Smad3 phospho-isoform signaling in hepatitis C virus-related chronic liver diseases

Takashi Yamaguchi, Katsunori Yoshida, Miki Murata, Koichi Matsuzaki

Department of Gastroenterology and Hepatology, Kansai Medical University, Osaka 570-8506, Japan

Author contributions: Yamaguchi T, Yoshida K, Murata M and Matsuzaki K performed the research and analyzed the data; Yamaguchi T and Matsuzaki K wrote the paper.

Correspondence to: Takashi Yamaguchi, MD, PhD, Department of Gastroenterology and Hepatology, Kansai Medical University, 10-15 Fumizonocho, Moriguchi, Osaka 570-8506, Japan. yamaguct@takii.kmu.ac.jp

Telephone: +81-6-69961001 Fax: +81-6-69964874
Received: November 27, 2013 Revised: February 22, 2014 Accepted: May 29, 2014 Published online: September 21, 2014

Abstract

The risk of hepatocellular carcinoma (HCC) development increases as hepatitis virus C (HCV)-related liver diseases progress, especially in patients with active inflammation. Insight into hepatic carcinogenesis have emerged from recent detailed analyses of transforming growth factor-β and c-Jun-N-terminal kinase signaling processes directed by multiple phosphorylated (phospho)-isoforms of a Smad3 mediator. In the course of HCV-related chronic liver diseases, chronic inflammation and host genetic/epigenetic alterations additively shift the hepatocytic Smad3 phospho-isoform signaling from tumor suppression to carcinogenesis, increasing the risk of HCC. Chronic inflammation represents an early carcinogenic step that provides a non-mutagenic tumor-promoting stimulus. After undergoing successful antiviral therapy, patients with chronic hepatitis C could experience a lower risk of HCC as Smad3 phospho-isoform signaling reverses from potential carcinogenesis to tumor suppression. Even after HCV clearance, however, patients with cirrhosis could still develop HCC because of sustained, intense carcinogenic Smad3 phospho-isoform signaling that is possibly caused by genetic or epigenetic alterations. Smad3 phospho-isoforms should assist with evaluating the effectiveness of interventions aimed at reducing human HCC.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Key words: Chronic inflammation; c-Jun N-terminal kinase; Hepatitis C virus; Hepatocellular carcinoma; Smad3 phospho-isoform signaling; Transforming growth factor β

Core tip: The risk of hepatocellular carcinoma (HCC) development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Our model suggests that the chronic inflammatory state and the hepatitis viruses themselves act in concert with genetic or epigenetic alterations to worsen human liver diseases by promoting hepatic carcinogenesis. The affected hepatocytes are subject to such interactions until their descendants acquire other genetic or epigenetic alterations. Even after the withdrawal of promoters such as chronic inflammation and hepatitis viruses, the hepatocytes could retain proliferative phenotypes, enabling some pre-neoplastic hepatocytes to develop into HCC.

Yamaguchi T, Yoshida K, Murata M, Matsuzaki K. Smad3 phospho-isoform signaling in hepatitis C virus-related chronic liver diseases. World J Gastroenterol 2014; 20(35): 12381-12390
Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i35/12381.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i35.12381

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is characterized by intrahepatic lipid accumulation (steatosis), variable degrees of progressive fibrosis, and long-term progression to cirrhosis and hepatocellular carcinoma (HCC). Approximately 5-10% of patients with chronic hepatitis C (CHC) develop HCC during the course of their disease, with the risk of HCC development increasing as CHC progresses. The risk of HCC development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Insight into hepatic carcinogenesis has emerged from recent detailed analyses of transforming growth factor-β (TGF-β) and c-Jun-N-terminal kinase (JNK) signaling processes directed by multiple phosphorylated (phospho)-isoforms of a Smad3 mediator. In the course of CHC-related chronic liver diseases, chronic inflammation and host genetic/epigenetic alterations additively shift the hepatocytic Smad3 phospho-isoform signaling from tumor suppression to carcinogenesis, increasing the risk of HCC. Chronic inflammation represents an early carcinogenic step that provides a non-mutagenic tumor-promoting stimulus. After undergoing successful antiviral therapy, patients with chronic hepatitis C could experience a lower risk of HCC as Smad3 phospho-isoform signaling reverses from potential carcinogenesis to tumor suppression. Even after HCV clearance, however, patients with cirrhosis could still develop HCC because of sustained, intense carcinogenic Smad3 phospho-isoform signaling that is possibly caused by genetic or epigenetic alterations. Smad3 phospho-isoforms should assist with evaluating the effectiveness of interventions aimed at reducing human HCC.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Key words: Chronic inflammation; c-Jun N-terminal kinase; Hepatitis C virus; Hepatocellular carcinoma; Smad3 phospho-isoform signaling; Transforming growth factor β

Core tip: The risk of hepatocellular carcinoma (HCC) development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Our model suggests that the chronic inflammatory state and the hepatitis viruses themselves act in concert with genetic or epigenetic alterations to worsen human liver diseases by promoting hepatic carcinogenesis. The affected hepatocytes are subject to such interactions until their descendants acquire other genetic or epigenetic alterations. Even after the withdrawal of promoters such as chronic inflammation and hepatitis viruses, the hepatocytes could retain proliferative phenotypes, enabling some pre-neoplastic hepatocytes to develop into HCC.

