**Name of journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO: 8374**

**Columns: TOPIC HIGHLIGHT**

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

**Linker phosphorylation of Smad3 promotes fibro-carcinogenesis in chronic viral hepatitis of hepatocellular carcinoma**

Murata M *et al*. Smad3 promotes fibro-carcinogenesis

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**Received:** December 26, 2013 **Revised:** March 8, 2014

**Accepted:** July 15, 2014

**Published online:**

**Abstract**

Epidemiologic and clinical data point to a close association between chronic hepatitis B virus infection or chronic hepatitis C virus infection and development of hepatocellular carcinoma (HCC). HCC develops in the progress of several decades and is associated with the fibrosis. This sequence suggests that persistent viral infection and chronic inflammation can synergistically induce liver fibrosis and hepatocarcinogenesis. The transforming growth factor-β (TGF-β) signaling pathway plays a pivotal role in diverse cellular processes and contributes to hepatic fibro-carcinogenesis under inflammatory microenvironments during chronic liver diseases. The biologic activities of TGF-β are initiated by the binding of the ligand to TGF-β receptors, which phosphorylates Smad proteins. TGF-β type I receptor activated Smad3 to create COOH-terminally phosphorylated Smad3 (pSmad3C), while pro-inflammatory cytokine-activated kinases phosphorylated Smad3 to create the linker phosphorylated Smad3 (pSmad3L). During chronic liver disease progression, virus components together with pro-inflammatory cytokines and somatic mutations convert to Smad3 signal from tumor-suppressive pSmad3C to fibro-carcinogenic pSmad3L pathways, accelerating liver fibrosis and increasing risk of HCC occurrence. The understanding of Smad3 phosphorylation profiles may provide new opportunities for effective chemoprevention and personalized therapy for patients with hepatitis virus-related HCC in the future.

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**Key words:** Chronic viral hepatitis;Transforming growth factor-β; Smad3; Phosphorylation; Fibro-carcinogenesis; Hepatocellular carcinoma

**Core tip:** Chronic hepatitis B and C infections are major causes of cirrhosis and hepatocellular carcinoma (HCC). Most patients with persistent viral infection remain asymptomatic, while some patients have poor prognosis by the development of HCC. Therefore, identifying persons at high-HCC risk among chronic hepatitis patients is crucial for preventing from HCC occurrence. Analyses of domain-specific phosphorylation of Smad3 in liver specimens can be helpful for understanding the stages of diseases and can become a marker for predicting HCC development.

Murata M, Yoshida K, Yamaguchi T, Matsuzaki K. Linker phosphorylation of Smad3 promotes fibro-carcinogenesis in chronic viral hepatitis and development of hepatocellular carcinoma. *World J Gastroenterol* 2014; In press

**INTRODUCTION**

The incidence of hepatocellular carcinoma (HCC) is an increasing tendency, and it is expected that the rates of the HCC development continues to increase for a while. Epidemiologic evidence clearly indicates that approximately 80% of HCC depend on chronically hepatitis B virus (HBV) or hepatitis C virus (HCV) infection worldwide. The remaining 20% patient is complicated with liver cancer for various causes such as excessive amounts of alcohol intake, fatty liver, hemochromatosis, and metabolic syndrome[1]. In natural progress of the viral hepatitis, there is persistence of a long latency HBV and HCV infection, but most of the patients with infected HBV and HCV are asymptomatic or pass for a light symptom without progression[2,3]. In the livers of some patients, the progress of various stages with active inflammation and fibrosis and eventually cirrhosis is seen. Cirrhosis precedes the liver-related complications including HCC. It usually takes 20 years more to lead to cirrhosis from viral infection. Subsequently, HCC often develops in the progress of another 10 years[4]. Therefore, liver carcinogenic process related to HBV or HCV infection tends to be insidious, most likely requiring multiple sequential genetic alterations and complex interactions between virus, host, and environment.

