TTL above or below this range. More recently, the survival time of patients with mean TTL < 5 ng/mL during the first four weeks after LT was observed to be significantly shorter than that of patients with higher mean TTL[9]. Despite these studies, the actual role of initial TTL on long-term outcomes after LT is difficult to assess. Retrospective studies did not report TTL during the follow-up period[3,9], and therefore the influence of potential differences among groups in tacrolimus exposure throughout the follow-up cannot be ruled out. In addition, in some reports TTL were maintained different in the study groups not only during the first month but throughout the whole follow-up, though not significantly, with the consequent difference of long-term tacrolimus exposure and the potential influence on the outcomes[4,6,8].

Our experience with the use of once-daily tacrolimus (Tac-QD) *de novo* after LT has been published[10]. Outstanding long-term patient and graft survival was achieved with the use of *de novo* Tac-QD in a minimizing immunosuppression protocol in LT recipients. With the aim of assessing the significance of the early post-transplant period in the outcomes of LT, we conducted this study to determine the real role of early TTL within the first month on long-term outcomes after LT.

## MATERIALS AND METHODS

### Design and patients

We conducted a retrospective analysis of a prospectively collected database of patients transplanted between April 2008 and May 2013. A total of 237 consecutive LTs were performed during the study period. Patients in the database with repeated LT ($n$ = 13) or combined transplantation ($n$ = 8) were excluded from this analysis, as were those who died within the first week after LT ($n$ = 5) and those who did not receive Tac-QD for various reasons ($n$ = 11). Patients who presented renal dysfunction prior to transplantation, defined as estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m$^2$, and/or those who needed induction therapy ($n$ = 45 overall) were also excluded to avoid bias in the early TTL measurements due to their particular immunosuppressive protocol with induction therapy and delayed initiation of tacrolimus. Finally, 155 adult LT recipients, whose immunosuppressive therapy was based on Tac-QD *de novo*, were eligible for this study and were followed up until December 31, 2015. Patients with HCC met the preoperative Milan criteria. To determine the effect of early exposure to tacrolimus on long-term outcomes and renal function, patients were classified into two groups according to their mean TTL during the first month after LT: ≤ 10 ng/mL or > 10 ng/mL. All TTL obtained during the first month were used to define the mean values.

The study was performed in accordance with relevant guidelines and regulation. No organs were procured from prisoners. The prospective database received the approval of the Research Ethics Committee of the Hospital Universitario Cruces, No. CEIC E13/08. All patients gave informed consent to be included in the prospective database; the requirement for specific informed consent was waived because of the retrospective nature of the study.

### Early post-transplantation immunosuppressive therapy

Initial immunosuppression included Tac-QD and steroids 20 mg/day, except in those patients with diabetes mellitus who were treated with Tac-QD and mycophenolate-mofetil (MMF), avoiding the use of steroids. Tac-QD was administered within the first 24 h after LT, either orally or *via* a nasogastric tube. Patients considered at risk of renal dysfunction received MMF at a daily dose of 1000-2000 mg. Initial Tac-QD dose was 0.15 mg/kg *per* day (or 0.1 mg/kg *per* day if combined with MMF). Subsequent doses were adjusted according to trough levels. Serum tacrolimus levels were monitored regularly every 48 h until discharge. Target TTL were 5-10 ng/mL during the first 3 mo; however, if trough levels were lower but liver function tests were normal, the TacQD dose was not preventively increased. Azathioprine was not used in our patients.

### Clinical follow-up and long-term immunosuppressive therapy

Biliary reconstruction in our patients is performed with end-to-end choledocho-choledochostomy with T-tube. When the patient progresses well, T-tube is closed on postoperative day 3 to avoid the potential effect that biliary diversion might have on TTL. Cholangiography is performed on day 7 and in the third postoperative month

before T-tube removal. During these three months, patients are monitored weekly at home after hospital discharge, and also seen every two weeks at the outpatient clinic. Patients are monitored with liver function tests and TTL monthly afterwards until completion of the first year, and every 2-3 mo for a further two years. Stable patients with no relevant comorbidities are seen every 4 to 6 mo from the third year on.

The treating physicians adjusted immunosuppressive treatment according to their clinical judgment. Target TTL were progressively reduced: 4-9 ng/mL from month 3 to 6, 3-8 ng/mL from month 6 to 12, < 7 ng/mL after the first year and < 5 ng/mL after the second year onwards. Immunosuppressive protocol included steroids withdrawal 3-4 mo after transplantation, except in case of autoimmune disease (in which low-dose prednisone 5 mg/day was maintained), and in patients with hepatitis C virus (HCV), in whom withdrawal was delayed until months 12-18. Duration of treatment with MMF depended on side effects and/or clinical requirements. Adherence to treatment was assessed at each visit by asking the patients regarding any deviations from the prescribed regimen.

### Endpoints and definitions

Outcome variables were: (1) Long-term renal function; (2) Immunosuppression-related morbidity; (3) Patient survival; and (4) Graft survival.

Long-term renal function was assessed by eGFR based on the modification of diet in renal disease formula. K/DIGO guidelines were used to define and classify chronic kidney disease[11]. Metabolic syndrome was defined according to already stablished definitions[12]. Fasting plasma glucose repeatedly > 126 mg/dL was used to define *de novo* diabetes whilst dyslipidemia was considered when treatment was prescribed for elevated blood cholesterol or triglycerides, and arterial hypertension when antihypertensive treatment was initiated. Patients with HCC met the Milan criteria. ACR was biopsy-proven acute rejection (BPAR) in all cases. BPAR were graded according to the Banff International Consensus Document[13]. Liver biopsy was not performed *per* protocol but indicated according to clinical evolution. In case of BPAR, tacrolimus exposure was further increased as the initial step. In case of severe rejection or if the graft dysfunction persisted after Tac-QD adjustments, three consecutive daily 500 mg corticosteroid boluses were used. Early graft dysfunction was defined according to previous specifications[14].

### Statistical analysis

Qualitative variables are summarized as percentages and quantitative variables using means and standard deviations or median and interquartile range. Comparisons between frequencies of characteristics among trough-level groups were performed using the Chi-squared test or Fisher test, and continuous variables were compared using the Kruskall-Wallis test. Patient and graft survival were analyzed using the Kaplan-Meier method, in which patients lost to follow-up were censored at their last recorded visit. Graft loss was defined as retransplantation or death with non-functioning graft. Death with functioning graft was censored for the analysis of graft survival. The log-rank test was used to compare survival among the three groups. A univariate Cox regression analysis was performed to identify clinical and treatment factors related with patient survival including all patients in the cohort. Those variables with a $P < 0.200$ were included in a multivariate Cox regression model. Variables with the higher $P$ value were excluded one by one until all variables had a $P < 0.05$. The proportional hazard assumption was tested. The statistical methods of this study were reviewed by Lorea Martinez-Indart from Bioinformatics and Statistics Platform, Biocruces Bizkaia Health Research Institute. Statistical analysis was performed using SPSS version 23.0.

## RESULTS

All patients were Caucasian and received whole grafts from donation after brain-death. Ninety-eight were included in the ≤ 10 ng/mL group and 57 in the > 10 ng/mL group. A median of 7 samples of TTL (range 5-12) were used to obtain the mean TTL during the first month after transplant. Donor and recipient characteristics of the two groups are summarized in Table 1. Recipient features were generally similar across groups, including age, cause of liver disease, model for end-stage liver disease (MELD) score, baseline kidney function and pre-transplant comorbidities. The only significant difference between groups was the age of the graft donor (older for recipients whose early TTL were > 10 ng/mL); consequently, stroke as the cause of death was more

**Table 1 Donors and recipients characteristics**

| | ≤ 10 ng/mL, *n* = 98 | > 10 ng/mL, *n* = 57 | *P* value |
|---|---|---|---|
| Donors | | | |
| Age, year (mean ± SD) | 54.7 ± 17.5 | 54.7 ± 17.5 | 0.002 |
| Male | 58 (59.2%) | 35 (61.4%) | 0.786 |
| Cause of death | | | 0.004 |
| Stroke | 57 (58.2%) | 48 (84.2%) | |
| Trauma | 27 (27.6%) | 6 (10.5%) | |
| Other | 14 (14.3%) | 3 (5.3%) | |
| Graft steatosis | 19 (19.6%) | 12 (21.1%) | 0.827 |
| Recipients | | | |
| Age, years (mean ± SD) | 55.3 ± 8.4 | 53.2 ± 9.8 | 0.227 |
| Male | 81 (82.7%) | 48 (84.2%) | 0.802 |
| MELD (mean ± SD) | 13.1 ± 5.6 | 12.7 ± 5.3 | 0.618 |
| Hepatocellular carcinoma | 45 (45.9%) | 29 (50.9%) | 0.551 |
| Cause of liver disease | | | 0.283 |
| Alcohol abuse | 45 (45.9%) | 24 (42.1%) | |
| HCV | 40 (40.8%) | 18 (31.6%) | |
| HBV | 3 (3.1%) | 5 (8.8%) | |
| Cho/estatic liver disease | 3 (3.1%) | 4 (7%) | |
| Other | 7 (7.1%) | 6 (10.5%) | |
| Medical history (*pre* LT) | | | |
| MDRD-4 (mean ± SD) | 107.8 ± 35.7 | 16.7 ± 33.7 | 0.223 |
| Diabetes mellitus | 18 (18.4%) | 9 (15.8%) | 0.683 |
| Arterial hypertension | 12 (12.2%) | 10 (17.5%) | 0.362 |
| Mean tacrolimus trough levels 1 mo (ng/mL) | 7.38 ± 1.68 | 12.62 ± 2.25 | NA |
| Corticosteroids | 80 (82.5%) | 49 (86.0%) | 0.571 |
| Mycophenolate mofetil | 32 (32.7%) | 9 (15.8%) | 0.024 |

MELD: Model for end-stage liver disease; MDRD-4: Modification of diet in renal disease; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

frequent among those donors. Corticosteroids were similarly used in all groups; however, MMF use was significantly more common in the group with TTL ≤ 10 ng/mL.

Evolution of mean TTL during the follow-up of the two groups is shown in Figure 1. Mean levels within the first month post-transplant were 7.4 ± 1.7 and 12.6 ± 2.2 ng/mL in the ≤ 10 and > 10 groups, respectively (Table 1). Levels decreased in the > 10 mg/mL group within the first three months and were similar in both groups by the third month. From the third month on, a steady decrease in TTL was observed in both groups. Of note, for the purpose of this study, TTL were significantly different among groups only during the first month after LT, but not during the rest of the follow-up ($P$ = 0.65).

