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Review

Perturbation of transforming growth factor-β signaling during human hepatic fibro-carcinogenesis: from bench to bedside

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ABSTRACT

Insights into hepatic fibrogenesis and carcinogenesis (fibro-carcinogenesis) have come to light in recent analyses of transforming growth factor (TGF)-β signaling directed by multiple phosphorylated (phospho-) isoforms of Smad mediators. Clinical observations suggest synergy between persistent hepatitis viral infection and chronic inflammation during human fibro-carcinogenesis. Considering basic research together with clinical outcomes, we first outline how hepatitis viruses and chronic inflammation additively promote hepatic fibro-carcinogenesis in hepatitis virus-related liver diseases, focusing on perturbation of Smad phospho-isoform signaling. We then consider the reversibility of Smad phospho-isoform signaling from fibro-carcinogenesis to tumor suppression after anti-viral therapies. Recent progress in Smad phospho-isoform signaling should permit the use of Smad phosphorylation as a prognostic indicator and as a biomarker in assessing effectiveness of interventions against human hepatic fibro-carcinogenesis.

KEYWORDS: carcinogenesis, chronic inflammation, hepatic fibrosis, hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, Smad phospho-isoforms, transforming growth factor-β

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ABBREVIATIONS

ECM	Extracellular matrix
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
LC	Liver cirrhosis
MFB	Myofibroblast
PAI-1	Pasminogen activator inhibitor-1
pSmadC	C-terminal phosphorylated Smad
pSmadL	Linker phorsporylated Smad
SVR	Sustained virological response
TGF-β	Transforming growth factor-β

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, accounting for an estimated 500,000 deaths annually [1, 2]. Chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) appear to be the most significant causes of HCC [3]. Chronic HCV and HBV infections rarely resolve spontaneously. The associated ongoing inflammation can cause liver fibrosis and damage DNA in regenerative hepatocytes. Regeneration in the presence of inflammation thus increases the likelihood of genetic alternations that promote hepatic fibrogenesis and carcinogenesis (fibro-carcinogenesis) [4].

Previous studies have shown that successful anti-viral therapy can improve biochemical liver function parameters as well as histological findings [5, 6]. Patients with mild liver fibrosis

are likely to show histologically evident decreases in fibrosis and inflammation after a sustained virological response (SVR) in response to interferon (IFN) treatment against HCV infection [7]. Furthermore, treated patients show marked reductions in decompensated liver disease [8] and HCC occurrence [9]. Patients with advanced fibrosis, however, retain relatively low but still considerable risks of HCC occurrence [8, 9] and hepatic decompensation [8] despite having attained SVR.

Transforming growth factor (TGF)-β is a pivotal regulator in fibro-carcinogenesis [10, 11]. Within inflammatory micro-environment, TGF-β, secreted by platelets and Kupffer cells, up-regulates extracellular matrix (ECM) production [12]. Responsiveness of ECM production to TGF-β occurs transiently, in tissue repair processes such as liver regeneration after acute liver injury [13, 14], suggesting that regulatory mechanisms for TGF-β signaling are operating in activated mesenchymal cells such as hepatic stellate cells (HSC). On the other hand, TGF-β can terminate proliferation of hepatocytes that has been induced by pro-inflammatory cytokine-mediated mitogenic signaling. During chronic liver diseases, TGF-β signaling is perturbed in injured hepatocytes by pro-inflammatory cytokines [10].

Progress over the past 10 years has disclosed important details of how the TGF-β family elicits its responses [15, 16, 17, 18]. Smads, central mediators that convey signals from receptors for TGF-β superfamily members to the nucleus, are modular proteins with conserved Mad-homology (MH)1, intermediate linker, and MH2 domains [17]. In cell-signaling pathways, various transcription factors are phosphorylated at multiple sites by upstream kinases. Catalytically active TGF-β type I receptor (TβRI) phosphorylates COOH-tail serine residues of receptor-activated Smads (R-Smads), which include Smad2 and the highly similar protein Smad3 [16]. Mitogenic signals alternatively cause phosphorylation of R-Smads at specific sites in their middle linker regions [19, 20, 21, 22, 23]. After phosphorylated R-Smads rapidly oligomerize with Smad4, this complex translocates to the nucleus, where it regulates transcription of target genes.