Yamaguchi T, Yoshida K, Murata M, Matsuzaki K. Smad3 phospho-isoform signaling in hepatitis C virus-related chronic liver diseases. World J Gastroenterol 2014; 20(35): 12381-12390
Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i35/12381.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i35.12381

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is characterized by intrahepatic lipid accumulation (steatosis), variable degrees of progressive fibrosis, and long-term progression to cirrhosis and hepatocellular carcinoma (HCC). Approximately 5-10% of patients with chronic hepatitis C (CHC) develop HCC during the course of their disease, with the risk of HCC development increasing as CHC progresses. The risk of HCC development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Insight into hepatic carcinogenesis has emerged from recent detailed analyses of transforming growth factor-β (TGF-β) and c-Jun-N-terminal kinase (JNK) signaling processes directed by multiple phosphorylated (phospho)-isoforms of a Smad3 mediator. In the course of CHC-related chronic liver diseases, chronic inflammation and host genetic/epigenetic alterations additively shift the hepatocytic Smad3 phospho-isoform signaling from tumor suppression to carcinogenesis, increasing the risk of HCC. Chronic inflammation represents an early carcinogenic step that provides a non-mutagenic tumor-promoting stimulus. After undergoing successful antiviral therapy, patients with chronic hepatitis C could experience a lower risk of HCC as Smad3 phospho-isoform signaling reverses from potential carcinogenesis to tumor suppression. Even after HCV clearance, however, patients with cirrhosis could still develop HCC because of sustained, intense carcinogenic Smad3 phospho-isoform signaling that is possibly caused by genetic or epigenetic alterations. Smad3 phospho-isoforms should assist with evaluating the effectiveness of interventions aimed at reducing human HCC.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Key words: Chronic inflammation; c-Jun N-terminal kinase; Hepatitis C virus; Hepatocellular carcinoma; Smad3 phospho-isoform signaling; Transforming growth factor β

Core tip: The risk of hepatocellular carcinoma (HCC) development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Our model suggests that the chronic inflammatory state and the hepatitis viruses themselves act in concert with genetic or epigenetic alterations to worsen human liver diseases by promoting hepatic carcinogenesis. The affected hepatocytes are subject to such interactions until their descendants acquire other genetic or epigenetic alterations. Even after the withdrawal of promoters such as chronic inflammation and hepatitis viruses, the hepatocytes could retain proliferative phenotypes, enabling some pre-neoplastic hepatocytes to develop into HCC.

Yamaguchi T, Yoshida K, Murata M, Matsuzaki K. Smad3 phospho-isoform signaling in hepatitis C virus-related chronic liver diseases. World J Gastroenterol 2014; 20(35): 12381-12390
Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i35/12381.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i35.12381

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is characterized by intrahepatic lipid accumulation (steatosis), variable degrees of progressive fibrosis, and long-term progression to cirrhosis and hepatocellular carcinoma (HCC). Approximately 5-10% of patients with chronic hepatitis C (CHC) develop HCC during the course of their disease, with the risk of HCC development increasing as CHC progresses. The risk of HCC development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Insight into hepatic carcinogenesis has emerged from recent detailed analyses of transforming growth factor-β (TGF-β) and c-Jun-N-terminal kinase (JNK) signaling processes directed by multiple phosphorylated (phospho)-isoforms of a Smad3 mediator. In the course of CHC-related chronic liver diseases, chronic inflammation and host genetic/epigenetic alterations additively shift the hepatocytic Smad3 phospho-isoform signaling from tumor suppression to carcinogenesis, increasing the risk of HCC. Chronic inflammation represents an early carcinogenic step that provides a non-mutagenic tumor-promoting stimulus. After undergoing successful antiviral therapy, patients with chronic hepatitis C could experience a lower risk of HCC as Smad3 phospho-isoform signaling reverses from potential carcinogenesis to tumor suppression. Even after HCV clearance, however, patients with cirrhosis could still develop HCC because of sustained, intense carcinogenic Smad3 phospho-isoform signaling that is possibly caused by genetic or epigenetic alterations. Smad3 phospho-isoforms should assist with evaluating the effectiveness of interventions aimed at reducing human HCC.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Key words: Chronic inflammation; c-Jun N-terminal kinase; Hepatitis C virus; Hepatocellular carcinoma; Smad3 phospho-isoform signaling; Transforming growth factor β

Core tip: The risk of hepatocellular carcinoma (HCC) development increases in patients persistently infected with hepatitis viruses, especially in patients with active viral replication and inflammation. Our model suggests that the chronic inflammatory state and the hepatitis viruses themselves act in concert with genetic or epigenetic alterations to worsen human liver diseases by promoting hepatic carcinogenesis. The affected hepatocytes are subject to such interactions until their descendants acquire other genetic or epigenetic alterations. Even after the withdrawal of promoters such as chronic inflammation and hepatitis viruses, the hepatocytes could retain proliferative phenotypes, enabling some pre-neoplastic hepatocytes to develop into HCC.
progression to cirrhosis and hepatocellular carcinoma (HCC)\(^{[3,4]}\). This progression is attributed to the continuous activation of the immune response, with increased production of pro-inflammatory cytokines\(^{[5]}\). Although the causal relationship between chronic inflammation and hepatic carcinogenesis is widely accepted, the molecular and cellular mechanisms explaining this relationship remain unknown. Moreover, the polyprotein encoded by the HCV genome contributes to hepatic carcinogenesis. Over-expression of the HCV core protein induces HCC in transgenic mice, and the nonstructural NS5A protein has trans-activating properties that might alter patterns of host gene expression to favor neoplastic transformation\(^{[6-9]}\). In addition, a full-length, infectious clone of HCV cDNA stably replicating in hepatocytes has been shown to stimulate hepatocytic growth and survival in vitro and to accelerate tumor formation in vivo\(^{[10]}\).