Median follow-up was 52.8 mo (range 2.8-81.1) for those patients with early levels ≤ 10 ng/mL and 52.6 mo (10.8-79.1) for patients with tacrolimus mean levels > 10 ng/mL. Patient outcomes after transplantation are summarized in Table 2. There were no statistically or clinically relevant differences among groups. Mean TTL during the early post-transplant period did not affect renal function. Creatinine clearance fell in parallel in both groups ($P$ = 0.67), decreasing similarly during the first 6 mo to remain steady thereafter until the end of follow-up, at mean levels of approximately 80

**Table 2 Recipients outcomes after liver transplantation**

| | ≤ 10 ng/mL, *n* = 98 | > 10 ng/mL, *n* = 57 | *P* value |
|---|---|---|---|
| Biopsy-proven acute rejection | 12 (12.2%) | 5(8.8%) | 0.505 |
| Arterial complications | 12 (12.2%) | 7(12.3%) | 0.995 |
| Biliary complications | 13 (13.3%) | 8 (14%) | 0.893 |
| Infection (any) | 49(50.0%) | 26 (45.6%) | 0.598 |
| Cytomegalovirus infection | 26 (26.5%) | 12 (21.1%) | 0.445 |
| Retransplantation | 5 (5.1%) | 5 (8.8%) | 0.500 |
| HCC recurrence[1] | 1 (2.3%) | 0 | 0.999 |
| HCV recurrence[2] | 35 (87.5%) | 14 (77.8%) | 0.438 |
| *De novo* tumor | 10 (10.2%) | 5 (8.8%) | 0.771 |
| New-onset arterial hypertension | 35(36.1%) | 19 (36.5%) | 0.827 |
| New-onset diabetes | 21 (21.6%) | 6 (12.7%) | 0.127 |
| Tacrolimus withdrawal. Causes: | 18 (18.4%) | 8 (14.0%) | 0.486 |
| Kidney failure | 7 | 1 | |
| Neurotoxicity | 1 | 2 | |
| Metabolic syndrome | 6 | 4 | |
| Metabolic synd + kidney failure | 1 | - | |
| Other | 3 | 1 | |
| MDRD-4 at 5 yr (mean ± SD) | 82.5 ± 19.4 | 80.32 ± 14.7 | 0.686 |
| Deaths. Causes: | 10 (10.2%) | 8 (14.0%) | 0.827 |
| HCV recurrence | 5 | 3 | |
| *De novo* tumor | 1 | 2 | |
| Sepsis | 2 | 1 | |
| Stroke | 0 | 1 | |
| Other | 2 | 1 | |

[1]Including variables with $P < 0.2$ in univariate analysis, highlighted in bold.
[2]Renal dysfunction during hospitalization. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; MDRD-4 stands for: Modification of Diet in Renal Disease.

mL/min/1.73 m$^2$ in all groups (Figure 2). Patients with higher levels within the first month after LT did not present more immunosuppression-related toxicity including new-onset diabetes, hypertension, HCC recurrence or *de novo* tumors. BPAR occurred with low and similar frequency in all groups (12.2%, and 8.8% in ≤ 10 and > 10 mg/mL, respectively; $P = 0.50$). Only 10 patients were treated with corticosteroid boluses (8 (66.7%) and 2 (40.0%), respectively; $P = 0.99$), and the rest responded to tacrolimus dose escalation. There was no relationship between the decision to withdraw tacrolimus during follow-up and the initial trough level.

Eighteen patients died during the follow-up and were evenly distributed across groups ($P = 0.83$) (Table 2). The most common cause of death was HCV recurrence. Five-year patient survival in the study groups was 90.5% and 84.9%, respectively ($P = 0.44$) (Figure 3A), while 5-year graft survival was 88.2% and 85.8%, respectively ($P = 0.42$) (Figure 3B).

### *Univariate and multivariate analysis*

All patients were included in a univariate and multivariate Cox regression analysis to study factors associated with patient mortality. Multiple variables from donor and recipients were considered in the univariate analysis, as well as various outcomes and adverse events. This analysis was performed considering the two mean TTL groups described in methods, and also dividing the sample into two groups using the cut-off

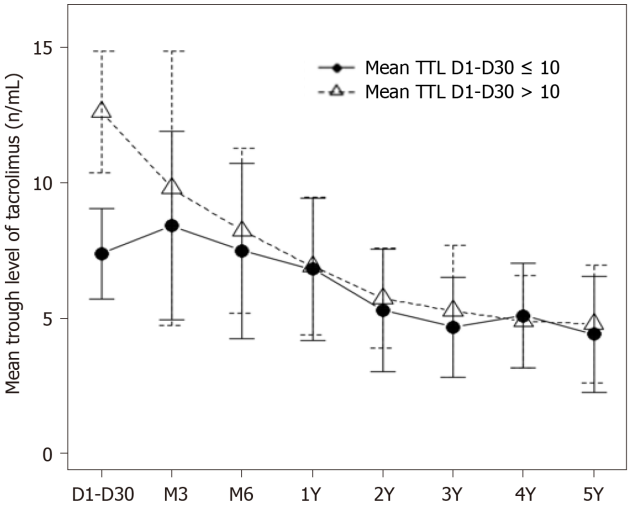


**Figure 1 Mean serum tacrolimus levels according to the mean tacrolimus trough levels for each group within 1 mo after transplantation (mean ± SD).** *P* = 0.65 comparing means from month 3 (M3) to year 5 (5Y). TTL: Tacrolimus trough levels.

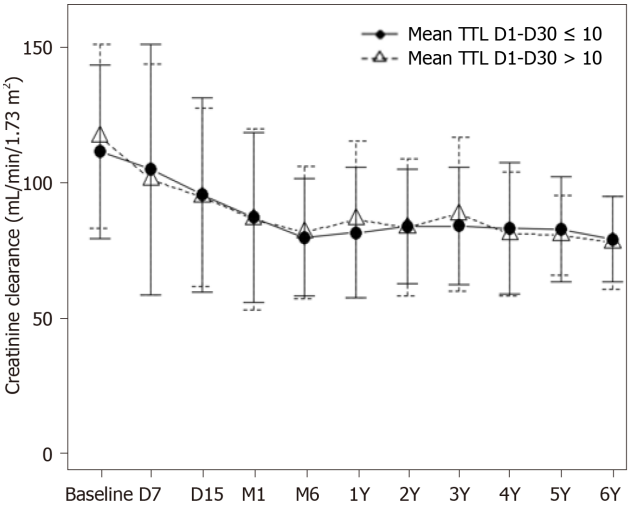


**Figure 2 Mean creatinine clearance according to the mean tacrolimus trough levels for each group within 1 mo after transplantation (mean ± SD) (*P* = 0.67).** TTL: Tacrolimus trough levels.

level 8 ng/mL or three groups using cut-off levels of < 7 ng/mL, 7-10 ng/mL and > 10 ng/mL. Multivariate analysis revealed that factors independently related with patient mortality were *de novo* tumor (HR = 13.8; 95%CI: 4.1-46.9; *P* < 0.001), MELD score ≥ 20 (HR = 6.1; 95%CI: 1.9-19.6; *P* = 0.003), HCV infection as the cause of liver disease (HR = 4.9; 95%CI: 1.7-14.1; *P* = 0.003) and arterial complications (HR = 3.7; 95%CI: 1.1-12.6; *P* = 0.03) (Table 3). Early TTL was not an independent factor for patient mortality.

## DISCUSSION

This analysis aimed to further explore factors related to long-term clinical outcomes in our LT patients treated *de novo* with Tac-QD, with particular interest in the effect of mean TTL during the early post-transplant period. In order to have an adequate follow-up time to study long-term outcomes, patients transplanted between 2008 and 2013 were included in the study. Considering the time when LTs were performed, we followed a policy of immunosuppression minimization with target TTL of 5-10 ng/mL during the first 3 mo; however, a significant number of patients in this cohort were outside our target levels during the first month after LT, although this was corrected afterwards, as shown in Figure 1. We divided our cohort into two groups of early TTL (within 1 mo) as previously done by Rodríguez-Perálvarez *et al*[7,8] who found a

**Table 3 Univariate and multivariate analysis of factors associated with patient mortality**

| | Univariate analysis | | Multivariate analysis[1] | |
|---|---|---|---|---|
| | *P* value | HR (95%CI) | *P* value | HR (95%CI) |
| Age of donor ≥ 70 years | 0.55 | 0.73 (0.26-2.05) | | |
| Recipient | | | | |
| Liver steatosis | 0.1 | 0.408 (0.09-1.79) | | |
| Age ≥ 60 years | 0.94 | 1.04 (0.39-2.78) | | |
| HCV infection as cause of liver disease | 0.02 | 3.02 (1.17-7.81) | 0.003 | 4.94 (1.72-14.17) |
| Presence of hepatocellular carcinoma | 0.57 | 1.31 (0.52-3.34) | | |
| MELD score ≥ 20 | 0.02 | 3.16 (1.12-8.91) | 0.003 | 6.06 (1.88-19.56) |
| Diabetes before transplantation | 0.63 | 1.32(0.43-4.01) | | |
| Hypertension before transplantation | 0.05 | 2.78 (0.98-7.90) | - | - |
| MDRD-4 at baseline | 0.35 | 0.99 (0.97-1.01) | | |
| Mycophenolate mofetil at initial therapy | 0.89 | 1.07 (0.38-3.02) | | |
| Outcomes and complications | | | | |
| BPAR | 0.20 | 2.08 (0.67-6.43) | | |
| Arterial complications | 0.06 | 2.91 (0.94-9.06) | 0.03 | 3.76 (1.12-12.62) |
| Biliary complications | 0.59 | 1.41 (0.40-4.92) | | |
| Renal dysfunction early after transplant[2] | 0.08 | 2.40 (0.90-6.38) | - | - |
| Renal hypertension | 0.82 | 1.12 (0.42-3.02) | | |
| *De novo* diabetes | 0.02 | 3.25 (1.24-8.55) | - | - |
| Cardiovascular | 0.14 | | | |
| Arterial hypertension | 0.08 | 0.32 (0.09-1.15) | - | - |
| Heart failure | 0.26 | 0.31 (0.04-2.37) | | |
| *De novo* tumor | 0.005 | 4.20 (1.56-11.32) | < 0.001 | 13.82 (4.06-46.98) |
| HCV recurrence | 0.22 | 1.79 (0.70-4.53) | | |
| HCC recurrence | 0.008 | 16.61 (2.10-131.07) | - | - |
| Any infection | 0.71 | 1.12 (0.47-3.03) | | |
| Bacterial infection | 0.04 | 2.71 (1.04-7.07) | - | - |
| Viral infection | 0.39 | 0.61 (0.20-1.87) | | |
| Fungal infection | 0.87 | 1.19 (0.16-9.03) | | |
| Cytomegalovirus infection | 0.79 | 0.86 (0.28-2.62) | | |
| Normal renal function at last visit (MDRD-4 ≥ 60 mL/min/1.73 $m^2$) | 0.92 | 1.08 (0.23-5.08) | | |
| Mean tacrolimus levels at days 1-30 after LT | | | | |
| > 10 ng/mL *vs* ≤ 10 ng/mL | 0.44 | 1.44 (0.57-3.65) | | |
| < 7 ng/mL (reference)[3] | 0.32 | | | |
| 7-10 ng/mL | 0.31 | 0.49 (0.12-1.96) | | |
| > 10 ng/mL | 0.59 | 1.33 (0.47-3.73) | | |
| > 8 ng/mL *vs* < 8 ng/mL[3] | 0.78 | 1.14 (0.44-2.95) | | |
| Early graft dysfunction | 0.08 | 2.44 (0.890-6.63) | < 0.001 | 6.02 (2.34-15.49) |