In the last few decades, research has provided significant insight into the transforming growth factor (TGF)-β signal transduction network, which regulates biologic processes widely. In a normal system, TGF-β is a protein inhibiting the physiological activity of epithelial cellgrowth, having a tumor suppressive function. At later stages, however, TGF-β can promote cancer progression[5-7]. As normal epithelial cells progress toward cancer cells, oncogenic potential of TGF-β in malignant cells rises together with selective reduction of tumor-suppressive activity[6]. In chronic hepatitis, cytostatic TGF-β effect for hepatocytes attenuates as liver disease progressing from cirrhosis to HCC under persistent inflammatory microenvironments[8]. Under pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-β (IL-β), TGF-β promotes extracellular matrix (ECM) accumulation, while cell proliferative pro-inflammatory cytokine signal has a contradicting relation with cytostatic signal through TGF-β[8,9]. By these regulatory actions, hepatic TGF-β signaling with pro-inflammatory cytokines can promote fibro-carcinogenesis.

TGF-β signal is transmitted mainly by a transcription factor called Smad. Smads proteins are comprised of Mad-homology (MH) 1 and MH2 domains, and linker part existing between these domains[10]. Activated type I receptors (TβRI) phosphorylate a C-terminus of Smad2 and Smad3 directly and forms pSmad2C and pSmad3C[11]. Mitogenic signals are generated by the phosphorylation of their linker regions in substitution for C-terminal phosphorylation[12-18]. After formation of hetero-oligomers with Smad4, a common partner, Smad2 and Smad3 translocate from the cytoplasm to the nucleus, lead to regulate transcription of target genes[19].

Mechanisms linking fibrosis and HCC remain largely unsettled. Considering that roles of phospho-Smads are greatly influenced by differential phosphorylation, better understanding of mechanisms of phospho-Smads signaling should help in design of approaches to diagnosis, prevention, and treatment of hepatitis virus-related hepato-carcinogenesis. In this review, we summarize Smad3 phosphorylation profile and consider how Smad3 affect human fibro-carcinogenesis at a molecular level.

**TGF-Β AND JNK SIGNALING INVOLVEMENT IN CHRONIC LIVER DISEASE PROGRESSION**

TGF-β appears highly important in hepatic fibro-carcinogenesis in patients with chronic liver diseases. TGF-β participates in a great variety of cell functions such as tissue and organ development, cell proliferation, differentiation, cell survival, and the control of the apoptosis[20]. TGF-β, the most potent hepatic pro-fibrogenic cytokine, is deeply involved in chronic liver diseases, especially liver cirrhosis and HCC[21], and is produced mostly by activated mesenchymal cells in the chronic liver damage[22]. TGF-β plays an important role in in epithelial-mesenchymal transition during fibrogenesis[23]. HCC usually occurs in cirrhotic livers where more TGF-β existed in comparison with healthy livers, which suggests that TGF-β has a role of pro-oncogenesis[24]. TGF-β regulates a large number of genetic expressions in conjunction with the carcinogenesis, which based on the results of recent accumulating analysis[25].

Another important factor influencing, HCC development in chronic HBV or HCV infection is c-Jun-N-terminal kinase (JNK) activity[26,27]. JNK is a mitogen-activated protein kinase (MAPK) family member that is activated by diverse stimuli, including cytokines such as IL-1β and TNF-α. These cytokines are transcriptionally activated in injured liver. Upon activation, JNK induces multiple biologic events through several transcription factors and transcription-independent control of effector molecules[28].

Smads mediate the intracellular TGF-β signal as transcription factors. Eight kinds of Smads are known in mammals, and they are classified in signal-specific Smad2/3, common type Smad4 and inhibitory type Smad7 by the function[29]. These Smads are comprised of MH1 domain and MH2 domain stored between Smad families, and linker lesion existing between these domains. SXS motif in the C-terminus of Smad3 is phosphorylated by TβRI upon TGF-β binding, leading to activation of Smad3 signaling (Figure 1A)[10,29]. On the other hands, signal-specific Smad2/3 at the linker lesion is phosphorylated directly by extracellular signal-regulated kinase (ERK), which is representative for downstream molecular of the Ras cascade, JNK, p38 MAPK, cyclin-dependent kinase (CDK), glycogen synthase kinase 3-β, Ca2+-calmodulin-dependent protein kinase II, and G protein-coupled receptor kinase-2 (Figure 1B)[12-17,30-35]. In substitution for pro-inflammatory cytokines and TGF-β, TGF-β activated kinase (TAK) 1 plays an important role in the signal reaction in the cell through non-Smad cascade. TAK1 mediates activation of JNK and p38 MAPK signaling *via* mitogen-activated kinase (MKK) 4/7 and MKK3/6[36,37]. In addition, JNK and p38 MAPK interfere with TGF-β signal by pro-inflammatory cytokines through their regulation of many physiological functions[38].