The roles of HBV and HCV in tumor formation appear complex, involving both direct and indirect mechanisms [24]. Integration of HBV DNA into the host genome occurs in early stages of clonal expansion. Alternatively, chronic liver inflammation and hepatocytic growth induced by host cellular immune responses can increase the risk of HCC development. Considering basic research findings together with clinical outcomes, we first outline how hepatitis viruses and chronic inflammation additively promote hepatic fibro-carcinogenesis in hepatitis virus-related liver diseases, focusing on alteration of domain-specific phospho-Smad signaling. We then consider the reversibility of phospho-Smad signaling after anti-viral therapy, where fibro-carcinogenic signals are replaced by other signals favoring tumor suppression.

Clinical features of HCC

HBV is known to integrate into the host genome, where the virus can contribute to HCC development [25]. In addition, hepatocytes with integrated HBV-DNA may express viral gene products which ultimately may cause carcinogenesis [26]. Even further, HCV increases the risk of HCC by promoting inflammation and fibrosis in the infected liver, culminating in cirrhosis. Recent genome-wide association studies have suggested that HCV infection additionally might modify the genetic background of the host. In this complex manner, both host and viral factors are involved in the process of fibro-carcinogenesis.

Most HCC arise from underlying liver disease, either chronic hepatitis or cirrhosis. Prognosis of HCC depends on the stage of the disease at the time of diagnosis as well as the remaining liver function. As HCC is often diagnosed at advanced stages, only 30% to 40% of patients are eligible for potential curative surgical resection or percutaneous therapeutic interventions such as radiofrequency ablation and ethanol injection. Approximately 70% of these treated patients develop recurrent tumors within 5 years [27]. HCC recurrence is generally dictated by the persistence of pro-tumorigenic signals within the damaged milieu of the fibrotic or cirrhotic livers. Distinct molecular subgroups of HCC have been identified and linked to poor prognosis [28, 29, 30, 31]. Once pro-tumorigenic signals are established,

no effective chemoprevention strategies are available to attenuate development of HCC. These barriers to cure reflect the involvement of a complex network of nontumoral cells, soluble factors and other molecules in creating a supportive and permissive environment for initiation and progression of HCC.

Fibro-carcinogenesis in human hepatitis virus-related chronic liver diseases

Several conditions in chronically damaged livers favor human hepatocarcinogenesis, mostly resulting from recurrent cycles of cellular proliferation, inflammation, and fibrosis. Within this anomalous environment, certain clones of hepatocytes acquire proliferative and survival advantages, eventually forming dysplastic nodules, the histological substrate of HCC [32]. A complex interplay among the various hepatic cell types takes place in injured livers. Hepatocytes are targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and chemical toxins [33]. Damaged hepatocytes induce recruitment of white blood cells by local inflammatory cells. Apoptosis of damaged hepatocytes stimulates fibrogenesis by Kupffer cells, the resident macrophages of the liver. Activated Kupffer cells secrete pro-inflammatory cytokines and TGF-β. Intensive studies have shown that HSC are the major cell type responsible for matrix production in damaged liver tissues [12]. HSC, characterized by retinoid droplets in the cytoplasm, are present in the space of Disse [34]. After liver injury, activated HSC secrete large amounts of ECM proteins. Hepatocytes are replaced with abundant ECM, mainly in the form of fibrillar collagen. Affected hepatocytes also participate in liver fibrogenesis by stimulating deposition of ECM proteins (Figure 1). Several soluble factors, including growth factors, cytokines, chemokines, and oxidative stress products take part in the activation of HSC and hepatocytes. In the presence of chronic liver tissue damage and inflammation, these factors directed at specific cell targets are simultaneously active in the tissue and are partly, perhaps largely, responsible for the fibro-carcinogenic process.

Tissue environment plays a critical role in tumor formation and development [35]. Carcinogenesis is a process that involves transition of a normal cell into a pre-neoplastic lesion that develops into a malignant tumor [36]. As a result of chronic liver damage, HSC undergo progressive activation to become myofibroblasts (MFB), which produce components of the ECM that promote fibrosis. This process is associated with distortion of the parenchyma characterized by deposition of basement membrane components within the space of Disse. Interaction of different cell types in the ECM results in acquisition of an abnormal phenotype that causes transformation. The stromal components support tumor growth and promote invasion through stimulation of hepatocyte proliferation, migration, and invasion, which together promote the transformation of normal hepatocytes into pre-neoplastic hepatocytes.

