Chapter 7

Early Chronic Inflammation and Subsequent Somatic Mutations Shift Phospho-Smad3 Signaling from Tumor-Suppression to Fibro-Carcinogenesis in Human Chronic Liver Diseases

Miki Murata, Katsunori Yoshida and
Koichi Matsuzaki

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http://dx.doi.org/10.5772/56739

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide [1]. HCC is strongly associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, which are implicated in about 80% of HCCs in certain geographic area [2]. Risk of HCC is increased 5- to 15-fold in chronic HBV carriers [1] and 11.5- to 17-fold in HCV-infected patients [3]. In addition, epidemiological studies have shown that chronic inflammation of the liver predisposes individuals to HCC. Most HCCs are associated with severe fibrosis or cirrhosis caused by unresolved inflammation. Both HBV and HCV show a wide spectrum of clinical manifestations, ranging from a healthy carrier state to chronic hepatitis, cirrhosis and HCC. Notably, HCC occurs less often in chronic viral hepatitis without cirrhosis. As liver fibrosis progresses from chronic hepatitis to cirrhosis, HCC occurrence increases [4]. Thus, unresolved inflammation with long-term viral infection leads to HCC associated with cirrhosis. Approaches to understanding how human HCC develops in chronic inflammatory liver diseases should therefor focus on molecular mechanisms shared between liver fibrosis and carcinogenesis (fibro-carcinogenesis).

Transforming growth factor (TGF)-β is a key regulator of many important biologic processes. TGF-β can inhibit epithelial cell growth, physiologically acting as a tumor suppressor, but it also can promote neoplasia. TGF-β has been shown to play both tumor-suppressive and tumor promoting roles [5-7]. As disease progresses toward malignancy, cancer cells gain advantage

by selective reduction of the tumor-suppressive activity of TGF-β together with augmentation of TGF-β oncogenic activity [6]. In concert with mitogens, TGF-β induces accumulation of extracellular matrix (ECM), while mitogenic signaling antagonizes cytostatic TGF-β function [8,9]. These results indicate that perturbation of TGF-β signaling by mitogens can promote hepatic fibro-carcinogenesis.

The TGF-β superfamily includes many multifunctional cytokines including TGF-β, activin, and others [6,10]. Progress over the past 10 years has disclosed important details of how the TGF-β family elicits its responses [11-14]. Smads, central mediators conveying 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 [13]. 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 [12]. Mitogenic signals alternatively cause phosphorylation of R-Smad at specific sites in their middle linker regions [15-20]. After a phosphorylated R-Smad rapidly oligomerizes with Smad4, this complex translocates to the nucleus, where it regulates transcription of target genes.

Monitoring phosphorylation status of signaling molecules is a key step in dissecting their pathways. In Smad signaling, phosphorylation of not only the COOH-tail but also the linker regions of R-Smads are likely to be important in regulating Smad activity under physiologic and pathologic conditions [21]. Understanding of molecular mechanisms underlying hepatitis virus-induced fibro-carcinogenesis can help to guide early management and improve therapy for patients with chronic liver diseases. This review describes current knowledge of the molecular pathogenesis of human fibro-carcinogenesis, especially concerning Smad3 phosphorylation profiles. We further consider how enhanced understanding of phospho-Smad3 signaling could lead to more effective prevention of human fibro-carcinogenesis.

2. Smad3 phosphoisoforms

The canonical TGF-β pathway involves Smad2 and Smad3 signaling through direct serine phosphorylation of COOH termini by TβRI upon TGF-β binding (Figure 1A), [10,13]. TβRI-mediated phosphorylation of Smad2 and Smad3 induces their association with the shared partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes [10-14]. Smad2 and Smad3 proteins contain a conserved Mad-homology (MH)1 domain that binds DNA, and a conserved MH2 domain that binds to receptors, Smad4, and transcription co-activators.

More divergent linker regions separate the two domains [13]. The linker domain undergoes regulatory phosphorylation by Ras/mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and cyclin-dependent kinase (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 (Figure