**ANTAGONISTIC SMAD3 PHOSPHO-ISOFORM SIGNALING: TUMOR-SUPPRESSIVE (CYTOSTATIC) PSMAD3C *VS* ARCINOGENIC (MITOGENIC) PSMAD3L**

Resent reports suggested that each step of the Smad3 signal transmission, including the ability of complex with Smad4, the local existence in the cell, binding to target gene promoter and the resolution, are controlled by phosphorylation[29]. Activation of TβRI with TGF-β binding and Ras-related kinase including JNK and CDK differentially phosphorylate Smad3 and form pSmad3C and the pSmad3L. These two domain-specific phosphorylation forms cause different action. Phosphorylation of Smad3C activates cytostatic signal, while phosphorylation at the linker region up-regulates mitogenic signal. Notably, as pSmad3L is produced during fibro-carcinogenesis, leading to structure changes, phosphorylation of Smad3C is inhibited by pro-fibrogenic and pro-tumorigenic non-Smad pathways[39].

In normal epithelial cells, TGF-β regulates growth and proliferation of cells. In particular, after pSmad3C shifted into nucleus, cell proliferation stops by activation of the p15*INK4B* and p21*WAF* gene transcription or suppressing c-Myc gene, resulting in apoptosis with inhibition of Bcl-2 expression (Figure 1A)[40-43].

Traditionally, the cytostatic effects of TGF-β have been thought to oppose mitogenic signaling in normal cells, while in cancer cells, potent mitogenic actions of certain oncogenes can overwhelm the anti-mitogenic capacity of the TGF-β signaling. Virus component including the HBx protein, inflammatory cytokines such as IL-1β and TNF-α, growth factor through a tyrosine kinase type receptor including hepatocyte growth factor and platelet-derived growth factor (PDGF), and Ras mutation additively up-regulate phosphorylation of Smad3L by activated JNK[16,44,45]. After pSmad3L moves into the nucleus, nuclear pSmad3L induces the growth of hepatocytes and ECM deposition by up-regulating c-Myc and PAI-1 transcription. Importantly, phosphorylation of Smad3 at the linker region inhibit C-terminal phosphorylation induced by TβRI[13,16,32,45,46]. When Smad3L phosphorylation is promoted with activation of mitogenic signaling, phosphorylation of pSmad3C is suppressed indirectly, that is suspected the cytostatic system (Figure 1B)[13,16].

A principal finding is shifting between JNK/pSmad3L and TRβI/pSmad3C signaling, representing a delicate balance between carcinogenesis and tumor-suppression (Figure 1C). Specifically blocking phosphorylation of Smad3 at the linker lesion with Smad3 mutants lacking linker phosphorylation sites or inhibition of JNK activity, can recover to the pathway going through tumor-suppresive pSmad3C pathway[16,45,46]. Therefore, we suggest that the cytostatic TβRI/pSmad3C signal has a contradicting relation with mitogenic JNK/pSmad3L signal.

**MODULATION OF SMAD3 SIGNALING FAVORING CARCINOGENESIS**

TGF-β activates cytostatic and apoptotic processes to maintain homeostasis in normal epithelial cells. In the human intestine, apoptosis induced by the TGF-β/pSmad3C pathway, which is essential to normal homeostasis, acts to inhibit human colorectal cancer cell proliferation. Similarly, hepatocytes illustrate the balance between proliferation and differentiation during liver regeneration. Proliferation of normal hepatocytes to compensate for partial hepatectomy or diffuse liver injury is constructed by the interaction of polypeptide cytokines and growth factors.