Recent insights into integrative systems are improving the global understanding of genetic alterations and molecular profiles in HCC. These studies have demonstrated a variety of genetic aberrations and marked heterogeneity of gene expression profiles among HCC cases, suggesting that HCC might be among the most complex and heterogeneous human cancers [37, 38, 39, 40, 41]. Such heterogeneity is consistent with the multiple etiologies of HCC and the long preceding period of chronic inflammatory disease that can foster accumulation of genetic and epigenetic defects. In various ways, an inflammatory microenvironment can increase mutation rates in addition to enhancing proliferation of mutated cells. Activated inflammatory cells serve as sources of reactive oxygen species and reactive nitrogen intermediates capable of inducing DNA damage and genomic instability, resulting in acquisition of malignant phenotype [42]. In addition to genetic alterations, many lines of evidence have indicated that epigenetic changes also contribute very importantly to hepatocarcinogenesis. DNA methylation occurs in the early stage of HCC development. Genomic hypomethylation increases chromosome instability, while localized hyper-methylation decreases tumor suppressor gene expression, which would increase the risk of HCC development [43]. Aberrant methylation of RASSF1A (Ras association domain family member 1) is thought to be an early event in the development of HCC [44]. Such processes, in the context of inflammation and oxidative DNA damage,

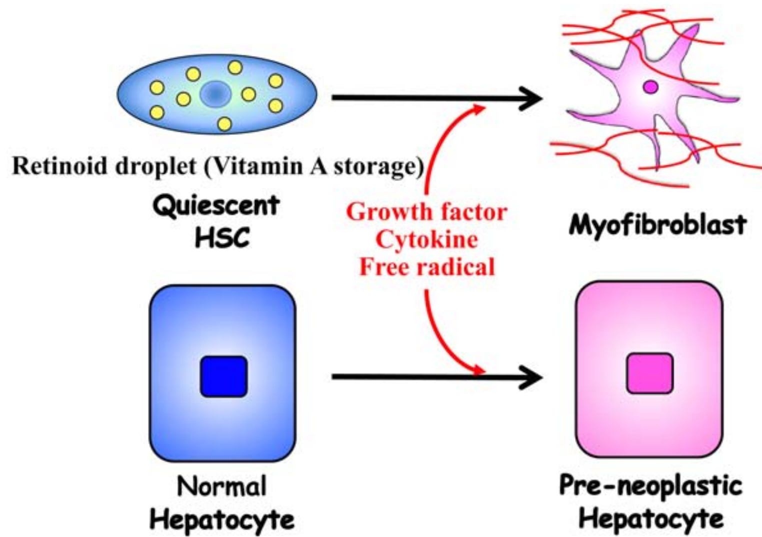

Figure 1. Hepatic stellate cells and hepatocytes with fibro-carcinogenic properties in the course of hepatitis virus-related chronic liver diseases. Quiescent hepatic stellate cells (HSC) characterized by retinoid droplets in the cytoplasm, are present in the space of Disse. Following chronic liver injury, HSC transdifferentiate into myofibroblasts (MFB), which display increased synthesis of cytokines, chemokines, growth factors, and extracellular matrix (ECM). MFB show increased proliferation and migration, participating in liver fibrosis. During progression of chronic liver disease, hepatocytes are persistently affected by pro-inflammatory cytokines, growth factors, and free radicals, eventually becoming pre-neoplastic hepatocytes. As a result of wound-healing responses to repeated injury in chronic liver disease, both MFB and pre-neoplastic hepatocytes undergo phenotypic activation, with acquisition of fibro-carcinogenic properties.

favor accumulation of mutations and epigenetic aberrations in pre-neoplastic hepatocytes or liver stem cells, thereby promoting development of dysplastic nodules and their malignant transformation to early HCC [45].