[1]Including variables with $P < 0.2$ in univariate analysis, highlighted in bold.
[2]Renal dysfunction during hospitalization.

[3]Additional analysis modifying cut-off values. MELD: Model for end-stage liver disease; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; BPAR: Biopsy-proven acute rejection; MDRD-4 stands for: Modification of Diet in Renal Disease; LT: Liver transplantation.

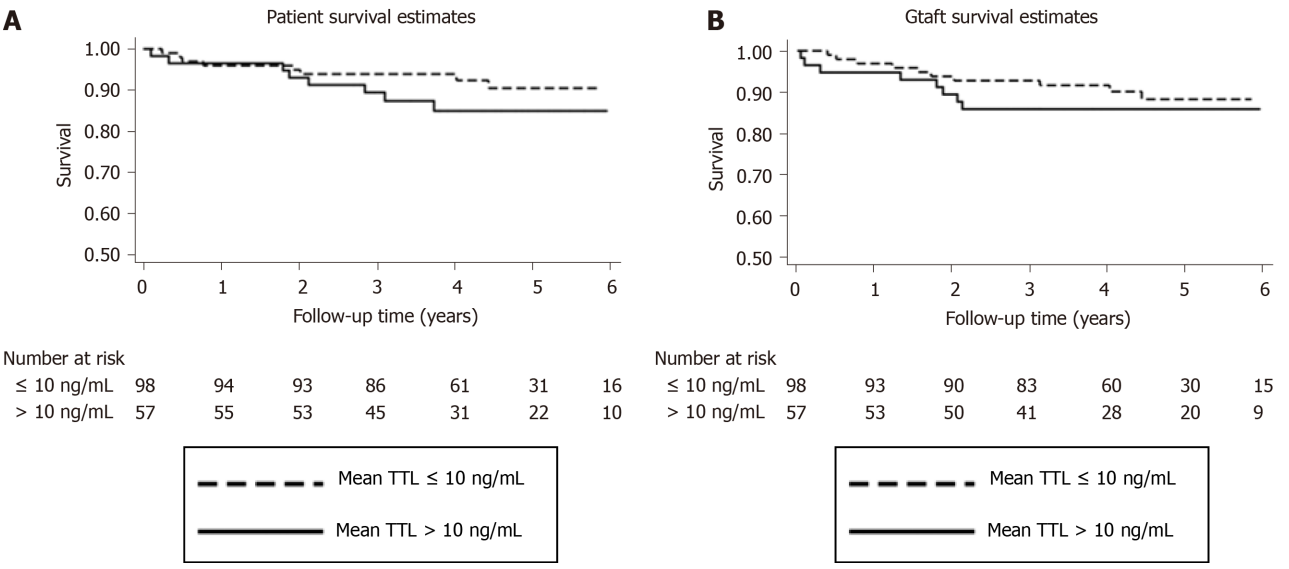


**Figure 3 Kaplan-Meier survival curves after liver transplantation according to the mean tacrolimus trough levels for each group within 1 mo after transplantation.** A: Patient survival ($P$ = 0.44); B: Graft survival ($P$ = 0.42). TTL: Tacrolimus trough levels.

significant improvement of outcomes when mean TTL within the first month post-LT were ≤ 10 ng /mL, compared with patients with > 10 ng/mL[7,8]. Of note, patients treated with induction therapy and delayed introduction of low-dose tacrolimus, namely those with pretransplant renal dysfunction, were excluded in our study to avoid bias as most of these patients would have probably ended in the low mean TTL group. In contrast to the published studies, we did not find significant differences in long-term renal function, HCC recurrence, immunosuppression-related toxicity or patient and graft survival in both groups of early TTL. In addition, multivariate analysis in our study, performed three times with different cut-off values for early TTL, demonstrated the lack of influence of early TTL on long-term patient survival.

In our study, donor age was significantly higher in the group with high TTL. Aging is characterized by a decline of liver cellular function that could determine alterations in immunosuppressants liver metabolism and pharmacokinetic. In this sense, it has been suggested that aged donor livers might exhibit lower drug clearance with consequently higher TTL[15]. Nevertheless, this circumstance was not detrimental in our experience as both TTL groups achieved comparable long-term outcomes.

According to the literature, the relative risk of death more than 1 year after LT suffers a 4-fold to 5-fold increase when renal dysfunction is present[16,17]. In our study, renal function evolved similarly in the two groups, with an expected 20% decrease in eGFR during the initial period after LT-as already described by other authors[18]-and maintenance of renal function from month 6 onwards. This contrasts with the progressive decline in renal function in the Mid/long-term repeatedly reported in literature[19-21]. Although, some authors have found no relationship between TTL within 15 d after LT and chronic renal impairment[3,9], high TTL within the first month after LT has been associated with worse renal function in different studies[7,20]. Karie-Guigues *et al*[20] found that the introduction of MMF significantly reduced the TTL at the end of the first month after LT, and this was associated with a significantly less marked reduction of the eGFR at 12 and 60 mo. Rodríguez-Perálvarez *et al*[7] also observed in a meta-analysis that reduced TTL (< 10 ng/mL) within the first month after LT were associated with less renal impairment at 1 year[7]. Nevertheless, both studies can be discussed. In the former study, TTL were shown at months 1, 12 and 60 after LT; however, no data were shown on the evolution of TTL between those time points and so, results could be biased due to different exposition to tacrolimus in both groups[20]. In the latter study, only two clinical trials were used in the meta-analysis and TTL were maintained higher in both study groups along the whole follow-up although differences did not achieve significance[7].

We can hypothesize that TTL early after LT have little effect on the evolution of long-term renal function when a tacrolimus minimization policy is implemented during long-term follow-up, as in our case. A longer period of high TTL in the post-transplant period might be needed to negatively affect the mid/long-term renal function. In accordance with this idea, the role of cumulative exposure to tacrolimus in eGFR decline after LT has been recently addressed[22]. In this study, conventional/high exposure to tacrolimus within the first 3 mo resulted in a more pronounced eGRF decline as compared with minimization (23.3 mL/min *vs* 9.5 mL/min; $P \leq 0.001$).

The role of tacrolimus exposure in HCC recurrence has been also addressed in different studies. High TTL (> 10 ng/mL) within the first month after LT but not thereafter was associated with increased risk of HCC recurrence at 5 years by Rodríguez-Perálvarez *et al*[8] (RR = 2.8; $P = 0.005$). Of note, in this study, tacrolimus levels were consistently lower during the 3-year follow-up in the non-recurrence group, although differences did not achieve significance. In another study, high exposure to tacrolimus was followed by a 50% recurrence rate *vs* 9.1% in patients with low exposure ($P = 0.001$)[23]. In this study, high exposure was described as > 10 ng/mL during the first year and not only during the first month reflecting a significant higher exposure to tacrolimus along the follow-up. In our study, overall HCC recurrence rate was extremely low and no differences were found between groups. Low exposure to tacrolimus not only during the early post-transplant period but in the long term, and our strict selection policy, all patients fulfilled Milan criteria prior to transplantation, might have positively influenced these remarkable results in our study. Recently, other authors have also reported the lack of effect of the first fifteen days of calcineurin inhibitor exposure in the development of HCC recurrence or *de novo* tumors after LT[24]. Again, it seems that longer periods of high exposure to tacrolimus-and not only during the first month after transplant-are needed to influence the development of *de novo* tumors or HCC recurrence.

Early TTL were not related with an increase in BPAR rates in our study. Reduction in early TTL was associated with the use of MMF and this could explain why the BPAR rate was not higher in patients with lower early TTL. Immunosuppression therapy with tacrolimus, MMF and steroids is currently the most common combination following LT[1], and has been demonstrated to be effective in reducing TTL while maintaining or even reducing the acute rejection rate[4,6].

We observed a relatively low rate of immunosuppression-related toxicity in terms of *de novo* diabetes or arterial hypertension and no differences were seen according to early TTL. In addition, development of *de novo* tumors was not influenced by TTL during the first month in our study.

In our study, factors associated with patient survival in multivariate analysis were *de novo* tumor, higher severity of liver disease (MELD score > 20), baseline HCV infection and arterial complications after LT. These factors have been repeatedly reported to be related to patient and graft survival after LT in the pre-direct-acting antivirals era[16,25,26]. Of note, early TTL were not an independent risk factors for patient survival in our study.

We recognize some limitations in our study. It is retrospective, although the data were retrieved from a prospective database. Indeed, the number of patients included in the different groups are limited and hence the number of patients who experienced adverse events of interest such as impairment of renal function or HCC recurrence are also limited. In addition, MMF was more frequently used in the lower TTL group although immunosuppression-related morbidity is more likely related with tacrolimus exposure rather than to the use of MMF. Nevertheless, our study has several strengths: (1) Median follow-up was more than 4 years in both groups, which seems sufficient to assess the long-term outcomes and draw meaningful conclusions; and (2) Regarding TTL, our study groups were significantly different only within the first month after LT, which was the target period of time in the study, but not during the rest of the follow-up, what reinforces the adequacy of the study for our purpose and avoids the significant potential bias of having not only different early TTL but different TTL during the study period.