Hepatic carcinogenesis is a multistep process involving multiple cellular signaling pathways. Transforming growth factor (TGF-\(\beta\)) signaling has important roles in this process. HCV infection is associated with significant increases in TGF-\(\beta\) in serum and liver samples\(^{[11]}\). Dysregulated, persistent TGF-\(\beta\) actions are thought to lead to pathological states. TGF-\(\beta\) is a potent growth inhibitor of hepatocytes, and is also considered the most potent pro-fibrogenic cytokine; TGF-\(\beta\) accelerates liver fibrosis by triggering the proliferation and transformation of hepatic stellate cells (HSCs) in HCV-infected patients\(^{[12-14]}\). A series of studies have shown that c-Jun N-terminal kinase (JNK) signaling communicates closely with TGF-\(\beta\) signaling to regulate hepatic carcinogenesis\(^{[15-18]}\). A consensus emerging from these studies is that the JNK pathway could antagonize TGF-\(\beta\) induced growth inhibition and favor the pro-invasive function of TGF-\(\beta\)\(^{[19]}\). Positive and negative regulation occurs between the two pathways.

Recently, the majority of investigations into TGF-\(\beta\) have concerned downstream regulatory events, particularly the components of TGF-\(\beta\) signaling\(^{[20]}\). Here, we outline how TGF-\(\beta\) signaling is regulated by the JNK pathway. We consider how influences such as chronic inflammation, HCV, and somatic mutations additively promote tumor formation. Finally, we discuss the reversibility of TGF-\(\beta\) signaling profiles between tumor suppression and carcinogenesis in human HCV-related chronic liver diseases.

**JNK PATHWAY INDUCES HEPATIC REGENERATION AND CARCINOGENESIS**

The JNK pathway participates in physiological cell growth and in malignant transformation. JNK activates activator protein (AP)-1, which promotes the expression of cyclin D and initiates the G0-G1 transition in the cell cycle\(^{[21]}\). Upon partial hepatectomy, the pharmacologic inhibition of JNK blocks c-Jun phosphorylation, AP-1 activation, and cyclin D1 expression, with the effect of delaying regeneration\(^{[22]}\). Consistently, Jnk1-/- mice show decreased liver regeneration following partial hepatectomy, and these mice have increased p21\(^{\text{WAF1}}\), which is a cell-cycle inhibitor, and reduced expression of c-Myc, which is a negative regulator of p21\(^{\text{WAF1}}\). A deficiency in p21\(^{\text{WAF1}}\) was found to reverse the regeneration defect in Jnk1-/-livers, indicating that JNK1 promotes hepatocyte proliferation by inhibiting p21\(^{\text{WAF1}}\)\(^{[23,24]}\). The most compelling evidence for a role of JNK in cancer initiation comes from studies of HCC development in animal and human models. JNK1-null mice exhibit impaired liver carcinogenesis with reduced tumor masses, sizes, and numbers caused by increased expression of p21\(^{\text{WAF1}}\) and reduced expression of c-Myc\(^{[25]}\). JNK1 is essential for mediating the development of HCC that accompanies a loss of hepatic nuclear factor-\(\kappa\)B activity in mice\(^{[26-28]}\). High JNK1 activation is associated with a poorer patient prognosis and the over-expression of several hepatic progenitor cell markers, such as CD133\(^{[29]}\).

**ONCOPROTEINS ENCODED BY THE HCV GENOME PROMOTE HEPATIC CARCINOGENESIS THROUGH THE JNK PATHWAY**

HCV, a single-stranded RNA virus that could not integrate into the host genome, has a predominantly cytoplasmic life cycle. All of the potentially pro-oncogenic events are likely to be restricted to the cytoplasm, suggesting indirect mechanisms of hepatic carcinogenesis\(^{[30]}\). The HCV genome has a long open reading frame that encodes a polyprotein precursor. This polyprotein is cleaved by host and viral proteases to generate 4 structural proteins (C, E1, E2, and P7) and 6 nonstructural proteins (xlink, NS3, NS4A, NS4B, NS5A, and NS5B)\(^{[31,32]}\). Although the immune response to the virus and the consequent nonspecific chronic inflammation is central to the development of HCC, the HCV core protein has been shown to induce HCC in transgenic mice and is suggested to play a role in HCC development in cases of chronic hepatitis C\(^{[33]}\). Exactly how the core protein acts in the development of HCC remains unclear\(^{[34]}\). However, the HCV core protein has been shown to promote oxidative stress, steatosis, and apoptosis, all of which might promote carcinogenesis. The HCV core protein induces activation of the JNK pathway through a regulatory mechanism involving vascular endothelial growth factor\(^{[35]}\). Cells over-expressing the HCV core protein have decreased p21\(^{\text{WAF1}}\) protein half-life and increased c-Myc promoter in tissue culture and in HCV core-expressing transgenic mice\(^{[36]}\). In addition, NS5A acts as a positive regulator of the JNK signaling pathway by interacting with tumor necrosis factor receptor associated factor (TRAF), which might play a key role in HCV pathogenesis\(^{[37]}\). In an HCV infection model, Lin et al\(^{[38]}\) demonstrated that HCV directly induced TGF-\(\beta\) release from hepatocytes in a manner that is dependent on the abundance of reactive oxygen species (ROS) and JNK.
CHRONIC INFLAMMATION INDUCED BY HCV INFECTION PROMOTES HEPATIC CARCINOGENESIS THROUGH THE JNK PATHWAY

A positive correlation between the amounts of pro-inflammatory cytokines, the intensity of non-inflamatory activity, and the degree of liver fibrosis has been reported. Injury-induced apoptosis and necrosis of hepatocytes activate Kupffer cells (KCs). In activated KCs, the translocation of pro-inflammatory cytokines is induced, most notably tumor necrosis factor (TNF-α and TGF-β).