Both TGF-β and pro-inflammatory cytokines have been implicated in fibro-carcinogenesis [46]. Activated Kupffer cells secrete pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as well as TGF-β. IL-1β promotes HSC proliferation, activation, and transdifferentiation to a myofibroblastic phenotype [47]. TNF-α modulates nuclear factor (NF)-κB and the MAPK pathway, and is involved in tumor development and progression [46]. These data suggest that cross-talk between TGF-β and pro-inflammatory cytokines is important for hepato-carcinogenesis. A key signal transducer for TGF-β and pro-inflamamatory cytokines, c-jun N-terminal kinase (JNK), has emerged as a principal endogenous tumor promoter. JNK, a serine/threonine kinase, affects cell proliferation, differentiation, survival, and migration [48, 49].

Canonical Smad pathway

Receptors for TGF-β consist of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic kinase domain. The latter phosphorylates serine and threonine residues. Upon ligand binding, the type II TGF-β receptor subunit, which has a constitutively active serine/threonine kinase, is brought into close proximity with the type I TGF-β receptor subunit, which then phosphorylates cytosolic R-Smads. R-Smads are phosphorylated by the activated TβR1 on the C-terminal SXS motif. Once within the nucleus, these Smads induce expression of $p15^{ink4B}$ and $p21^{waf1}$, both representing cyclin-dependent kinase (CDK) inhibitors [15, 16, 17, 50]. In normal hepatocytes, TGF-β shuts down cell cycle progression and triggers apoptosis.

Non-Smad pathway

Activation of Ras involves a sequence of phosphorylation events that ends with MAPK translocation into the nucleus, which modifies expression of proliferation-related genes [51].

In particular, Ras/JNK is frequently activated in pre-neoplastic hepatocytes and HCC [52]. Especially important among genes induced by the Ras/JNK pathway are the 2 immediate-early genes encoding the Fos and c-Jun transcription factors. Once synthesized, these proteins can associate with one another to form activator protein (AP)-1, a widely acting heterodimeric transcription factor that is often found in hepatocarcinogensis and liver fibrosis [53]. TGF-β and pro-inflammatory cytokines elicit signaling responses through JNK/non-Smad pathway [54]. In JNK1$^{-/-}$ mice, both fibrosis and HCC development are prevented. Collagen deposition is marked in wild-type and JNK2$^{-/-}$ mice but is less dense in JNK1$^{-/-}$ mice, suggesting an importance of JNK1 in the development of liver fibrosis [55]. JNK1$^{-/-}$ mice exhibit impaired liver carcinogenesis, with smaller and fewer tumor masses [56]. Importantly, JNK1$^{-/-}$ mice displayed decreased HCC proliferation in a carcinogenic model and decreased hepatocytic growth in a model of liver regeneration. In both instances, impaired proliferation is caused by increased expression of p21^{WAF1}, a cell-cycle inhibitor, and reduced expression of c-Myc, a negative regulator of p21^{WAF1}.

Non-canonical Smad pathway

Smad2 and Smad3 proteins contain a conserved MH1 domain that binds DNA, and a conserved MH2 domain that binds receptors, a partner Smad4, and transcription co-activators [57]. More divergent linker regions separate the two domains [17]. The linker domain undergoes regulatory phosphorylation by MAPK including extracellular signal regulated kinase (ERK), JNK, p38 MAPK, and CDK-2/4, as well as glycogen synthase kinase 3-β, Ca (2+)-calmodulin-dependent protein kinase II, and G-protein-coupled receptor kinase-2 [19, 20, 22, 23, 58, 59, 60, 61, 62, 63].

Antibodies (Abs) specifically reactive with structurally related, but differently phosphorylated peptides are emerging as valuable tools for determining phosphorylation sites *in vivo*, and for investigating distinct signals via phosphorylated domains. To elucidate how pro-inflammatory cytokines modulate TGF-β signaling through R-Smad linker phosphorylation, we generated numerous Abs against phosphorylated domains of R-Smads. Domain-specific phospho-R-Smad Abs allowed us to determine that TβRI and JNK/CDK4 differentially phosphorylate R-Smad to create 3 phosphorylated forms (phosphoisoforms): COOH-terminally phosphorylated R-Smad (pSmad2C and pSmad3C), linker phosphorylated R-Smad (pSmad2L and pSmad3L), and dually phosphorylated R-Smad (pSmad2L/C and pSmad3L/C) [22, 64, 65, 66]. While pSmad2L shows cytoplasmic localization [19, 67], all other phospho-isoforms are localized to cell nuclei [21, 22, 59, 60, 63, 67, 68, 69, 70, 71, 72]. Linker phosphorylation can modify COOH-terminally phosphorylated R-Smad signaling [19, 20, 21, 22, 60, 61].