In quiescent hepatocytes, Smad3C phosphorylation *via* activin type I receptor (ActRI) persist[47]. At the acute liver damage, mitogenic pSmad3L signaling induced by TNF-α becomes more dominant than pSmad3C signaling. As decreases in TNF-α and pSmad3L allowed increased sensitivity to phosphorylation at Smad3C by TβRI, hepatocytic proliferation ceases. Such competitive manner in hepatocytic Smad3 signaling is protective reaction which may prevent malignant transformation from normal cell. In this manner, hepatocytes with non-proliferative pSmad3C signaling may be destined to undergo apoptotic cell death[48].

On the other hand, constitutive pSmad3L (Ser-213)/c-Myc signaling accelerates cancer development[49]. Hepatocytes keep the potential of the cell proliferation by Smad3 linker phosphorylation and inhibition of anti-apoptotic action by Smad3C, which make the environment that mutant genes are easy to accumulate. This step mainly contributes to progression of hepatocarcinofenesis.

**FIBRO-CARCINOGENESIS IN HUMAN CHRONIC LIVER DISEASES: RECIPROCAL CHANGES IN PSMAD3L AND PSMAD3C PATHWAYS**

Around 80% to 90% of HCCs arise as a complication of long-standing symptomatic cirrhosis[50]. Cirrhosis is seen in 20% to 30% of patients with persistent HCV infection, after several decades passed after viral infection. As fibrosis progresses, the risks of the HCC increase, in particular, HCC develops at an annual rate of 1% to 7% in HCV-infected patients with cirrhosis[51], and 0.02% to 3.7% in HBV-infected patients with cirrhosis[52,53]. These epidemiologic findings indicate that hepato-carcinogenesis in chronic viral hepatitis is a sequential step from chronic liver injury through cirrhosis to HCC, with fibrosis being pivotal at the pre-neoplastic stage.

During progression of liver injury, hepatic stellate cell (HSC) undergo a complex transformation or activation process losing lipid droplets retaining retinoid and changing in myofibroblast (MFB)-like cells which progress ECM accumulation[54]. This step is a beginning of fibrosis. If this process being repeated, the liver shifts to irreversible cirrhosis. Activation of HSC promote fibrogenesis and control intracellular signaling networks by proliferative PDGF[55,56] and fibrogenic TGF-β cytokines[57]. Moreover, our *in vivo* model indicated that pSmad3C-mediated signal decreased while the pSmad3L pathway predominated during transdifferentiation in culture[31]. In chronic hepatitis C, α-smooth muscle actin (SMA)-positive MFB in portal area is strongly affected by pSmad3L signal rather than pSmad3C signal[44]. Increase of α-SMA in HSC creates scar-forming MFB, lead to liver fibrosis[58]. In the same as MFB, pSmad3L is predominantly located in hepatocytic nuclei in portal tracts, in sharp contrast to pSmad3C[44]. Kupffer cells in portal tract produce and release TGF-β and a variety of pro-inflammatory cytokines, which provoke JNK activation[59,60]. According to these findings, JNK activated pro-inflammatory cytokine, is able to transform Smad3 into pSmad3L in both hepatocytes and MFB during the course of chronic hepatitis.

Our studies compared pSmad3L with pSmad3C distribution in biopsy specimens from patients chronically infected with HBV or HCV. These results demonstrated reciprocal changes in pSmad3L and pSmad3C pathways during hepato-carcinogenesis with pSmad3L being seen predominantly occupation in hepatocytes with progress of liver disease (Figure 2, left panel)[44,45]. In contrast, pSmad3C staining decreased in hepatocytes during fibrosis progression (Figure 2, right panel)[44,45].Furthermore,JNK/pSmad3L-mediated pathway in hepatocytes and activated HSC involves hepatic fibrosis during a long cancerous process. This is a principal mechanism in the development of liver fibrosis toward HCC in both HBV- and HCV-related liver diseases.