http://dx.doi.org/10.5772/56739

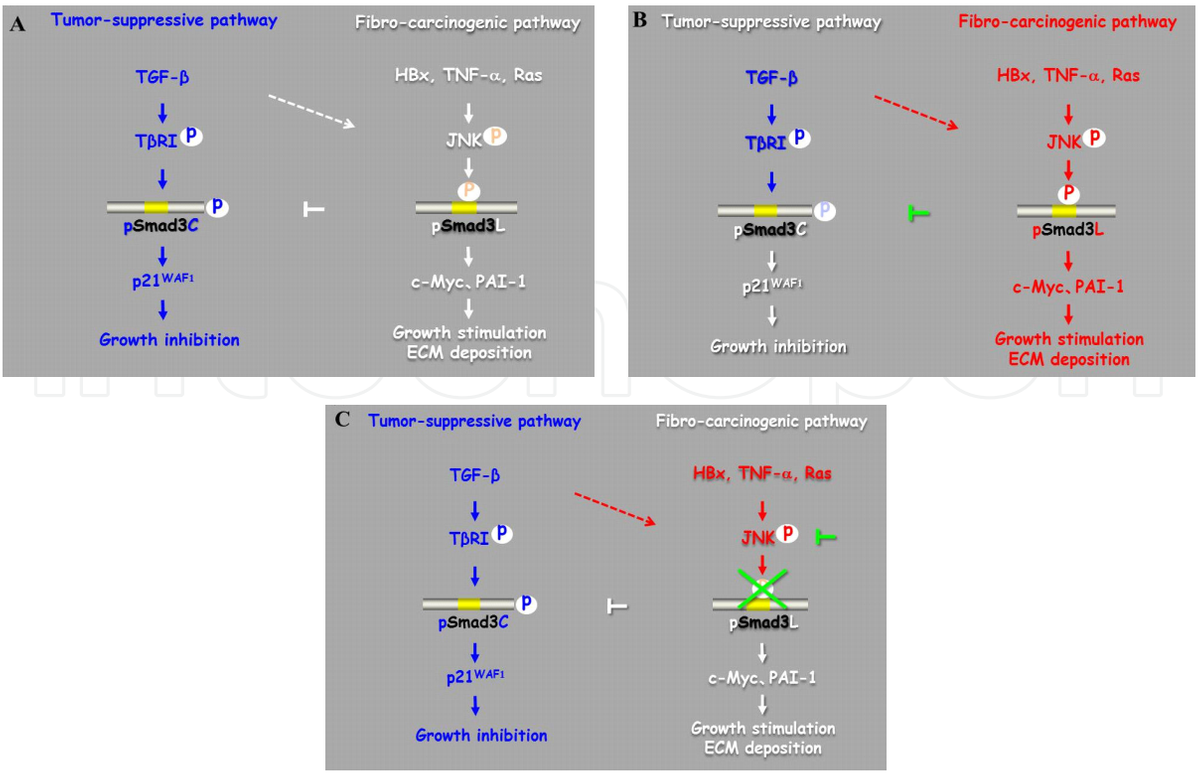

Figure 1. Reversible phospho-Smad3 signaling between tumor-suppression and fibro-carcinogenesis A) TGF-β treatment activates TβRI, further leading to direct phosphorylation of Smad3C, which inhibits normally hepatocytic growth by up-regulating $p21^{WAF1}$ transcription. B) Mitogens drastically alter phospho-Smad3 signaling via the JNK pathway, increasing basal nuclear fibro-carcinogenic pSmad3L activity while shutting down TGF-β-dependent cytostatic pSmad3C. Although TGF-β signal weakly phosphorylates Smad3L in normal hepatocytes (dotted line), hepatitis viral components including HBx, pro-inflammatory cytokines including TNF-α, and somatic mutations such as Ras additively transmit 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 $p21^{WAF1}$ transcriptions 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.

1B), [15-26]. TGF-β alternatively elicits signaling responses through non-Smad pathways representing important effectors for TGF-β activated kinase (TAK) 1 in response to pro-inflammatory cytokines. TAK1 activates JNK and p38 MAPK signaling through mitogen-activated kinase kinase (MKK) 4/7 and MKK3/6 [27,28]. JNK and p38 MAPK have been linked to modification of TGF-β signaling by pro-inflammatory cytokines through their regulation of distinct processes such as cytoskeleton organization, cell growth, survival, migration and invasion [29]. Imbalances between signaling through non-Smad and Smad pathways may occur during fibro-carcinogenesis, with interaction between these pathways mediating pro-fibrogenic and pro-tumorigenic effects of TGF-β [30]

Findings in mice with targeted deletion of Smad3 and JNK1 indicate that both Smad3 and JNK1 pathways promote hepatic fibro-carcinogesis. When acute liver injury was induced by administration of CCl_4, *Smad3*$^{-/-}$ mice showed approximately half as much of the induction of collagen type I mRNA as seen in wild-type mice [31]. *JNK1*$^{-/-}$ mice resisted not only liver fibrosis

but also HCC development. Remarkable collagen deposition in wild-type and *JNK2*$^{-/-}$ was less evident in *JNK1*$^{-/-}$ mice, suggesting importance of JNK1 in development of liver fibrosis [32]. *JNK1*$^{-/-}$ mice exhibited impaired liver carcinogenesis with reduced tumor mass, size, and number [33]. Importantly, *JNK1*$^{-/-}$ mice displayed decreased HCC proliferation in a carcinogenic model and decreased hepatocytic growth in a model of liver regeneration. In both cases, the impaired proliferation was caused by increased expression of $p21^{WAF1}$, a cell-cycle inhibitor, and reduced expression of c-Myc, a negative regulator of $p21^{WAF1}$.