The binding of TNF-α to TNF receptor 1 leads to the rapid formation of complex I, comprising TNFR associated death domain, receptor interacting protein 1 (RIP1), TRAF2, cellular inhibitor of apoptosis (cIAP)-1, cIAP2, and a dimeric ubiquitin (Ub)-conjugating enzyme composed of Ubc13. The cIAP-mediated K63 ubiquitination of RIP1 recruits and activates TGF-β activated kinase 1 (TAK1). TAK1 activates JNK through mitogen-activated kinase kinase 4/7 (MKK4/7). Complex I also contributes to ROS production through reduced nicotinamide adenine dinucleotide phosphate oxidase 1 and Rac1. ROS accumulation promotes the prolonged activation of JNK by inactivating JNK phosphatases.

The inflammatory response triggered by HCV infection precedes tumor development and part of the normal host defenses. In most cases, HCV subverts host immunity and establishes persistent infections associated with chronic inflammation. Chronic inflammation drives a maladaptive reparative reaction and stimulates liver cell death and regeneration, which is associated with the eventual development of dysplastic nodules and cancer. Accordingly, HCV-related liver disease progression is promoted by the synergistic action of HCV and chronic inflammation, with chronic inflammation appearing to play the predominant role.

JNK DEPENDENT SMAD3 SIGNALING THROUGH LINKER PHOSPHORYLATION

The receptor-associated activation of Smads, which include Smad2 and the very similar protein Smad3, is an important intracellular mediator of TGF-β signaling. Previous studies have shown mutual interactions between the TGF-β-related JNK and Smad pathways. The JNK pathway could antagonize TGF-β-induced growth inhibition, allowing for the acquisition of an invasive phenotype. The nuclear function of Smad complexes could be regulated by JNK1, which promotes binding to DNA and supports diverse gene responses to TGF-β, leading to an invasive phenotype. TGF-β also induces the activation of non-Smad signaling pathways involving TAK1 and JNK.

In addition to the modulation of transcriptional responses, linker phosphorylation of cytoplasmic Smad proteins is critical for the integration of JNK signaling with the TGF-β pathway. Linker phosphorylation of Smad3 permits translocation into the nucleus and allows for further consequences of JNK signaling.

Thus, JNK signaling modulates or competes for a Smad-mediated signaling pathway. JNK simultaneously activates linker-phosphorylated Smad and nuclear transcription factors binding the Smad complex, and these changes typically occur in parallel. Accordingly, the linker-phosphorylated Smad pathway is difficult to assess in isolation. To address this problem, we produced domain-specific antibodies (Abs) that are able to distinguish between the phosphorylated linker region and the C-terminal region of Smad3. These Abs allowed us to determine the phosphorylation sites of Smad3 and the cellular location of Smad3 in liver tissue, providing better understanding of phospho-Smad3 signaling regulation in hepatocytes (Figure 1).

**SMAD3 PHOSPHO-ISOFORM SIGNALING: TUMOR SUPPRESSIVE TGF-β VS CARCINOGENIC JNK/PSMAD3L PATHWAYS**

Cell proliferation is limited by extracellular signals that maintain tissue homeostasis. TGF-β signaling is a potent supplier of such signals. TGF-β catalytically activates TβRI, which phosphorylates the C-terminal serine residues of Smad3. C-terminal phosphorylated Smad3 (pSmad3C) accumulates in the nucleus and participates in the induction of p21WAF1, which inhibits cyclin-dependent kinase (CDK) and represses the expression of c-Myc (Figure 2A).

Liver regeneration induced by necrogenic doses of carbon tetrachloride (CCL4) is a well-studied model of cell cycle control and signal transduction because of the robust kinetic profile of hepatocyte necrosis and proliferation following CCL4-induced liver injury. Pro-inflammatory cytokines, such as TNF-α, are important components of the mitogenic pathways leading to regeneration after an acute liver injury. During liver regeneration, hepatocytes acquire temporary resistance to growth inhibition mediated by TGF-β, allowing the cells to proliferate in response to pro-inflammatory cytokine. Because TGF-β is also increased in the damaged liver, other mechanisms appear to play critical roles in hepatocytic growth regulation. The phosphorylation pattern of Smad3 in regenerative hepatocytes after an acute liver injury suggests an important participation of Smad3 phospho-isofrom signaling in this regulation. After CCL4 poisoning, linker-phosphorylated Smad3 (pSmad3L) molecules were located in the nuclei of regenerating hepatocytes within 8 h. After a liver injury, pro-inflammatory cytokines activate JNK to induce the phosphorylation of Smad3, which undergoes translocation to the nucleus to stimulate c-Myc transcription (Figure 2B), which increases the proliferation of hepatocytes and opposes the cyostatic TGF-β signaling. As pSmad3L eventually diminishes, pSmad3C dramatically increases in...
Figure 1  Two distinct hepatocytic Smad3 phospho-isoforms in chronic hepatitis.

Liver specimens with moderate fibrosis and inflammation from a patient with chronic hepatitis C were immunostained using domain-specific antibodies (Abs) that are able to distinguish between the phosphorylated linker region and the C-terminal region of Smad3. Each section was counterstained with hematoxylin (blue). The brown color indicates specific Ab reactivity. The distribution of pSmad3C and pSmad3L showed a sharp contrast in the liver specimens. Whereas the hepatocytes adjacent to the collagen fibers of the portal tract showed nuclear localization of pSmad3L (upper column), pSmad3C was predominantly located in the hepatocytic nuclei distant from the portal tract (lower column).

Figure 2  Smad3 phospho-isoform signaling: Tumor suppressive TβRI/pSmad3C pathway vs carcinogenic JNK/pSmad3L pathway.