**PROMOTION OF HEPATO-CARCINOGENESIS BY CHRONIC VIRAL INFECTION TOGETHER WITH INFLAMMATION IN HUMAN LIVER**

A number of pathogenic mechanisms have been proposed for HCC associated with chronic inflammatory diseases, including chronic viral hepatitis B and C, alcoholic hepatitis, non- alcoholic fatty liver disease, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson’s disease, and others. Although the cell division is very rare in normal hepatocytes, in viral chronic liver disease, hepatocyte turnover rate is significantly accelerated under repeated hepatocytic necrosis and regeneration. The cell proliferation fundamentally does not have a function to transform cells, but cell proliferation is a necessary process in progress of the liver carcinogenesis[61]. By accelerating hepatocyte turnover, chronic inflammatory liver is exposed to risk of cell progression into a fully malignant phenotype, acting as a potent tumor promoter. Moreover, stepwise accumulation of mutations in cancer-related genes and molecular alterations also take part in human carcinogenesis[62]. Eventually, somatic mutations in hepatocytes stimulate hepatocytic growth, and HCC occur as a result of malignant cell transformation.

In comparison with other influences, chronic infection with HBV or HCV represents the most important risk factor for development of HCC. Hepato-carcinogenesis in HBV and HCV infection has been studied extensively with necrosis and chronic inflammation, followed by fibrosis and cell proliferation playing a role. Nevertheless, HCC only occurs in a small proportion of HBV and HCV carriers. Since hepato-carcinogenic process involves the interplay between hepatitis viruses and host inflammatory responses, both factors may contribute to the final malignant outcome, either individually or synergistically[63-67].

**CHRONIC INFLAMMATION AND HEPATITIS VIRUS ALTER THE HEPATOCYTIC TGF-Β SIGNALING**

Hepatitis viruses and inflammation influence the fibro-carcinogenic TGF-β pathway, which leads to development of HCC. We investigated the correlation between hepatocytic pSmad3L positivity and plasma HBV DNA concentration in chronic hepatitis B patients, because HBV itself can up-regulate phosphorylation of hepatocytic Smad3L[45]. pSmad3L dominantly located in the hepatocytes nucleus adjoining inflammatory cells in portal tracts[44]. Furthermore, Smad3L phosphorylation showed strong correlation with inflammatory activity[44].

In the onset of HCC, chronic inflammation, cell death and persistent of viral infection in liver cells, play a central role. Hepatocytes with much pSmad3L and little pSmad3C can survive during the progress of chronic hepatitis, resulting in accumulation of acquired various mutationsin sequence.These mutations are associated with genes in Ras pathway[68] that activate phosphorylation of Smad3L constantly in pre-neoplastic hepatocytes, resulting in eventual development into overt HCC[69]. These observations are consistent with the general idea of additive promotion of hepato-carcinogenesis by persistent hepatitis virus infection and chronic inflammation.

**SMAD3 PHOSPHORYLATION FOR PREDICTING RISK OF HCC**

Identification of high-risk patients among virus hepatitis carriers is of great importance. Well-planned treatment allocation and effective screening for HCC in chronic hepatitis patients are essential. Several factors have been identified as associated with higher risk of development of HCC, including male gender, advanced age[70], virologic factors[71-73], and disease factors including alanine aminotransferase concentrations and presence of cirrhosis[70,74].

Our retrospective study using pSmad3L and pSmad3C in human liver have provide physiopathogenic insights. In the chronic hepatitis B patients, HCC develop from the group with high pSmad3L but low pSmad3C in the hepatocytes. In contrast, HCC do not occur from the group with high pSmad3C but little pSmad3L in the hepatocytes[45]. The similar tendency is observed in human HCV-related hepato-carcinogenesis[44]. Striking differences in cumulative HCC incidence have been observed between the two groups during 12 years follow-up. These findings clarify pSmad3C play as a tumor suppressor, while pSmad3L play a tumor promoter during human hepto-carcinogenesis. In addition, according to a result of multivariate analysis, high pSmad3L and low pSmad3C in hepatocyte were each independent risk factors of the liver carcinogenesis[45]. These observations suggest that Smad3 phosphorylation profile has an excellent discriminating ability for triage of chronic hepatitis patients for the most appropriate treatment.

**CONCLUSION**

We tried to deepen understanding about mechanism of the hepato-carcinogenesis during the last decade, while HCC associated with chronic with HBV and HCV infections remains is associated with poor prognosis. Nonetheless, our studies clearly demonstrate that as the stage of viral chronic liver disease are progress, fibrogenic and oncogenic pSmad3L signaling is gradually increased in pre-neoplastic hepatocytes persistently affected by TGF-β together with pro-inflammatory cytokines, lead to liver fibrosis and arising HCC (Figure 3). The model focusing on phospho-Smad signaling we proposed may be understood as a crucial molecular mechanism by which most HCC develops in the context of severe fibrosis[75]. Thus, understanding of Smad phospho-isoform signaling is important for comprehension of mechanisms underlying hepato-carcinogeneis.