## CONCLUSION

In summary, TTL within the first month after LT had no significant effect on long-term renal function, immunosuppression-related morbidity and 5-year patient or graft survival in our study. Early post-transplant tacrolimus level was not an independent factor for long-term patient in multivariate analysis. We conclude that relatively small

differences in mean tacrolimus levels restricted to the first month after LT do not determine differences in long-term immunosuppression-related morbidity and patient survival and therefore, larger exposure to tacrolimus seems to be needed to influence long-term outcomes. Larger studies should be advisable to confirm our results; however, these studies should be done on the basis of different TTL only during the early post-transplant period and not along the follow-up to avoid potential biases.

## ARTICLE HIGHLIGHTS

### *Research background*

Immunosuppression is a cornerstone in liver transplantation (LT) and current immunosuppressive regimens are mostly based on tacrolimus. At present, side effects relating anticalcineurin inhibitors are one of the main concerns for long-term outcomes after LT. Side effects are commonly related with drug dose and trough levels.

### *Research motivation*

Tacrolimus trough levels (TTL) above 10 ng/mL during the first weeks after liver transplant have been related with mid and long-term outcomes including impairment of renal function and an increased rate of hepatocellular recurrence, *de novo* tumors and new-onset diabetes.

### *Research objectives*

The aim of this study was to assess the influence of the TTL during the early post-transplant period in the long-term outcomes of LT.

### *Research methods*

This was a retrospective study of 155 consecutive liver transplants treated with an immunosuppressive regimen based on *de novo* once-daily tacrolimus. Patients were classified into 2 groups according to their mean TTL within the first month after transplantation: ≤ 10 ng/mL ($n$ = 98) and > 10 ng/mL ($n$ = 57). All TTL obtained during the first month were used to define the mean values. Multivariate analyses were performed to assess risk factors for patient mortality.

### *Research results*

TTL were significantly different among groups only during the first month after transplantation, but not during the rest of the follow-up After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in the evolution of the mean estimated glomerular filtration rate, hepatocellular carcinoma recurrence, development of *de novo* tumors, new-onset diabetes, new-onset arterial hypertension or biopsy-proven acute rejection rate. Five-year patient and graft survival were comparable. Early tacrolimus trough level was not an independent factor for patient mortality in multivariate analyses.

### *Research conclusions*

Differences in tacrolimus levels restricted to the first month after transplantation did not result in significant differences in long-term outcomes of liver transplant recipients.

### *Research perspectives*

Mid and long-term calcineurin inhibitors-related side effects after LT should be studied considering the cumulative exposure to tacrolimus along the follow-up and not only the trough levels observed during the early post-transplant period.

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META-ANALYSIS

# Efficacy and safety of once daily tacrolimus compared to twice daily tacrolimus after liver transplantation

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## Abstract

### *BACKGROUND*

Once daily tacrolimus regimen was found to exhibits similar bioavailability, safety and efficacy properties compared to twice-daily tacrolimus in kidney transplantation patients.

### *AIM*

To compare the efficacy and safety of once-daily prolonged release tacrolimus compared to twice-daily tacrolimus in liver transplantation patients.

### *METHODS*

MEDLINE, EMBASE, CENTRAL databases were searched for clinical trials until December 2020. Efficacy outcome measured as the rate of treatment failure indicated by biopsy-proven acute rejection, Serum creatinine, graft loss, or death. Two reviewers independently selected studies, collected data and assessed risk of bias. The results are reported as risk ratio with 95% confidence interval (CI) for dichotomous data.

### *RESULTS*

Seven studies included with 965 patients. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of

any other conflict of interest is associated with this work.

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Bias tool. Biopsy-proven acute rejection was reported in four studies, and pooled analysis of those studies indicated similar rejections in both twice daily and once daily tacrolimus groups (risk ratio: 1.06, 95%CI: 0.84-1.34, $n$ = 758, $I^2$ = 0%) and also we found no significant difference between both groups for renal outcome (serum creatinine; mean difference, 0.001 mg/dL, 95%CI: -0.042 to 0.043, $n$ = 846, $I^2$ = 18.6%). Similarly, there was similar number of adverse events such as hypertension, headache, back pain, blood related disorders, infections and nausea observed in both groups.

*CONCLUSION*
The analysis findings confirm that both once daily and twice daily tacrolimus formulations are comparable in terms of efficacy and safety outcomes.

**Key Words:** Prolonged release; Tacrolimus; Liver transplantation; Graft rejection; Renal impairment; FK level



**Core Tip:** Tacrolimus, a calcineurin inhibitor is an important component of the immunosuppressive regimens post liver transplantation. Compliance to immunosuppression treatment generally is important and non-adherence is a major risk factor of graft rejection and loss. Compliance to medication declines over the course of time in patients after liver transplantation due to several factors and this contributes to about 20% of late acute rejection. The efficacy of once daily tacrolimus regimens has been reported in many studies and this systematic review/meta-analysis confirmed the evidence of comparable efficacy and safety of prolonged release tacrolimus to the twice daily immediate release formulation.

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## INTRODUCTION

Advances in immunosuppression regimens after solid-organ transplantation have significantly improved patient and graft survival. Tacrolimus, a calcineurin inhibitor is an important component of the immunosuppressive regimens widely used following liver transplantation (LT). Compliance to immunosuppression treatment however is important and non-adherence is a recognized contributing factor in rejection and graft loss[1,2].

Compliance to medication declines over the course of time in patients after LT due to several factors including the number of drugs to consume and the rate of rejection/infections increases. Previous reviews directed at recipients transplanted between the late 1980s and mid-2000s showed that the prevalence of non-adherence to immunosuppressive medications averaged about 25%. This non-adherence to medications was felt to contribute to about 20% of late acute rejection episodes and 16%-36% of graft losses[3]. To maintain good adherence, less frequently administering regimen were proved to be effective[4].

Recently, tacrolimus once-daily prolonged-release (PR) formulation was developed. Based on the previous literature, it was evident that conversion from the twice-daily, immediate release (IR) to PR tacrolimus was well tolerated, safe and conveniently used in stable patients after LT[5,6]. However, there is no systematic review that has been conducted till date to confirm the efficacy and safety of PR tacrolimus compared to IR tacrolimus.

## MATERIALS AND METHODS

### Database search

This systematic review and meta-analysis was performed according to Cochrane Collaboration[7] and Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement[8].

We searched MEDLINE, EMBASE, CENTRAL databases since inception to December 2020 using an extensive search strategy to identify relevant literature. We used the following terms: Tacrolimus, liver transplantation and dosage forms (Supplementary file) while searching databases with human and English language restrictions. In addition, we also searched clinicaltrials.gov.in and Google Scholar and references of previously published relevant papers to find more relevant trials.

### Eligibility criteria

Clinical trials conducted on adult (> 18 years) patients who received a primary LT from a deceased or living donor, having an average serum tacrolimus level of 1-10 ng/mL for more than 6 wk, that compared once daily tacrolimus to twice daily tacrolimus in LT patients were included.

### Exclusion criteria

Studies were excluded if they had patients with a previous organ transplant other than liver and multiple organ transplantations. Studies also conducted on paediatric population and lack of a control group (the study had only included patients who received once daily tacrolimus. We also excluded studies only assessed pharmacokinetics of tacrolimus. Finally, studies without full-text such as conference proceedings, editorials, reviews, secondary analyses and letters excluded.

**Outcomes:** Efficacy was measured as the rate of treatment failure indicated by biopsy-proven acute rejection (BPAR), liver graft loss, or death while safety was assessed by the incidence of adverse events.

### Study selection and data extraction

Two reviewers independently (KB and RT) screened the identified studies according to the aforementioned criteria and excluded studies that were found to be clearly irrelevant. We obtained the full text of the remaining studies and the same two reviewers screened full texts and selected trials for inclusion. The same two reviewers independently extracted data from included trials into the predesigned and validated data collection form. Disagreements were resolved by arbitration, and consensus was reached after discussion. We collected study characteristics (type of design with duration of intervention and methods), baseline demographics, and efficacy and safety outcome data from each included trial.

### Quality assessment

Two reviewers (KB and RT) independently assessed quality of included studies using Cochrane Risk of Bias tool[9], and disagreements were resolved by discussion. If a consensus could not be reached, any discrepancy was resolved by a senior author. Seven domains of quality assessment included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias.

### Statistical analysis

We performed statistical analysis using Comprehensive Meta-analysis Version 3.0[10]. We reported the results as risk ratio with 95% confidence interval (CI) for dichotomous data and continuous data as mean difference. We used a random-effects model to combine individual results regardless whether there was significant heterogeneity or not. We tested heterogeneity among trial results using the $I^2$ statistic[7]. We considered a value greater than 50% as substantial heterogeneity. Publication bias was not assessed due to limited number of included studies in this review.

## RESULTS

A total of 701 articles from databases search and 15 from additional searches identified. After removing duplicates 543 studies remained for screening. Upon

screening titles and abstracts, 490 clearly irrelevant articles removed. The remaining 48 articles subjected to full text screening. Finally, seven clinical trials met the inclusion criteria[11-17]. The flow of the randomised controlled trial included in our analysis is shown in Figure 1.

### Study characteristics

A total of seven clinical trials were included with 965 patients. Study characteristics were summarized in Table 1. Studies included are conducted in various countries including United States, Japan, United Kingdom, and one study in another 16 countries.

The mean age of included patients was 52.8 years and majority (71%) of them were males. Four studies had follow-up for one year while the other two had follow up for 3 and 6 mo. In four studies, concomitant treatment with mycophenolate mofetil and steroids was allowed. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of Bias tool (Figure 2).

### Efficacy outcomes

Acute rejection confirmed by biopsy was reported in four studies[12,15-17], and pooled analysis of those studies indicated similar rejection rate in both twice daily and once daily tacrolimus groups (risk ratio 1.06, 95%CI: 0.84-1.34, $n$ = 758, $I^2$ = 0%; Figure 3).

## DISCUSSION

This systematic review and meta-analysis compared PR tacrolimus to IR tacrolimus in LT recipients. The efficacy and safety outcomes were found to be similar for both regimens.

Adherence to the immunosuppressant regimen post-LT is important for preventing rejection and graft loss. The reported rate of non-adherence to immunosuppressant regimens is 15%-40%, which could lead to significantly higher rate of graft rejection, graft loss and severe impact on long-term survival[18]. It was observed that once daily tacrolimus is safe and is associated with better adherence and low variability of liver function tests[18,19].