A: In normal hepatocytes, transforming growth factor (TGF)-β activates the TGF-β type I receptor (TβRⅠ), leading to the direct phosphorylation of the C-terminal region of Smad3. The C-terminal phosphorylated Smad3 (pSmad3C) moves to the nucleus and transmits cytostatic signals by up-regulating p21WAF1. The TβRI/pSmad3C pathway is crucial for homeostasis and is a tumor suppressor; B: In hepatocytes affected by mitogenic stimuli, pro-inflammatory cytokines activate c-Jun N-terminal kinase (JNK), drastically altering the Smad3 signaling to induce the phosphorylation of Smad3 at its linker region. The linker-phosphorylated Smad3 (pSmad3L) undergoes translocation to the nucleus to stimulate c-Myc transcription, resulting in a proliferative state. During hepatitis C virus-related chronic liver disease progression, the JNK/pSmad3L pathway is constitutively activated and promotes carcinogenesis.
the nuclei of hepatocytes at 36 h after a chemical insult, a time when proliferation stops\[^{52}\]. The cytostatic T\(\beta\)RI/ pSmad3C signaling contributes to the termination of hepatocyte proliferation.

In human liver specimens with chronic hepatitis C, the distribution of pSmad3L and pSmad3C showed a sharp contrast (Figure 1). Whereas the hepatocytes adjacent to the collagen fibers of the portal tract showed nuclear localization of pSmad3L, pSmad3C was predominantly located in the hepatocytic nuclei distant from the portal tract. During the transdifferentiation of HSCs to myofibroblasts (MFBs) phenotype, the pSmad3C-mediated signal decreases, and the pSmad3L pathway predominates\[^{64}\]. The MFBs in the portal tracts of chronically HCV-infected liver specimens also exhibit the pSmad3L pathway. These observations indicate that HCV-infected hepatocytes exhibit the same pSmad3L signaling as MFBs, accelerating liver disease progression\[^{9,67}\].

During carcinogenesis, the physiological balance between proliferation and differentiation in normal hepatocyte homeostasis is lost. The cytostatic TGF-\(\beta\) signaling appears to maintain such balance in the early stages of carcinogenesis\[^{33}\]. Some reports showed a low frequency of mutations of T\(\beta\)RII and other TGF-\(\beta\)-pathway genes in HCC, which are frequently found to be mutationally inactivated in other gastrointestinal cancers\[^{56-60}\]. When T\(\beta\)fr2 was deleted in hepatic tissue, the p53 knockout mice showed inhibition of hepatocarcinogenesis\[^{61}\]. Furthermore, in HCC cell lines, the knockdown of T\(\beta\)RII or Smad4 attenuated the nuclear accumulation of pSmad3L and caused growth inhibition\[^{62}\]. These reports indicate that carcinogenic TGF-\(\beta\) signaling is likely mediated in part by linker phosphorylation of Smad3. This is demonstrated by the fact that a high level of linker Smad3 phosphorylation was observed in HCC specimens and in human HCC cell lines\[^{63,65}\].

We previously showed that HBV- and HCV-induced HCC subsequently develops in patients whose liver specimens show abundant Smad3L and limited Smad3C phosphorylation in hepatic nuclei; however, the HBV- and HCV-induced HCC does not develop in patients whose liver specimens show abundant pSmad3C but limited pSmad3L\[^{63,64}\]. Taken together, during the progression of HCV-related chronic liver diseases, pre-neoplastic hepatocytes lose the tumor suppressive T\(\beta\)RI/pSmad3C signaling pathway to constitutively activate the carcinogenic JNK/pSmad3L signaling pathway.

**As HCV Infections of the Liver Progresses from Chronic Hepatitis Through Cirrhosis to HCC, Hepatocytic Smad3 Phospho-isoform Signaling Shifts from Tumor Suppression to Carcinogenesis**

To clarify the relationship between Smad3 phospho-isoform signaling and liver disease progression, we studied patients with HCV-related chronic liver disease and HCC. We immunostained serial liver sections using anti-pSmad3L and anti-pSmad3C Abs, paired with staining for p21\(^{WAF1}\) and c-Myc\[^{63,64}\]. The immunostaining of a normal liver with an Ab specific to pSmad3C shows slight phosphorylation of Smad3C throughout the liver and scant phosphorylation of Smad3L. In cirrhotic and HCC livers, pSmad3C is less abundant than in chronic hepatitis C, and pSmad3L gradually increases during liver disease progression. As chronic hepatitis C progresses through cirrhosis to HCC, c-Myc staining increases, whereas p21\(^{WAF1}\) staining decreases in the hepatocytic nuclei. Moreover, double-immunofluorescence studies on the liver specimens representing HCV-related chronic liver diseases confirm that pSmad3L co-localizes with c-Myc, and pSmad3C co-localizes with p21\(^{WAF1}\). These observations indicate that hepatocytic Smad3 phospho-isoform signaling shifts from tumor suppressive pSmad3C/p21\(^{WAF1}\) signaling to carcinogenic pSmad3L/c-Myc signaling during chronic liver disease progression.

We previously reported that positivity of hepatocytic nuclei for pSmad3L in chronic hepatitis C specimens showed no significant relationship with the plasma HCV-RNA level in chronic hepatitis C patients\[^{63}\]; in chronic hepatitis B patients, the positivity of hepatocytic nuclei for pSmad3L increased in proportion to the amount of HBV-DNA\[^{64}\]. The hepatocytic phosphorylation of Smad3L in chronic hepatitis C increased in proportion to necroinflammatory activity\[^{64}\], and there was no correlation between pSmad3L and necroinflammatory activity in chronic hepatitis B patients\[^{64}\]. These results suggested that HCV contributed indirectly to the development of HCC through chronic inflammation. In the early stages of chronic hepatitis, HBV played a more important role than HCV in hepatocarcinogenesis. Because HCC gradually develops in transgenic mice expressing the core protein or the HCV full-length polyprotein\[^{67,69}\], hepatitis virus itself, together with the host immune response, might promote human hepatocarcinogenesis via the JNK/pSmad3L pathway during the late stages of the carcinogenic process in HCV-related chronic liver disease.