Numerous reports have suggested that fibro-carcinogenic effects of TGF-β involve a pathologic switch of TGF-β signaling from canonical Smad pathway to non-Smad pathway [73, 74]. However, Smad signaling itself drives collagen gene expression [75] and invasive behavior [76]. Linker phosphorylation can explain these long-standing paradoxes concerning Smad signaling, since such phosphorylation occurs apart from the canonical Smad signaling, instead promoting cell growth, invasion, and fibrosis via JNK pathway [77]. TGF-β and pro-inflammatory cytokines simultaneously activate linker-phosphorylated R-Smad and non-Smad signaling through JNK. Imbalance might occur between signaling through non-Smad and Smad pathways during fibro-carcinognensis, and interaction betweens these pathways can mediate pro-fibrogenic and pro-tumorigenic effects of TGF-β and pro-inflammatory cytokines. Collectively, Smad signaling through linker phosphorylation should be recognized as a major non-canonical Smad pathway [10].

Non-canonical Smad signaling in MFB

Differential localization of kinases and phosphatases in the cytoplasm or nucleus raises the intriguing possibility of different temporal dynamics for cytoplasmic or nuclear R-Smad phospho-isoforms, which can add to the repertoire of signaling responses that determine cell-fate decisions [78]. As a result of chronic liver damage, HSC undergo progressive activation to become MFB-like cells [34]. MFB constitutively

produce TGF-β [12]. In MFB, TGF-β and pro-inflammatory cytokine-dependent Smad3 phosphorylation at the linker region was stimulated via activated JNK, in turn suppressing the cytostatic pSmad3C pathway (Figure 2, left). JNK-mediated phosphorylation led to hetero-complex formation between Smad3 and Smad4 [21, 59, 69, 72].

On the other hand, activated JNK retains most Smad2 proteins in the cytoplasm [19]. Smad2 can accumulate in the nucleus only if its C-terminus is phosphorylated under conditions of sustained linker phosphorylation by JNK [21]. Phospho-Smad2L/C undergoes translocation to the nucleus, where it binds to the pSmad3L and Smad4 complex [22, 59] (Figure 2, right), which in turn stimulates PAI-1 transcription [59]. These altered responses are fully consistent with the finding of pSmad3L rather than pSmad3C in the nuclei of α-Smooth muscle actin (SMA)-immunoreactive MFB in portal tracts of chronically HCV-infected liver specimens [69]. The presence of α-SMA is associated with transdifferentiation of HSC into scar-forming MFB, a pivotal event in the fibrogenic response [12].

Non-canonical Smad signaling in pre-neoplastic hepatocytes

Similar to MFB, hepatocytes in chronically HCV-infected livers exhibit phosphorylation at Smad3L, particularly in hepatocytes adjacent to inflamed portal tracts [69]. There, hepatocytes are regulated by the same pSmad3L pathway as are MFB. The extent of phosphorylation at Smad3L is less in hepatocytes distant from portal tracts, in sharp contrast to pSmad3C, which is located predominantly in hepatocytic nuclei distant from portal tracts [69]. Pro-inflammatory cytokines are released from infiltrating Kupffer cells in the portal tract to activate JNK [79, 80]. These findings suggest that pro-inflammatory cytokine-dependent JNK can convert pSmad3C to pSmad3L in both affected hepatocytes and MFB in chronic hepatitis (Figure 2).

In damaged hepatocytes and MFB under inflammatory microenviroment, TGF-β can act together with pro-inflammatory cytokines to induce fibrogenic signaling via the pSmad2L/C pathway. As a consequence, these cells exhibit proliferative and pro-fibrogenic TGF-β/Smad signaling, but have lost the capacity to respond to TGF-β with growth arrest and apoptosis.