A study by Muduma *et al*[20] looked at the cost effectiveness of PR tacrolimus in LT recipients. Based on a United Kingdom specific analysis of the projected cost-utility of PR tacrolimus relative to IR tacrolimus and cyclosporin, once daily tacrolimus was cost-effective, improved life expectancy and quality adjusted life year and incremental cost effectiveness ratio below £20000 per a quality adjusted life year gained. Over a 3-year time horizon, one graft would be saved for every 14 patients treated with PR tacrolimus with minimal impact on cost when compared to IR tacrolimus.

The results of recently published systematic review showed that PR tacrolimus when compared to the IR tacrolimus resulted in no significant difference in the glomerular filtration rate, BPAR and the safety outcomes among the kidney transplant recipients[21]. The findings of our review are also in congruent with the previous review. In contrast, another meta-analysis based on combination of two clinical trials and four observational studies found that once daily tacrolimus is effective for the first year after liver transplantation, however, there was no significant difference in 1-year mortality and adverse events between once daily and twice daily tacrolimus groups[22].

PR tacrolimus has been introduced as helpful therapeutic option to increase the patient adherence to immunosuppressive treatment. Studies with short follow-up and pharmacokinetic evaluation were not included in this review, however one study which evaluated pharmacokinetic outcomes along with efficacy outcomes showed similar BPAR, graft losses and safety outcomes such as hypertension, infections and blood related disorders[16] between groups. Of the included studies in our systematic review, four reported concomitant immunosuppressant therapies administration such as corticosteroids, and mycophenolate mofetil. It was evident that those concomitant drugs have negative association with occurred adverse events with tacrolimus[23]. An eight years long-term follow up study based on European Liver Transplant Registry has recently been published study and the findings were in favour of PR in terms of graft losses and acute rejections. This very large population study also reported better outcome in those converted from IR to PR tacrolimus after 1 mo compared to those maintained on IR tacrolimus-based immunosuppression. They concluded that patients on PR tacrolimus continues to provide ongoing benefits for graft and patient survival beyond 3 years post transplantation[24]. The major limitation of the study, were the lack of data on the dosages and the trough levels of tacrolimus were not captured. In

**Table 1 Characteristics of the included studies**

| Ref. | Year | Country | Study design | Follow-up period | Sample size | Donor type | Mean age | Concurrent therapy |
|---|---|---|---|---|---|---|---|---|
| Alloway *et al*[11] | 2014 | United States | Phase-II, 3-sequence, open-label, multicenter, prospective study | 1 yr | 59 | NR | 49.8 | Mycophenolate mofetil |
| Kim *et al*[13] | 2016 | South Korea | 2-armed, parallel group, prospective, randomized, open-label, phase IV | 1 yr | 79 | Deceased | 54 | NR |
| Sańko-Resmer *et al*[14] | 2012 | United Kingdom | Multicentre, open-label, single-sequence, crossover, phase IIIb | 3 mo | 98 | NR | 55 | None |
| Shin *et al*[17] | 2018 | South Korea | Phase IV, randomized, open-label, comparative, single-center study | 6 mo | 100 | NR | 52 | Corticosteroid, mycophenolate mofetil, and basiliximab. |
| Trunečka *et al*[15] | 2010 | 16 countries | 1:1-randomized, double-blind, double-dummy, two-arm, parallel-group phase III, comparative study | 1 yr | 471 | NR | 52 | Mycophenolate sodium |
| DuBay *et al*[12] | 2019 | United States | Phase II, open label, multicenter, randomized trial | 1 yr | 29 | Deceased | 54.4 | Mycophenolate mofetil, mycophenolic acid sodium, prednisone, or azathioprine |
| Fischer *et al*[16] | 2010 | Germany | Randomized, phase II, multicenter, open-label, prospective trial | 6 wk | 129 | NR | 47 | Anti-fungal, antibiotics, anti-hypertensives, antiepileptics and rifampicin) |

NR: Not reported.

addition, the lack of clarity on the IR tacrolimus preparations the cohort received. The retrospective design of the study was the main reason for its exclusion in our analyses as it did not meet the eligibility criteria.

### *Strengths and limitations*

The major strength of our study is that, we have only included clinical trials of long-term follow-up to address efficacy and safety of PR tacrolimus. There was no heterogeneity found for all the outcomes assessed, except for any adverse events. One of the limitations of our review is that, we have only included studies published in English language, which means some of the studies published in other language might have been missed. Publication bias assessment was also not assessed due to less than ten studies included in the analysis, however due to our intense search effort it was evident that we did not miss any study meeting this review's eligibility criteria. Majority of the studies were of open-label design, that could have introduced bias, however this could not be avoided due to the nature of administration. In addition, the paucity of studies of PR tacrolimus in Asian patients renders data from this review of high interest to the transplant community.

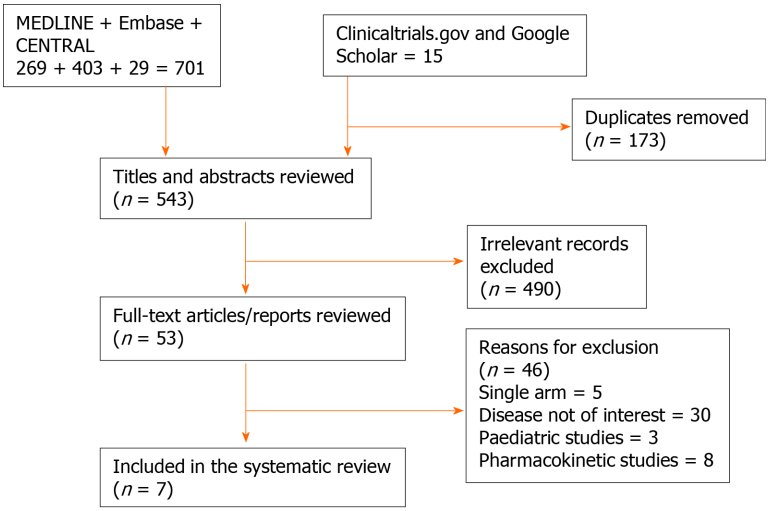


**Figure 1 Preferred reporting items for systematic reviews and meta-analyses flow chart.**

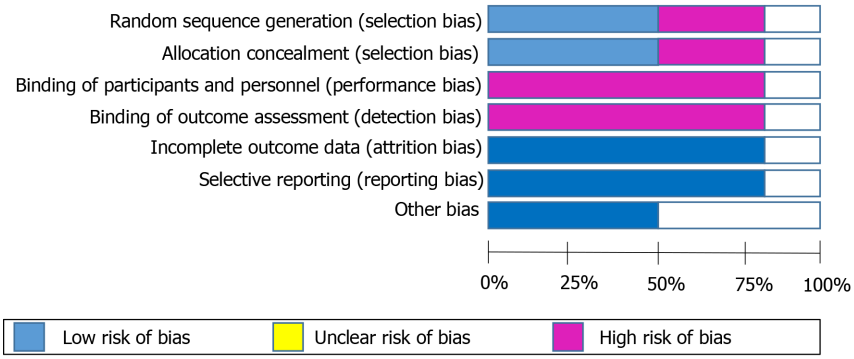


**Figure 2 Risk of bias assessment according to Cochrane risk of bias tool.**

## CONCLUSION

Our systematic review and meta-analysis indicate that both PR and IR tacrolimus formulations are comparable in terms of efficacy and safety outcomes. However, to confirm these findings, long-term follow-up randomized controlled trials with large sample sizes are required. Also, to assess acceptability by patients, quality of life and economic evaluations should be conducted.

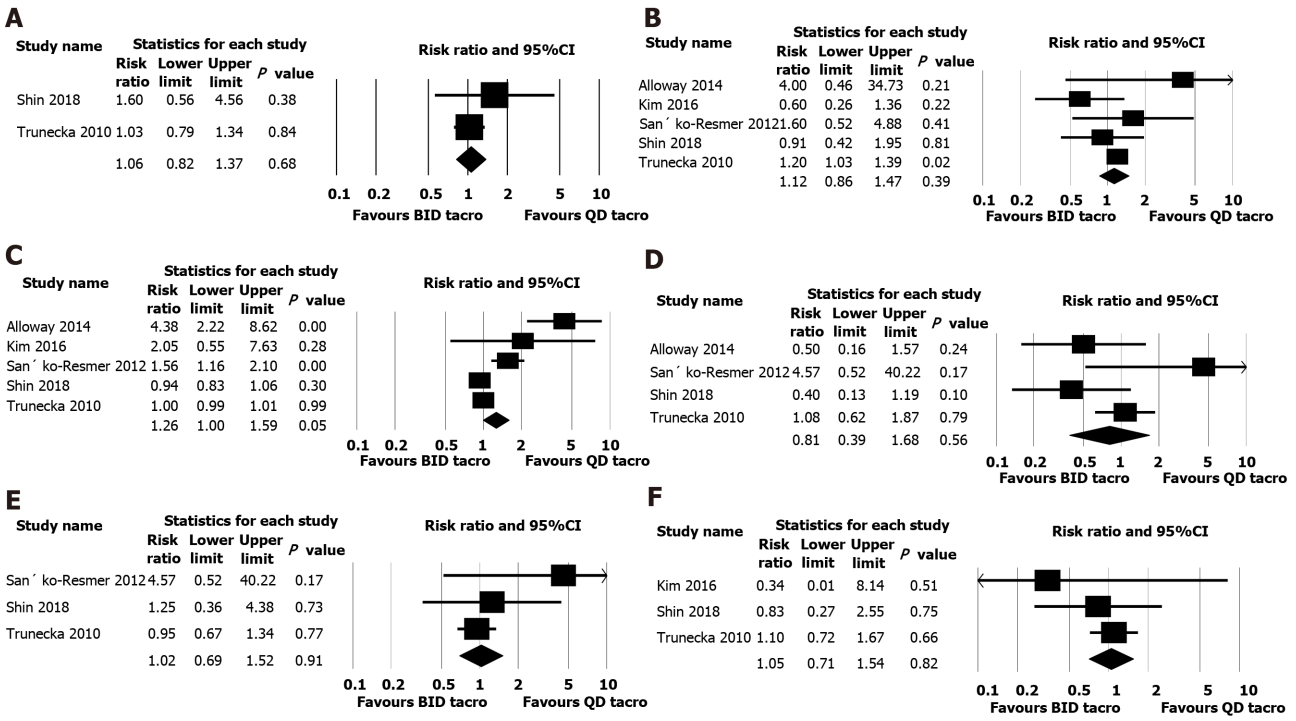


**Figure 3 Efficacy outcomes.** A: Acute graft rejection; B: Infection; C: Any adverse drug reaction; D: Headache; E: Back pain; and F: Blood disorders. CI: Confidence interval.