Because the T\(\beta\)RI/pSmad3C pathway is required for the maintenance of genomic stability, the induction of replicative senescence, and the suppression of telomerase\[^{60,71}\], escaping the tumor suppressive T\(\beta\)RI/pSmad3C pathway is a critical step for progression to full malignancy in cancers; this step must overcome multiple fail-safe genetic controls\[^{72}\]. The chronic inflammation associated with HCV infection appears to be the primary initial requirement for the carcinogenic JNK/pSmad3L pathway in earlier stages of chronic hepatitis C. Genomic alterations appear to develop randomly, beginning in pre-neoplastic hepatocytes, and their development escalates in dysplastic hepatocytes and HCCs. Synergy between chronic inflammation and persistent HCV infection leads to the accumulation of events necessary for the malignant transformation of hepatocytes. HCV promotes genetic instability by several mechanisms that
compromise the ability of the hepatocytes to mediate DNA repair\cite{68}. HCV upregulates DNA methyltransferases, which decrease the expression of genes involved in DNA repair\cite{73}. HCV core proteins compromise several p53 functions, including nucleotide excision repair and transcription-coupled repair\cite{74,75}. HCV also blocks DNA repair through the ataxia telangiectasia mutated DNA repair pathway\cite{76}. HCV core proteins suppress the CDK inhibitors through promoter methylation, resulting in the inactivation of the retinoblastoma tumor suppressor protein\cite{77}. JNK signaling might be capable of affecting the epigenetic landscape of the genome, which could render hepatocytes more susceptible to carcinogens and DNA-damaging influences, such as oxidative stress. Increased cell proliferation occurring in the presence of oxidative stress leads to the accumulation of DNA damage that is thought to compromise gene and chromosome stability and forms the genomic basis for the malignant transformation of hepatocytes. Hepatocytes with high expression levels of histone methyltransferases, especially EZH2, resulting from increased JNK activation or activity might be able to bypass the cellular senescence or apoptotic program, leading to malignant transformation\cite{78}. Furthermore, the accumulation of damaged DNA and cell senescence could give rise to tumor promoting chronic inflammation\cite{79}. Chronic inflammation and HCV additively shift hepatocytic Smad3 phospho-isoform signaling from tumor suppression to carcinogenesis, increasing the risk of hepatocellular transformation during HCV-related chronic liver diseases, and lead to further carcinogenic transformation of hepatocytes in later stages in cooperation with additional host genetic/epigenetic alterations (Figure 3A).

**AFTER SUCCESSFUL ANTIVIRAL THERAPY, PATIENTS WITH CHRONIC HEPATITIS C HAVE A LOWER RISK OF HCC BECAUSE HEPATOCYTIC SMAD3 PHOSPHO-ISOFORM SIGNALING HAS SHIFTED FROM CARCINOGENESIS TO TUMOR SUPPRESSION, ALTHOUGH PATIENTS WITH CIRRHOSIS REMAIN AT SERIOUS RISK**

Linker phosphorylation of Smad3 indirectly inhibits Smad3 C-terminal phosphorylation and subsequently suppresses pSmad3C signaling\cite{15,17}. Smad3 mutants that lack linker phosphorylation sites and JNK inhibitors could restore the tumor suppressive TβRI/pSmad3C signaling in Ras-transformed epithelial cells, HBx expressing hepatocytes, and N-diethylnitrosamine-treated rat livers\cite{15,17,19,64}. The TβRI/pSmad3C and JNK/pSmad3L signals oppose each other, and the balance could shift from tumor suppression to carcinogenesis.

We recently studied paired liver samples obtained from patients with chronic hepatitis C before antiviral therapy and after a sustained virological response (SVR) against HCV infection in response to antiviral therapy. Patients with SVR frequently show histologically evident decreases in fibrosis and inflammation\cite{80}. Furthermore, these patients rarely develop HCC\cite{81}. Immunohistochemical analyses confirmed that carcinogenic JNK/pSmad3L signaling gradually shifts to tumor suppressive TβRI/pSmad3C signaling after a SVR\cite{82} (Figure 3B). Based on these observations, the chronic inflammation caused by persistent HCV infection is hypothesized to represent an early carcinogenic step that provides a nonmutagenic tumor-promoting stimulus. In contrast to patients with chronic hepatitis C, patients with cirrhosis have relatively low, although significant, risk of HCC and do not show reductions in decompensated liver diseases despite HCV clearance\cite{83,84}. We studied the liver samples obtained from patients with cirrhosis who are at risk for the development of HCC despite a SVR. Reflecting these clinical observations, the hepatocytes maintaining high carcinogenic JNK/pSmad3L signaling could not return to tumor suppressive TβRI/pSmad3C-signaling even after HCV clearance\cite{85}. Because the majority of these patients display decreased inflammatory activity, their disease progression appears to have become independent of chronic inflammation. These patients appear to have evolved beyond dependence on inflammation and are unable to reverse carcinogenic JNK/pSmad3L signaling because the hepatocytes have acquired genetic or epigenetic alterations (Figure 3B). Understanding the molecular mechanisms of inflammation-independent carcinogenesis requires further genetic and epigenetic analyses in patients who develop HCC despite HCV clearance.

**CONCLUSION**

To date, reliable predictive markers that could accurately estimate the risk of chronic hepatitis C disease progression are unavailable. The identification of robust molecular predictive markers for HCC that could supplement conventional pathological staging systems is needed. Our approach identified pSmad3L as a potentially clinically useful prognostic marker for HCC.