In our recent studies, HCV clearance at an early stage by anti-viral therapy restored hepatocytic TGF-β signaling from fibro-carcinogenesis toward the tumor-suppression[76]. Moreover, low pSmad3C and high pSmad3L positivity were significantly predictive of human HCC development[44,45]. We conclude that pSmad3L and pSmad3C can serve as a useful markers determining whether you can defend development of viral related HCC by antiviral therapy. Using an individual’s phospho-Smad3 markers, clinicians may be able to achieve more effective chemoprevention and individualized therapy for HBV- and HCV-infected patients.

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**P-Reviewers:** Ferreira CN, Sipos F **S-Editor:** Gou SX **L-Editor: E-Editor:**

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**** **Figure 1 Reversibility of phospho-Smad3 signaling between tumor suppression and fibro-carcinogenesis.** A: Transforming growth factor-β (TGFβ) treatment activates TGF-β type I (TβRI), further leading to direct phosphorylation of Smad3C, which inhibits normally hepatocytic growth by up-regulating p21WAF1 transcription; B: Mitogens drastically alter phospho-Smad3 signaling *via* the c-Jun N-terminal kinase (JNK) pathway, increasing nuclear fibro-carcinogenic pSmad3L activity while shutting down TGF-β-dependent cytostatic pSmad3C. Although the TGF-β signal weakly phosphorylates Smad3L in normal hepatocytes (dotted line), hepatitis viral components including HBx, pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-alpha), and somatic mutations such as Ras additively transmit a fibro-carcinogenic signal through the JNK-dependent pSmad3L pathway to participate in hepatocytic growth and ECM deposition, possibly by stimulating transcription of *c-Myc* and *PAI-1* genes. Linker phosphorylation of Smad3 indirectly prevents COOH-tail phosphorylation, pSmad3C-mediated p21WAF1 transcription and cytostatic function; C: Either various JNK inhibitors or a Smad3 mutation causing lack of JNK phosphorylation sites in the linker region can eliminate fibro-carcinogenic pSmad3L signaling, restoring or maintaining the tumor-suppressive pSmad3C signaling characteristic of mature hepatocytes.

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**Figure 2 Hepatic fibro-carcinogenesis: reciprocal change in linker phosphorylated and COOH-terminally phosphorylated Smad3 pathways.** The linker phosphorylated Smad3 (pSmad3L)/c-Myc pathway shows increasing prominence in hepatocytes, as hepatitis C virus (HCV)-infected liver progresses from chronic hepatitis through cirrhosis to HCC. In contrast to intense staining for pSmad3L and c-Myc, the COOH-terminally phosphorylated Smad3 (pSmad3C)/p21WAF1 pathway staining decreases in hepatocytes as liver disease progresses toward hepatocellular carcinoma (HCC).

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**Figure 3 Together, persistent hepatitis virus infection.** Together, persistent hepatitis virus infection and chronic inflammation shift hepatocytic phospho-Smad3 signaling from the tumor-suppressive transforming growth factor-β (TGFβ) type I (TβRI)/COOH-terminally phosphorylated Smad3 (pSmad3C) mode to the fibro-carcinogenic c-Jun N-terminal kinase (JNK)/linker phosphorylated Smad3 (pSmad3L) mode characteristic of median forebrain bundle, accelerating liver fibrosis while increasing risk of hepatocellular carcinoma (HCC).In the liver, persistent hepatitis virus infection and chronic inflammation contribute to fibro-carcinogenesis. In proportion to severity of fibrosis, mitogenic genetic or epigenetic alterations can drive fibro-carcinogenesis *via* the pSmad3L pathway. Escaping the cytostatic action of pSmad3C is a critical step for progression to full malignancy in cancers, which must overcome multiple fail-safe genetic controls. HBV: Hepatitis B virus; HCV: Hepatitis C virus.