## ARTICLE HIGHLIGHTS

### *Research background*

Tacrolimus, a calcineurin inhibitor is an important immunosuppressive medication post liver transplantation. Compliance to immunosuppression is important and non-adherence can lead to rejection and graft loss. To maintain good adherence, less frequently administering regimen were proved to be effective.

### *Research motivation*

Recently, tacrolimus once-daily prolonged-release (PR) formulation was developed. Several studies have shown evidence that conversion from the twice-daily, immediate release to PR tacrolimus was well tolerated, safe and conveniently used in stable patients after liver transplantation.

### *Research objectives*

Our objective was to conduct a metanalysis and systematic review of the published clinical trials that studied the safety and efficacy of PR tacrolimus compared to immediate release tacrolimus.

### *Research methods*

MEDLINE, EMBASE, CENTRAL databases were searched for clinical trials until December 2020. Efficacy outcome measured as the rate of treatment failure indicated by biopsy-proven acute rejection, Serum creatinine, graft loss, or death. Two reviewers independently selected studies, collected data and assessed risk of bias. The results are reported as risk ratio with 95%CI for dichotomous data.

### *Research results*

Seven studies included with 965 patients. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of Bias tool. Biopsy-proven acute rejection was reported in four studies, and pooled analysis of those studies indicated similar rejections in both twice daily and once daily tacrolimus groups. We also found no significant difference between both groups for renal outcome (serum creatinine; mean difference, 0.001 mg/dL, 95%CI: -0.042 to 0.043, $n$ = 846, $I^2$ = 18.6%). Similarly, there was similar number of adverse events such as hypertension, headache, back pain, blood related disorders, infections and nausea

observed in both groups.

### Research conclusions

The analysis findings confirm that both once daily and twice daily tacrolimus formulations are comparable in terms of efficacy and safety outcomes.

### Research perspectives

Long-term follow-up randomized controlled trials with large sample sizes are required. Also, to assess acceptability by patients, quality of life and economic evaluations should be conducted.

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CASE REPORT

# Conversion hepatectomy for hepatocellular carcinoma with main portal vein tumour thrombus after lenvatinib treatment: A case report

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**Informed consent statement:** Informed written consent was obtained from the patients for publication of this report and any accompanying images.

**Conflict-of-interest statement:** The

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## Abstract

### *BACKGROUND*

Hepatocellular carcinoma (HCC) accompanied by portal vein tumour thrombus (PVTT) presents an aggressive disease course, worsening liver function reserve, and a high recurrence rate. Clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion. However, to achieve longer survival in these patients, the treatment strategy should be concluded with removal of the tumour by locoregional therapy. We experienced a case of initially unresectable HCC with main PVTT converted to radical hepatectomy after lenvatinib treatment.

### *CASE SUMMARY*

A 59-year-old male with chronic hepatitis C infection visited our clinic as a regular post-surgery follow-up. Contrast-enhanced abdominal computed tomography revealed a liver mass diffusely located at the lateral segment with a massive PVTT extending from the umbilical portion to the main and contralateral third-order portal branches. With the diagnosis of unresectable HCC with Vp4 (main trunk/contralateral branch) PVTT, lenvatinib was started at 12 mg/d. The computed tomography taken 3 mo after starting lenvatinib showed regression of the PVTT, which had retreated to the contralateral first-order portal branch. He tolerated the full dose without major adverse effects. With cessation of lenvatinib

for 7 d, radical left lobectomy and PVTT thrombectomy were conducted. The patient's postoperative course was uneventful. Microscopically, the primary lesion showed fibrotic changes, with moderately to poorly differentiated tumour cells surrounded by granulation tissues in some areas. The majority of the PVTT showed necrosis. He was alive without recurrence for 8 mo.

*CONCLUSION*
This is the first case of HCC with Vp4 PVTT in which radical conversion hepatectomy was succeeded after lenvatinib treatment.

**Key Words:** Hepatocellular carcinoma; Lenvatinib; Portal vein tumour thrombus; Conversion hepatectomy; Case report



**Core Tip:** Patients with hepatocellular carcinoma (HCC) with portal vein tumour thrombus demonstrate an aggressive disease course, decreased liver function reserve, and higher recurrence rates after treatment. Clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion. However, to achieve longer survival in these patients, the treatment strategy should be concluded with removal of the tumour. We report the first case of HCC with main portal vein tumour thrombus, in which radical conversion hepatectomy was successfully performed after lenvatinib treatment. Lenvatinib has several strengths that validate its use for targeting conversion hepatectomy for unresectable HCC.

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## INTRODUCTION

Portal vein tumour thrombus (PVTT) is a condition of hepatocellular carcinoma (HCC) that leads to the wide dissemination of tumours throughout the liver and causes a deterioration of liver function, leading to poor prognosis. PVTT is classified as Vp1 (segmentary), Vp2 (secondary order branch), Vp3 (first order branch), and Vp4 (main trunk/contralateral branch)[1], and clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion[2,3]. Current systemic therapy for HCC consists of receptor tyrosine kinase inhibitors (TKIs) and checkpoint inhibitors[4]. As a newly introduced TKI, lenvatinib is a multitargeted TKI that inhibits vascular endothelial growth factor receptor 1-3, platelet-derived growth factor receptor-alpha, rearranged during transfection, and stem cell factor receptor. Lenvatinib is characterized by high tumour regression and tumour necrosis effects[4,5]. However, post progression survival is recognized as being short[6], and the post hoc exploratory analysis disclosed severe morbidities related to lenvatinib treatment in patients with HCC with Vp4 PVTT (data not shown). To achieve longer survival in patients with advanced HCC, the treatment strategy should be concluded with removal of the tumour by locoregional therapy (LRT) because of the limitation of systemic therapy alone[7-9]. Here, we present a case of initially unresectable HCC with Vp4 PVTT converted to radical hepatectomy after lenvatinib treatment.

## CASE PRESENTATION

### *Chief complaints*

A 59-year-old male presented to our clinic as a regular post-surgery follow-up for

authors declare that they have no conflict of interest.

**CARE Checklist (2016) statement:** The authors have read the CARE checklist (2016), and the manuscript was prepared and revised according to the CARE checklist (2016).



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HCC.

### History of present illness

The patient received segmentectomy 5 and cholecystectomy for a single HCC 2 years prior.

### History of past illness

He had hepatitis C virus infection with genotypes 1a which was treated with 24 wk of ledipasvir/sofosbuvir 5 years prior, and sustained virologic response rate was achieved. He received the radiofrequency ablation a year before the first hepatectomy.

### Personal and family history

The patient had a history of alcohol use with 200 mL daily intake for 35 years. Since HCC was diagnosed, the patient had quitted alcohol drinking. He had no family history of cancer.

### Physical examination

The patient's temperature was 36.5 °C, heart rate was 74 bpm, respiratory rate was 14 breath/min, blood pressure was 128/81 mmHg and oxygen saturation in room air was 98%. There was an operative scar for a J-shaped incision on the abdomen from the previous liver resection. No ascites and encephalopathy were detected.

### Laboratory examinations

Laboratory exams were normal except for a slight increase in aspartate aminotransferase levels of 52 U/L and protein induced by des-γ-carboxy prothrombin of 107 mAU/mL. Electrocardiogram, chest X-ray and arterial blood gas were also normal.

### Imaging examinations

Contrast-enhanced (CE) abdominal computed tomography (CT) revealed a liver mass diffusely located at the lateral segment with a massive PVTT extending from the umbilical portion to the main portal and the contralateral third portal branches (Vp4) (Figure 1A and B).

## MULTIDISCIPLINARY EXPERT CONSULTATION

### Amane Takahashi, MD, PhD, Chief Surgeon, Department of Gastroenterological Surgery, Saitama Cancer Center

As a treatment strategy, we should administer lenvatinib at a dose of 12 mg, following the clinical guidelines. The reason for choosing lenvatinib, not sorafenib was that lenvatinib demonstrated higher response rate compared with sorafenib in an open-label, phase III, multicentre, non-inferiority trial involving patients with advanced HCC (the REFLECT trial). If the PVTT exhibited shrinkage to the contralateral first portal branch, we would be able to remove the tumour surgically. We should be careful to follow the liver function during lenvatinib treatment, since the post hoc exploratory analysis revealed severe morbidities including liver failure in cases with Vp4 PVTT.

## FINAL DIAGNOSIS

With the diagnosis of unresectable HCC with Vp4 PVTT, lenvatinib was started at 12 mg/d. CT taken two weeks after starting lenvatinib showed regression of PVTT (by 11%) with partial disappearance of contrast enhancement, retreating to the contralateral second-order PV (Figure 2A). At 3 mo, the PVTT regressed further to the contralateral first-order branch with more loss of contrast enhancement (by 58%), meeting the definition of partial response according to the modified Response Evaluation Criteria in Solid Tumours criteria (Figure 2B and C). During lenvatinib treatment, liver function was maintained within Child-Pugh A (5 points), and the albumin-bilirubin (ALBI) score was -3.45 to -2.93 (Grade 1). He tolerated the full dose without treatment-related adverse effects (TRAEs).