Despite some success with our approaches using human liver tissue samples, several technical hurdles must be overcome before pSmad3L could become a reliable HCC risk indicator. First, patient characteristics often show a bias when samples are retrospectively collected from a tissue archive that was not intended for the particular study at the time of acquisition. Large-scale validation in a prospective multicenter study with the integration of long-term outcome data is needed. Second, confirming the accuracy of the variables reported in our recent study concerning pSmad3L positivity requires quantitative analyses. We recently developed a sandwich enzyme-linked immunosorbent assay (ELISA) to quantitatively evaluate the extent of phosphorylation of Smad3L. A preliminary
study using this system showed that the sandwich ELISA closely correlated with immunohistochemical scores in liver tissue that is chronically infected with HCV (unpublished observation). We hope that, in the future, many investigators will use our sandwich ELISA system as a rapid, specific, and quantitative method for the detection of the phosphorylation of Smad3 in various human tissues. Such predictive markers should allow us to identify groups of HCV patients at high or low risk for developing HCC, especially patients that have achieved a SVR. Such profiles are likely to permit the evaluation of the effectiveness of interventions aimed at reducing human cancer risks.

REFERENCES

1  Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, Everson GT, Lindsay KL, Lee WM, Bonkovsky
Arzumanany A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocarcinoma. Nat Rev Cancer 2013; 13: 123-135 [PMID: 23344543 DOI: 10.1038/nrc3449]

Chung RT, Kang M, Sherman KE, Koziel MJ, Peters MG. Role of JNK activity in the development of hepatocellular carcinoma. Cancer Cell 2009; 15: 21-62 [PMID: 16904831 DOI: 10.1016/j.ccr.2006.12.016]

Chung WJ, Zhang L, Zhao H, Jang JY, Chung RT. Hepatitis B virus regulates transforming growth factor beta1 production that promotes chemical hepatocarcinogenesis. J Hepatol 2009; 51: 810-820 [PMID: 19545926 DOI: 10.1016/j.jhep.2009.05.008]

Tsai WL, Chung RT. Viral hepatocarcinogenesis. Oncogene 2010; 29: 2309-2324 [PMID: 20228847 DOI: 10.1038/onc.2010.36]

Blackard JT, Moses HL, Dienstag JL, Ghany MG, Morishima C, Goodman ZD. Parallel epigenetic and genetic changes in the pathogenesis of hepatitis virus-associated hepatocellular carcinoma. Cancer Lett 2006; 239: 10-20 [PMID: 16154256 DOI: 10.1016/j.canlet.2005.07.009]

CHANG Q, Chang WJ, Zhang L, Zhao H, Huang J, Chen J, Castranova V, Shi X, Chen F. Inhibition of c-Jun NH2-terminal kinase switches Smad3 signaling from oncogenesis to tumor-suppression in rat hepatocellular carcinoma. Hepatology 2009; 49: 1944-1953 [PMID: 19181558 DOI: 10.1002/hep.22860]

Schwabe RF, Bradham CA, Uehara T, Hatanaka E, Bennett BL, Schoonhoven R, Brenner DA. Transient induction of c-jun during hepatic regeneration. Hepatology 1990; 11: 909-915 [PMID: 2114348 DOI: 10.1002/hep.1840110602]

Matsuzaki K, Uemoto S. Inhibition of c-Jun NH2-terminal kinase switches Smad3 signaling from oncogenesis to tumor-suppression in rat hepatocellular carcinoma. Hepatology 2009; 49: 1944-1953 [PMID: 19181558 DOI: 10.1002/hep.22860]
Cytostatic vs carcinogenic Smad signaling.

Vasilaki E, Siderakis M, Papakosta P, Skourti-Stathaki K, Marvidiou S, Kardassis D. Novel regulation of Smad3 oligomerization and DNA binding by its linker domain. Biochemistry 2009; 48: 8366-8378 [PMID: 19645436 DOI: 10.1021/bi0905489]

Matsuzaki K. Smad phosphoisoform signals in acute and chronic liver injury: similarities and differences between epithelial and mesenchymal cells. Cell Tissue Res 2012; 347: 225-243 [DOI: 21626291 DOI: 10.1007/s00441-011-1178-6]

Matsuzaki K. Smad phosphoisoforms direct context-dependent TGF-β signaling. Cytokine Growth Factor Rev 2013; 24: 385-399 [PMID: 23786109 DOI: 10.1016/j.cytogfr.2013.06.002]

Matsuzaki K. Smad3 phosphoisoform-mediated signaling during sporadic human colorectal carcinogenesis. Histol Histopathol 2006; 21: 645-662 [PMID: 15628675]

Massagué J. G1 cell-cycle control and cancer. Nature 2004; 432: 298-306 [PMID: 15540991]

Fausto N, Campbell JS, Riehle KL. Liver regeneration. Hepatology 2006; 43: 545-553 [PMID: 16447274 DOI: 10.1002/hep.20969]

Matsuzaki K. Smad phosphoisoform signaling specificity: the right place at the right time. Carcinogenesis 2011; 32: 1578-1588 [PMID: 21798854 DOI: 10.1009/carcin/bgr172]

Yakicier MC, Irnak MB, Romano A, Kew M, Ozturk M. Smad2 and Smad4 gene mutations in hepatocellular carcinoma. Oncogene 1999; 18: 4879-4883 [PMID: 10498821 DOI: 10.1080/sj.1202866]

Furuta K, Misao S, Takahashi K, Tagaya T, Fukuzawa Y, Ishikawa T, Yoshioka K, Kakumusu S. Gene mutation of transforming growth factor beta type II receptor in hepatocellular carcinoma. Int J Cancer 1999; 81: 851-853 [PMID: 10362218 DOI: 10.1002/(SICI)1097-0215]