Phospho-Smad signaling during progression of hepatitis virus-related chronic liver diseases

Immunohistochemical and immunofluorescence analyses using specific Abs in human tissues can examine clinical significance of context-dependent and cell type-specific signaling mediated by R-Smad phospho-isoforms, by comparison of tissue/cellular localization of these phospho-isoforms in tissue specimens [10]. Many clinical observations suggest that persistent hepatitis viral infection and chronic inflammation additively influence the development of HCC. For example, alcohol consumption is a recognized major cause of liver disease, and can contribute to progression to HCC. However, alcoholic liver disease progresses less frequently to HCC than HBV- or HCV-related hepatitis, and patients with both viral infection and alcohol consumption have a higher risk of developing HCC than those with alcohol consumption alone [81, 82, 83]. While autoimmune hepatitis (AIH) and primary billiary cirrhosis (PBC) are chronic inflammatory disorders that proceed to cirrhosis, HCC only rarely arises from AIH or PBC, particularly in the absence of HBV or HCV infection [84, 85].

As HBV contains partially double stranded-DNA, it can directly cause HCC by integrating its DNA into the host genome. HBV genomic integration is present in over 85% to 90% of livers developing HBV-related HCC, usually even before the development of HCC [86]. Integration of HBV DNA is not restricted to HCC but also is found in non-tumor tissue in patients with chronic HBV infection [87, 88]. HBV integration induces a wide range of genetic alterations within the host genome, including chromosomal deletions, translocations, production of fusion transcripts, amplification of cellular DNA, and generalized genomic instability [89, 90]. The HBx protein encoded by the X gene long has been suspected as a viral oncoprotein participating in hepato-carcinogenesis. HBx was shown to potentiate c-Myc-induced liver carcinogenesis in transgenic mice [91].

In transgenic models, HBx participates importantly in hepatocarcinogenesis via the pSmad3L/c-Myc pathway [70]. HBx transgenic mouse livers progressed through hyperplasia to HCC. Hepatocytic HBx, pSmad3L, and c-Myc increased as mouse liver progressed through hyperplasia to HCC. Positivity of hepatocytic nuclei for pSmad3L in early chronic hepatitis B specimens increases with the amount of HBV-DNA [70]. Taken together with results of *in vitro* experiments using HBx-expressing hepatocytes and HBx-transgenic livers, the more general human findings suggest that HBx oncoprotein participates directly in hepatocarcinogenesis by shifting hepatocytic Smad3 phospho-isoform signaling from the tumor suppressive pSmad3C/p21^{WAF1} pathway to the oncogenic JNK/pSmad3L/c-Myc pathway [70].

Unlike HBV, HCV is a positive-single-strand RNA virus, apparently incapable of integration into the host's genome. The HCV components modulate a number of cellular regulatory functions by targeting a wide spectrum of cellular signaling pathways [92, 93 ,94, 95, 96, 97, 98, 99]. HCV core expression has been shown to induce activation of the JNK pathway in regulation of vascular endothelial growth factor [99]. NS5A acts as a positive regulator of the JNK signaling pathway by interacting with TNF receptor-associated factor 2, which may be highly important in HCV pathogenesis [100]. In an HCV infection model, Lin *et al.* demonstrated that HCV directly induced TGF-β release from hepatocytes in a reactive oxygen species (ROS)-dependent and JNK-dependent manner [101]. Moreover, recent studies using transgenic mouse models indicated that HCV is involved in directly hepato-carcinogenesis. Three different HCV core transgenic lines develop liver steatosis and HCC [102, 103, 104]. HCV components and pro-inflammatory cytokine-dependent JNK can shift signaling from the tumor suppressive pSmad3C pathway to the carcinogenic JNK/pSmad3L pathway and fibrogenic pSmad2L/C pathway, accelerating liver fibrosis and increasing the risk of HCC [69].

Reversibility of phospho-Smad signaling between tumor-suppression and carcinogenesis

Chronic hepatitis B and C are now treatable diseases. Two types of therapy are available for HBV: interferon therapy and nucleoside analogues. Lamivudine, a nucleoside analogue, suppresses HBV replication through inhibition of reverse transcriptase and DNA polymerase [105]. Four other nucleoside and nucleoside analogues have been licensed: adefovir (in 2002) [106], entecavir (in 2005) [107], telbivudine (in 2006) [108], and most recently, tenofovir disoproxil fumarate (in 2008). These antiviral drugs act primarily by inhibiting reverse transcription of pregenomic RNA to HBV DNA. When effective suppression of viral replication in chronic hepatitis B patients has been achieved, alanine aminotransferase (ALT) levels decrease markedly and liver histology improves significantly. Antiviral therapies with interferon or a nucleoside analogue have proven to be useful in reduction of the incidence of HBV-related decompensated liver disease and rate of HCC occurrence [109].