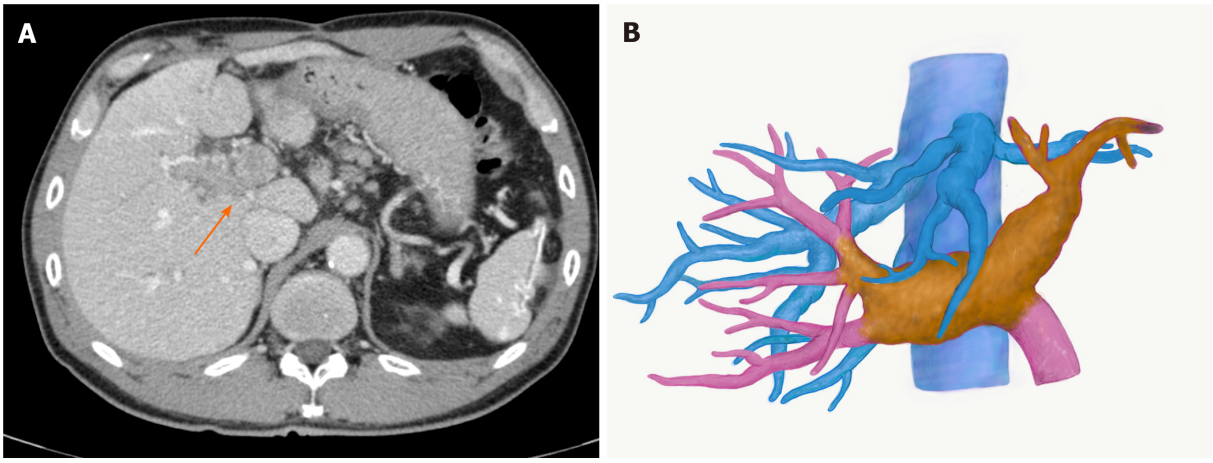


**Figure 1 Images of hepatocellular carcinoma with portal vein tumour thrombus before lenvatinib treatment.** A: Computed tomography image. An arrow indicates portal vein tumour thrombus; B: Three-dimensional image. The yellow mass demonstrates a viable portal vein tumour thrombus, extending to the contralateral third portal branch.

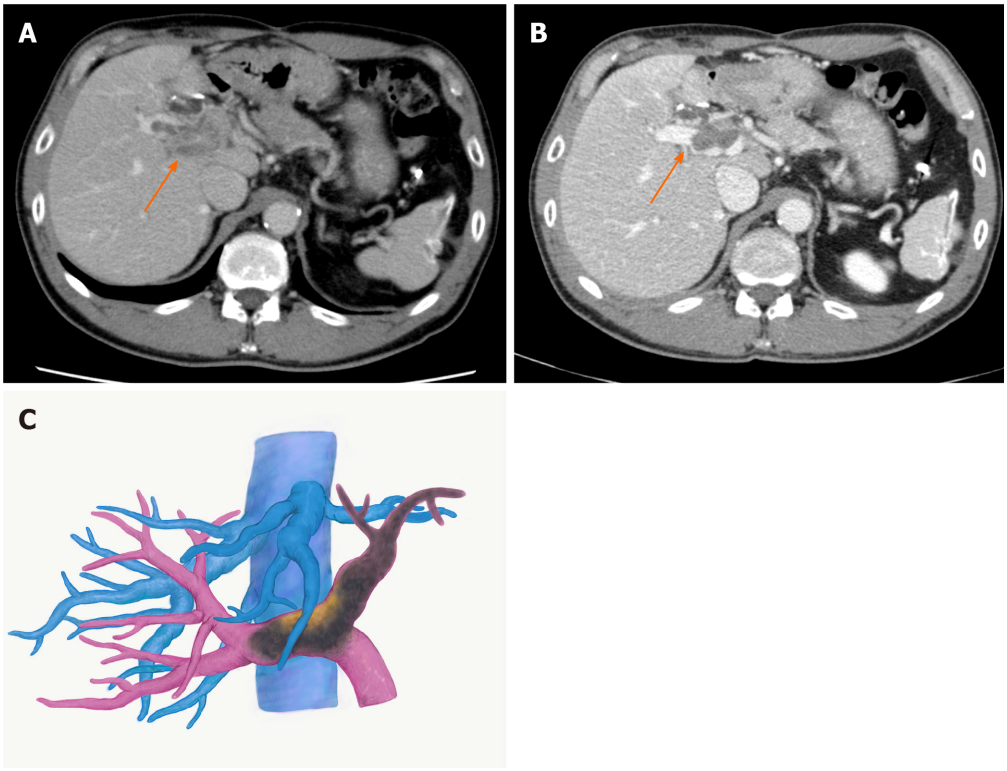


**Figure 2 Images of hepatocellular carcinoma with portal vein tumour thrombus after lenvatinib treatment.** A: Computed tomography image two weeks after the treatment. The portal vein tumour thrombus (PVTT) showed regression with partial disappearance of contrast enhancement; B: Computed tomography image three months after the treatment; C: Three-dimensional image three months after the treatment. The PVTT regressed to the contralateral first-order branch with loss of contrast enhancement. Arrows indicate PVTT.

## TREATMENT

After cessation of lenvatinib for 7 d, left lobectomy with PVTT thrombectomy was performed. Intraoperatively, no intrahepatic satellite lesions, ascites or disseminated nodules were identified. The left hepatic artery, left PV and left hepatic duct were isolated at the hilum. After ligating and disconnecting the left hepatic artery, the right, left and main PVs were exposed. After checking the PVTT by ultrasound, the PVs were clamped by Satinsky forceps (Figure 3A). Venotomy was placed at the bifurcation of the left PV, and the PVTT was thrombectomized (Figure 3B). After flushing the PV with normal saline and confirming that no PVTT remained, the left PV stump was closed by 6-0 proline (Figure 3C). Liver dissection was completed along the

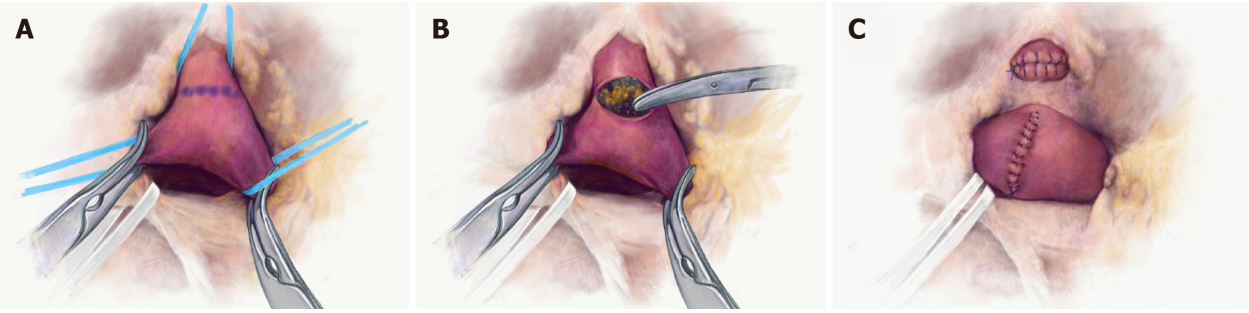


**Figure 3 Portal vein tumour thrombus thrombectomy.** A: The right and the main portal veins were clamped by Satinsky forceps. Venotomy was placed at the bifurcation of the left portal vein; B: The portal vein tumour thrombus was thrombectomized; C: The left portal vein stump was closed by 6-0 proline.

middle hepatic vein. The left hepatic duct and hepatic vein were cut and closed with 6-0 proline. The specimen was finally removed.

## OUTCOME AND FOLLOW-UP

The patient's postoperative course was uneventful. Macroscopically, the primary tumour at the parenchyma was obscure. The PVTT demonstrated a white to brownish nodule with a size of 60 mm × 30 mm × 25 mm (Figure 4A). Microscopically, the primary lesion demonstrated fibrotic changes with haemosiderin deposition. In some areas, moderately to poorly differentiated tumour cells and tumour cells with necrotic changes were surrounded by granulation tissues and fibrosis (Figure 4B and C). The majority of the PVTT showed necrosis (Figure 4D). According to the Union for International Cancer Control classification, the tumour was finally staged as T3 N0 M0 Stage IIIB. He is alive with no evidence of recurrence 8 mo post-surgery.

## DISCUSSION

Patients with HCC with PVTT usually have an aggressive disease course, decreased liver function reserve, limited treatment options, higher recurrence rates after treatment and poor overall survival (OS). The median OS is reported to be as poor as 2-4 mo with best supporting care[10]. The Barcelona Clinic Liver Cancer (BCLC) staging classifies patients with PVTT with Child-Pugh A or B liver function reserve as advanced HCC with BCLC stage C. The recommended treatment option for this group is systemic therapy with sorafenib or lenvatinib as the first-line treatment[4]. LRT including hepatectomy was not recommended over systemic therapy since there was inadequate evidence to inform the balance of benefit *vs* harm[2]. LRT could only be considered for HCC with Vp1/2, only as an option within research settings[2]. In a Japanese nationwide surveillance study consisting of more than 6000 patients with HCC with PVTT, propensity score matching analysis demonstrated a longer median OS in the surgical group than in the non-surgery group (2.87 years *vs* 1.10 years, $P < 0.001$)[11]. However, surgical benefit was acknowledged when PVTT was limited to the first-order branch (Vp3), and no surgical benefit was observed among patients with Vp4 PVTT. The problem in this study was that more than half of the patients with Vp3/4 underwent non-curative resection, and the impact of curative resection was not clarified in this cohort. Several retrospective studies have demonstrated survival benefits of curative hepatectomies with aggressive PV thrombectomy or *en block* resection for Vp4 PVTT[12-14]. Hatano *et al*[14] conducted a retrospective multi-institutional study regarding the outcome of macroscopically curative hepatic resection in 400 patients with HCC with Vp3/4 PVTT. The results demonstrated a median survival time and 5-year OS rate of 21.5 mo and 25.7%, respectively. OS time showed no statistically significant difference between Vp3 and Vp4.