Kawate S, Takenoshita S, Ohwada S, Mogi A, Fukusato T, Makita F, Kuvano H, Morishita Y. Mutation analysis of transforming growth factor beta type II receptor, Smad2, and Smad4 in hepatocellular carcinoma. Int J Oncol 1999; 14: 127-131 [PMID: 9863018]

Kawate S, Ohwada S, Hamada K, Koyama T, Takenoshita S, Morishita Y, Hagiwara K. Mutational analysis of the Smad4 and Smad7 genes in hepatocellular carcinoma. Int J Mol Med 2001; 8: 49-52 [PMID: 11408948]

Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev 2006; 17: 41-58 [PMID: 16310402 DOI: 10.1016/j.cytogfr.2005.09.009]

Morris SM, Baek JY, Koszarek A, Kangnun S, Knoblaugh SE, Grady WM. Transforming growth factor-beta signaling promotes hepatocarcinogenesis induced by p53 loss. Hepatology 2012; 55: 121-131 [PMID: 21885802 DOI: 10.1002/hep.24653]

Mu X, Lin S, Yang J, Chen C, Chen Y, Herzig MC, Washburn K, Halff GA, Walter CA, Sun B, Sun LZ. TGF-beta signaling is often attenuated during hepatotumorigenesis, but is retained for the malignancy of hepatocellular carcinoma cells. PLoS One 2013; 8: e65408 [PMID: 23704908 DOI: 10.1371/journal.pone.0065436]

Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, Kaidori M, Kamiyama Y, Nishimura M, Fujisawa J, Okazaki K, Seki T. Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor beta signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology 2007; 46: 48-57 [PMID: 17596875 DOI: 10.1002/hep.21672]

Murata M, Matsuzaki K, Yoshida K, Sekimoto G, Tahashi Y, Mori S, Uemura Y, Sakaida N, Fujisawa J, Seki T, Kobayashi K, Yokote K, Koike K, Okazaki K. Hepatitis B virus X protein shifts human hepatic transforming growth factor (TGF)-beta signaling from tumor suppression to oncogenesis in early chronic hepatitis B. Hepatology 2009; 49: 1203-1217 [PMID: 19263472 DOI: 10.1002/hep.22765]

Liu X, Yang Y, Zhang X, Xu S, He S, Huang W, Roberts

Each reference is numbered from 34 to 49, indicating that the text contains a comprehensive list of references cited in the document. The references cover a range of topics related to Smad signaling, liver injury, and cancer development.
MS. Compound Astragalus and Salvia miltiorrhiza extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell. J Gastroenterol Hepatol 2010; 25: 420-426 [PMID: 19793165 DOI: 10.1111/j.1440-1746.2009.05981]

Yamaguchi T, Matsuzaki K, Inokuchi R, Kawamura R, Yoshida K, Murata M, Fujisawa J, Fukushima N, Sata M, Kage M, Nakashima O, Tamori A, Kawada N, Tsuneyama K, Dooley S, Seki T, Okazaki K. Phosphorylated Smad2 and Smad3 signaling: Shifting between tumor suppression and fibro-carcinogenesis in chronic hepatitis C. Hepatol Res 2013; 43: 1327-1342 [PMID: 23458062 DOI: 10.1111/hepr.12082]

Moriya K, Komai L, Ou JH, Lai MM. Hepatitis C virus inhibits DNA damage repair through reactive oxygen and nitrogen species and by interfering with the ATM-NBS1/Mre11/Rad50 DNA repair pathway in monocytes and hepatocytes. J Immunol 2010; 185: 6985-6998 [PMID: 20974981 DOI: 10.4049/jimmunol.1000618]

Lim JS, Park SH, Jang KL. Hepatitis C virus Core protein promotes heparin binding EGF-like growth factor expression and activates Akt. Hepatol Res 2011; 41: 455-462 [PMID: 21418450 DOI: 10.1111/j.1872-034X.2011.00792.x]

Machida K, McNamara G, Cheng KT, Huang J, Wang CH, Comai L, Ou JH, Lai MM. Hepatitis C virus inhibits DNA damage repair through reactive oxygen and nitrogen species and by interfering with the ATM-NBS1/Mre11/Rad50 DNA repair pathway in monocytes and hepatocytes. J Immunol 2010; 185: 6985-6998 [PMID: 20974981 DOI: 10.4049/jimmunol.1000618]

Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta 2009; 1796: 242-251 [PMID: 19591900 DOI: 10.1016/j.bbcan.2009.06.005]

Shiratori Y, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, Kuroki T, Nishiguchi S, Sata M, Yamada G, Fujiyama S, Yoshida H, Omata M. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med 2000; 132: 517-524 [PMID: 10744587]

Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, Inoue O, Yano M, Tanaka M, Fujiyama S, Nishiguchi S, Kuroki T, Imazeki F, Yokosuka O, Kinoyama S, Yamada G, Omata M. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999; 131: 174-181

Kobayashi S, Takeda T, Enomoto M, Tamori A, Kawada N, Habu D, Sakaguchi H, Kuroda T, Kioka K, Kim SR, Kann S, Ueda T, Hirano M, Fujimoto S, Jomura H, Nishiguchi S, Seki S, Development of hepatocellular carcinoma in patients with chronic hepatitis C who had a sustained virological response to interferon therapy: a multicenter, retrospective cohort study of 1124 patients. Liver Int 2007; 27: 186-191 [PMID: 17311612]

Morgan TR, Ghanjy MG, Kim HY, Snow KK, Shiffman ML, De Santo JL, Lee WM, Di Bisceglie AM, Bonkovsky HL, Dienstag JL, Morishima C, Lindsay KL, Lok AS. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. Hepatology 2010; 52: 833-844 [PMID: 20564351 DOI: 10.1002/hep.23741]