The current treatment of hepatitis C, on the other hand, is pegylated IFN (PEG-IFN)-α, given by subcutaneous injection once weekly, and oral ribavirin (RBV) daily. RBV is a guanosine nucleoside analogue. This agent shows only modest activity against hepatitis C but it increases the activity of IFN-α when the 2 agents are used in combination. Efficacy of PEG-IFN and RBV has been investigated in several controlled trials that demonstrated an overall SVR rate of 40% to 50% [110]. However, limitations of IFN and RBV treatment have prompted a continuing search for improved therapies. Various molecular targets are now focus of anti-HCV drug development. Several new NS3 protease inhibitors, NS5b nucleoside polymerase inhibitors, and non-nucleoside polymerase inhibitors are being assessed in phase 3 studies [111, 112].

Patients with mild liver fibrosis have a significant likelihood of histologically evident decreases in fibrosis and inflammation after a SVR against HCV infection in response to IFN treatment [7]. Furthermore, these patients have marked reductions in decompensated liver disease and HCC occurrence [8, 9]. However, patients with advanced fibrosis have relatively low but appreciable risks of HCC occurrence and hepatic decompensation despite SVR [8]. Aleman *et al.* have pointed out that patients with cirrhosis from

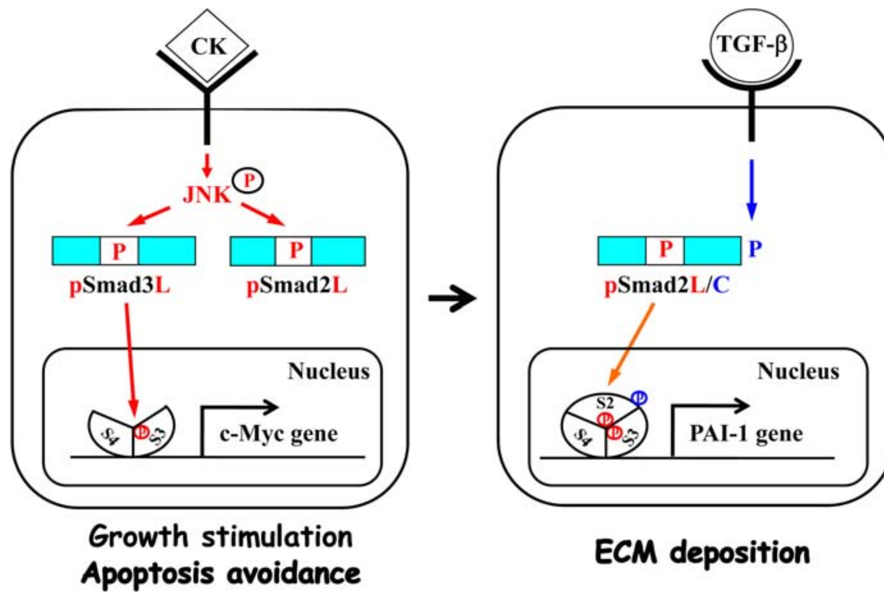

Figure 2. Fibro-carcinogenic signaling through non-canonical Smad. Pro-inflammatory cytokines (CK) such as TNF-α activate JNK, which phosphorylates Smad2L and Smad3L (left). After binding with Smad4, pSmad3L translocates with Smad4 to the nucleus and binds the PAI-1 promoter. After COOH-tail phophorylation of cytoplasmic pSmad2L by TβRI, pSmad2L/C translocate to the nucleus, where it interacts with pSmad3L. Both pSmad2L/C and pSmad3L stimulate PAI-1 transcription and ECM deposition (right). The Smad complex then stimulates PAI-1 transcription and ECM deposition, while it suppresses the pSmad3C-mediated tumor suppressive pathway.