Lenvatinib was initially approved as the first-line therapy for advanced HCC in Japan in 2018. The REFLECT trial met its primary endpoint of non-inferiority to sorafenib in OS[6]. Lenvatinib was superior to sorafenib in progression-free survival (PFS) and time to tumour progression (TTP). Although the complete response rate was low, the objective response rate (ORR) in the lenvatinib group was significantly higher than that in the sorafenib group (40.6% *vs* 12.4%, $P < 0.001$). TRAEs, including hand-

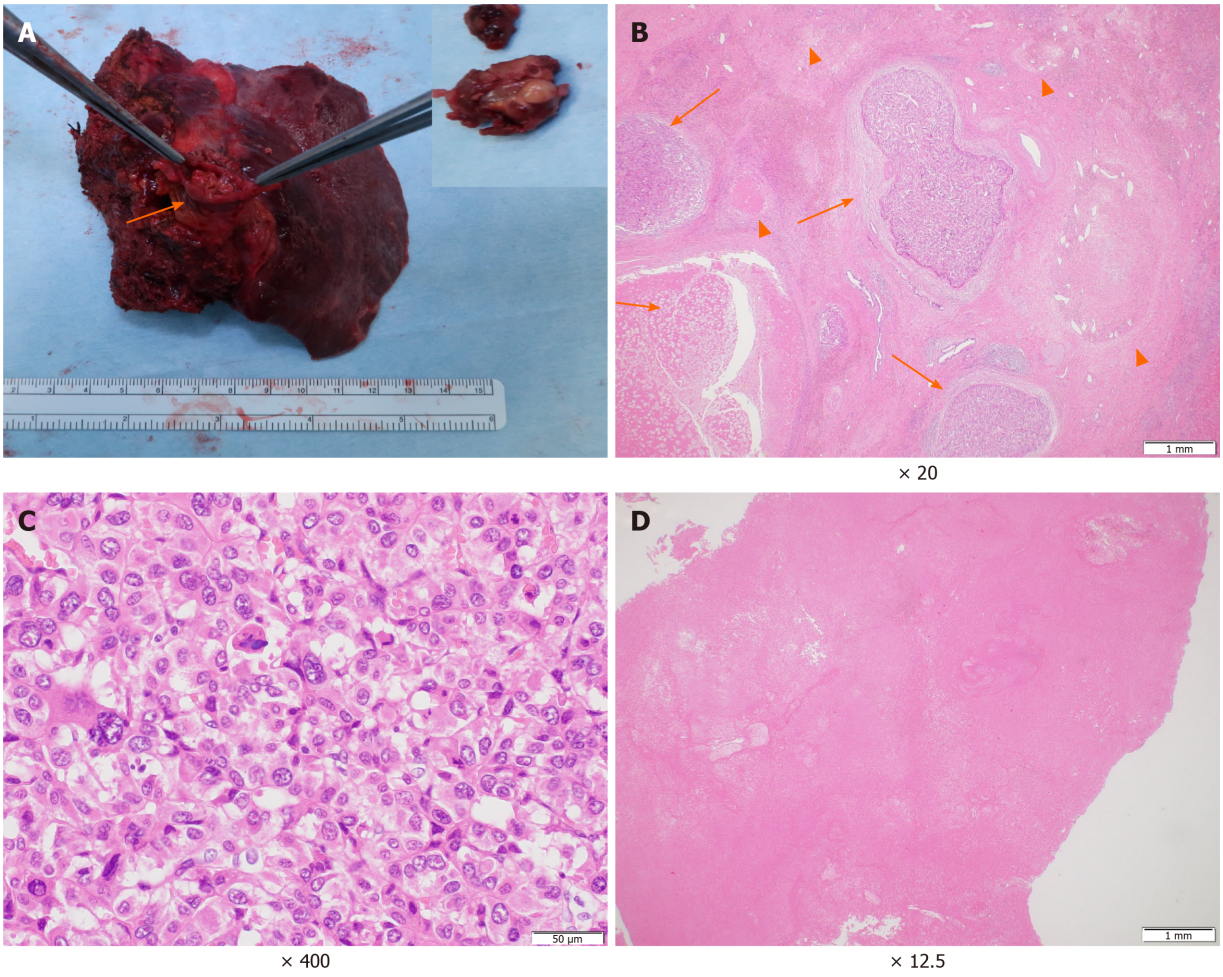


**Figure 4 Macroscopic and microscopic findings of the main tumour and the portal vein tumour thrombus.** A: A white to brownish nodule was found in the left portal vein (arrows). Inlet: close-up picture of the removed portal vein tumour thrombus; B: The primary lesion showed severe fibrotic change with haemosiderin deposition. In the fibrosis, the viable tumour cell nests (arrow) and the necrotic tumour lesions (arrowhead) were scattered; C: High magnification demonstrated moderately to poorly differentiated tumour cells; D: Most of the portal vein tumour thrombus showed necrotic changes.

foot syndrome, hypertension, proteinuria, and anorexia, were comparable between lenvatinib and sorafenib. These side effects are not life-threatening, and they can usually be controlled by supportive medical treatments. Subsequent studies have reported a relatively high ORR with lenvatinib of 29.4%-45.0%[15-17]. On the other hand, the REFLECT trial excluded HCC cases that had main PV invasion, and the outcomes of this cohort were unclear. The efficacy of lenvatinib treatment for unresectable HCC with major PVTT has been reported in some case reports and retrospective studies[18-20]. Kuzuya *et al*[20] compared the outcomes of advanced HCC with Vp3/4 PVTT between sorafenib and lenvatinib as the first-line systemic therapy. The ORR was significantly higher using lenvatinib (53.8% *vs* 14.3% $P = 0.0193$), and the median OS and TTP were significantly longer in the lenvatinib group than in the sorafenib group. No patient discontinued lenvatinib treatment secondary to TRAEs. These reports may characterize lenvatinib as having a relatively strong antitumour effect against HCC including PVTT, with less emergence of serious side effects.

Other characteristics of lenvatinib treatment are the rapid antitumour effects and preservation and fast recovery of liver function[20]. The antitumour effects of lenvatinib have been described as quick, which could be confirmed in 2 wk, and these early radiologic changes could be biomarkers to predict clinical outcomes, including OS[16]. Another group similarly stated that the changes in arterial tumour perfusion on CE-ultrasound at 1 wk were associated with the radiological antitumour response on CE-CT at 8 wk[21]. Regarding the preservation of liver function, patients treated with lenvatinib maintained liver functional reserves better than those treated with sorafenib[22]. Furthermore, ALBI scores in the lenvatinib group improved faster than those in the sorafenib group[20]. In our case, the patient tolerated the full dose while maintaining liver function without major side effects. The tumour including the PVTT showed early necrotic changes 2 wk after lenvatinib treatment.

Based on these reports, lenvatinib is characterized by the following strengths: (1) Relatively strong antitumour effect not only on the main tumour but also on PVTT; (2) Quick antitumour effects that could be noted in 1-2 wk; and (3) Preservation and early recovery of liver function with less incidence of life-threatening TRAEs. Because of these strengths, lenvatinib can be considered an optimal chemotherapeutic agent targeting radical conversion hepatectomy for unresectable HCC. The good indication might be unresectable HCC with a large size or with PV invasion. Multiple intra-extra hepatic HCC can be considered as long as curative resection is feasible, since pathological complete response is usually difficult to attain by lenvatinib alone, and the tumours can quickly regrow during the drug cessation period[19,23]. Lenvatinib demonstrates quick antitumour effects, and it deteriorates liver function temporally[17]. The treatment effects on the tumour and liver function reserve should be evaluated in a short period to avoid missing the best timing for conversion. Since severe morbidities related to lenvatinib treatment were reported in advanced HCC with PVTT (data not shown), physicians should be reminded to perform careful observation during the treatment period, especially in cases with Vp3/4 PVTT, since liver function could deteriorate quickly.

Identification of serum biomarkers for the prediction of lenvatinib response would be of significant benefit for the proper selection of patients for treatment. The post hoc exploratory analysis of the REFLECT trial revealed that the occurrence of hypertension, diarrhoea, proteinuria, or hypothyroidism was generally associated with longer OS in patients with unresectable HCC treated with lenvatinib[24]. Another group stated that maintaining a higher relative dose intensity (RDI) in the early period after starting lenvatinib was associated with a higher ORR and longer PFS[25]. In our case, the patient did not complain of any TRAEs that deteriorated his quality of life, and he could continue lenvatinib with RDI of 100% without decreasing the lenvatinib dose. It is reasonable to think that the high RDI might be the main reason for this significant antitumour effect, leading to PR and conversion hepatectomy.

Four conversion cases with lenvatinib treatment, including ours, were reported in the previous literature (Table 1)[23,25,26]. Three cases were treated with lenvatinib monotherapy, and one case was treated with a combination of lenvatinib and nivolmab. Unresectable factors in these cases were large tumour size with inadequate residual liver volume, lung metastasis, and Vp4 PVTT. The duration of lenvatinib treatment in cases of large tumours and PVTT cases was short, 3-6 mo, and RDIs before conversion were all high (over 70%) in these cases. All cases demonstrated good postoperative courses with no evidence of tumour recurrence. Since the length of its market use is still short, it is necessary to gain experience and cases to clarify which cohort is suitable for targeting conversion.

Recently, there have been reports regarding the efficacy of proton beam therapy for advanced HCC with PVTT[27,28]. Proton beam therapy has advantages in that it is less invasive to patients; however, it requires high medical expenses and a large-scale facility that is not widely available worldwide. Because of its strong and quick antitumour effects with fewer TRAEs, conversion hepatectomy using lenvatinib could be an ideal strategy. A clinical trial is currently underway in Japan regarding conversion surgery during lenvatinib administration for unresectable HCC. Several molecular targeting agents and checkpoint inhibitors are being developed and will be coming to the market soon. These sequential flows could explore a new strategy against unresectable HCC.

## CONCLUSION

In conclusion, we experienced the first case of HCC with Vp4 PVTT in which radical conversion hepatectomy was successfully performed after lenvatinib treatment. Lenvatinib has several strengths that validate its use for targeting radical conversion hepatectomy for unresectable HCC. A multicentre prospective trial is needed to clarify its clinical utility.

**Table 1 Case reports of conversion hepatectomy after lenvatinib treatment**

| Ref. | Age/sex | Background disease | Regimen | Reason for unresectivity | Former treatment | Child-Pugh classification | Duration | RDI (%) | Type of hepatectomy | Prognosis |
|---|---|---|---|---|---|---|---|---|---|---|
| Sato *et al*[23] (2019) | 66/F | HCV | Lenvatinib | Large size | TACE | 8 (B) | 6 mo | 70 | Extended right hepatectomy | 3 mo alive with no recurrence |
| Chen *et al*[26] (2019) | 69/F | HBV | Lenvatinib, nivolmab | Large size | Sorafenib TACE | 8 (B) | 3.5 mo | 100 | Extended right hepatectomy | 3 mo alive with no recurrence |
| Takahashi *et al*[25] (2019) | 82/F | Non B/C | Lenvatinib | Lung metastasis | None | 5 (A) | 13 mo | 38 | Extended posterior segmentectomy | 5 mo alive with no recurrence |
| Present study | 59/M | HCV | Lenvatinib | PVTT (Vp4) | None | 5 (A) | 3 mo | 100 | Left hepatectomy | 8 mo alive with no recurrence |

F: Female; M: Male; HCV: Hepatitis C virus; HBV: Hepatitis B virus; PVTT: Portal vein tumour thrombus; TACE: Transarterial chemoembolization; RDI: Relative dose intensity; Vp4: Main trunk/contralateral branch.

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