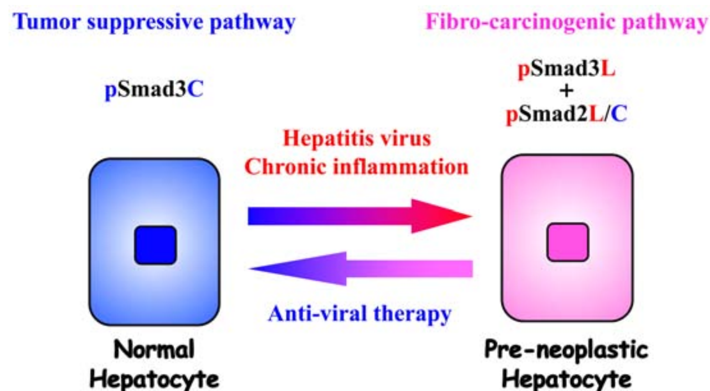

Figure 3. Reversible Smad phospho-isoform signaling between tumor suppression and fibro-carcinogenesis in human hepatitis virus-related chronic liver diseases. Chronic inflammation and hepatitis virus additively shift hepatocytic Smad phospho-isoform signaling from tumor suppressive pSmad3C to carcinogenic pSmad3L and fibrogenic pSmad2L/C pathways, accelerating liver fibrosis and increasing the risk of HCC. Patients with chronic liver diseases respond effectively to anti-viral therapy by successfully shifting Smad phospho-isoform signaling from fibro-carcinogenesis to tumor suppression.

HCV who achieve SVR should undergo long-term surveillance for HCC, while further studies aiming to better identify those with remaining long-term risk for HCC are needed [113].

We have found that HCV clearance restores human hepatocytic Smad phospho-isoform signaling from fibro-carcinogenenic to tumor suppressive signaling in early stages of chronic

HCV [114]. Even when SVR was achieved by patients with advanced fibrosis, hepatocytic positivity for pSmad3L increased, while pSmad3C positivity did not recover significantly. In addition, both linker and COOH-tail phosphorylation of Smad2 remained high in hepatocytic nuclei despite SVR. Consistently with that observations, these hepatocytes maintained high expression of c-Myc and PAI-1 (Yamaguchi T, unpublished data). A recent study demonstrated activation of the Ras pathway in 100% of HCC specimens examined in sharp contrast with non-neoplastic surrounding tissue and normal livers. Moreover, Ras inhibitors suppressed human HCC growth, supporting Ras pathway involvement in liver cancer [59]. Taken together, these results indicate that pre-neoplastic hepatocytes maintained fibro-carcinogenic JNK signaling by genetic and epigenetic alterations despite maintenance of SVR. Thus, the degree of chronic inflammation in preneoplastic hepatocytes no longer exerted a critical influence on Smad phospho-isoform signaling. Genetic and epigenetic alterations of major oncogenic pathway may maintain linker phosphorylation of Smad2/3, pSmad3L and pSmad2L/C, which can transmit fibro-carcinogenic signals even after chronic inflammation resolves. In this situation, HCV clearance cannot reverse acquired fibro-carcinogenic Smad signaling (Figure 3).

Pharmacologic interference with the JNK/pSmad2/3L pathway might interrupt carcinogenesis. In this respect Nagata *et al.* treated rats with a JNK inhibitor in order to reverse Smad3 signaling from the oncogenic pSmad3L pathway to the tumor suppressive pSmad3C pathway [71]. Collectively, the JNK/pSmad2/3L pathway should be an attractive target for adjuvant therapy against HCC.

CONCLUSION

IFN-based therapy has been shown to decrease the risk of hepatic decompensation [8] and HCC occurrence [9] among sustained virologic responders, while prevention of unfavorable disease outcome seems most effective when therapy is given before development of cirrhosis [8]. In this review we have focused on the reversibility and irreversibility of Smad phospho-isoform signaling between tumor suppression and fibro-carcinogenesis in hepatitis virus-related liver diseases. Detailed understanding of the molecular mechanisms involved in progression to HCC is of fundamental importance in guiding the development of effective prevention and treatment for HCC. Additionally, Smad phospho-isoform signaling can be used as a new predictive biomarker for early assessment of pharmacologic interventions intended to suppress human fibro-carcinogenesis.

CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest related to this article.

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