Mitogens simultaneously activate linker-phosphorylated R-Smad and non-Smad signaling, with both usually operating in parallel. Biologic significance of linker-phosphorylated R-Smad pathways is therefore difficult to assess in isolation. Here we will review recent work in this area with a particular focus on how mitogens modulate TGF-β signaling through Smad3 linker phosphorylation, using hepatic fibro-carcinogenesis as an example. Antibodies (Abs) reactive with structurally related phosphorylated peptides are emerging as valuable tools for determining phosphorylation sites *in vivo* and for investigating distinct signals via phosphorylated domains. Domain-specific phospho-Smad3 Abs have allowed us to reveal that TβRI and JNK differentially phosphorylate Smad3 to create 2 phosphorylated forms (phosphoisoforms): COOH-terminally phosphorylated Smad3 (pSmad3C) and linker phosphorylated Smad3 (pSmad3L) [34-37]. Linker phosphorylation can modify COOH-terminally phosphorylated R-Smad signaling [15-17,19-24]. Differential localization of kinases and phosphatases in the cytoplasm or nucleus raises the intriguing possibility of differences in temporal dynamics between cytoplasmic and nuclear R-Smad phosphoisoforms, adding to the repertoire of signaling responses that determine cell-fate decisions [8,9]. Immunohistochemical and immunofluorescence analyses using specific Abs in human tissues can examine the clinical significance of context-dependent and cell type-specific signaling mediated by R-Smad phosphoisoforms by comparison of their tissue and cellular localization in pathologic specimens.

3. Tumor-suppressive (cytostatic) TGF-β signaling: the pSmad3C pathway

TGF-β inhibits proliferation of normal hepatocytes, a crucial function in hepatic homeostasis [38]. In the context of cell cycle control, the most important targets of action by TGF-β are the genes encoding two CDK inhibitors (*p21*^{WAF1} and *p15*^{INK4B}) and *c-Myc* [39]. The pSmad3C signal induces expression of these CDK inhibitors and represses expression of c-Myc, shutting down cell cycle progression in the early to mid G_1 phase of the cell cycle (Figure 1A). Development of HCC is ordinarily blocked through actions of the pSmad3C pathway, which causes normal hepatocytes to cease growth and enter apoptosis after hepatocytic proliferation.

4. Carcinogenic (mitogenic) JNK signaling: the pSmad3L pathway

Mitogens strongly activate the JNK pathway, as TGF-β does more weakly (Figure 1B) [40]. Ras/MAPK signaling has been shown to induce phosphorylation of Smad2 and Smad3 at their

http://dx.doi.org/10.5772/56739

linker regions [15]. Smad2 phosphorylation at the linker region inhibits nuclear accumulation of Smad2 without interfering with TGF-β-induced phosphorylation of its COOH-tail [19,41-50]. In contrast, linker phosphorylation does not retain Smad3 in the cytoplasm, permitting further consequences of Ras/JNK signaling. Mechanisms underlying this difference between the two R-Smads are not known, but phosphorylation sites of Smad3 at clusters of 3 serine residues in its linker region (Ser^{204}, Ser^{208}, and Ser^{213}) somewhat differ in sequence location from the corresponding linker phosphorylation sites of Smad2 (Ser^{245}, Ser^{250}, and Ser^{255}).

Several lines of evidence indicate that JNK transmits carcinogenic (mitogenic) signal via the pSmad3L pathway. First, JNK can directly phosphorylate Smad3 linker sites *in vitro*, while JNK inhibitors block Smad3 linker phosphorylation *in vivo* [16,19]. Second, mitogens translocate pSmad3L into the nucleus [16,19,20]. Third, nuclear pSmad3L forms a hetero-complex with Smad4 [16,23]. Fourth, nuclear pSmad3L binds to the Smad-binding element in the promoter with high affinity and specificity [23,51-53]. Finally, mitogens induce growth of normal epithelial cells by up-regulating c-Myc, and such mitogenic effects are blocked in Smad3 mutants lacking linker phosphorylation sites and by JNK inhibitors [19,54]. These results strongly support the notion that JNK specifically signals via Smad3 [55].

5. Reversible shifts in phospho-Smad3 signaling between tumor-suppression and carcinogenesis

JNK/pSmad3L and TβRI/pSmad3C signals oppose each other; most importantly, the balance between carcinogenesis and tumor-suppression can shift (Figure 1C). Linker phosphorylation of Smad3 blocks COOH-tail phosphorylation induced by TβRI [16,19,24,54,56]. Mitogenic signaling accelerates nuclear transport of pSmad3L from the cytoplasm, while preventing Smad3C phosphorylation, pSmad3C-mediated transcription, and anti-proliferative effects of TGF-β [16,19]. Smad3 mutants lacking linker phosphorylation sites, as well as JNK inhibitors, can restore growth inhibitory and transcriptional responses to TGF-β in Ras-transformed cells and pre-neoplastic hepatocytes, both *in vitro* and *in vivo* [19,54,56]. Our model implies that the JNK pathway directly or indirectly modulates pSmad3C- and pSmad3L-mediated signaling to regulate target genes, resulting in an antagonistic relationship between carcinogenesis and tumor-suppression. Thus, effectiveness of tumor-suppressive TGF-β signaling can depend on extent of Smad3 phosphorylation at the linker region.

6. Homeostatic termination of mitogenic JNK/pSmad3L/c-Myc signaling after liver regeneration by hepatocytic TβRI/pSmad3C/$p21^{WAF1}$ signaling

Carcinogenesis is currently thought to occur as a sequence of steps termed initiation, promotion, and progression. Each step is characterized by disruption of normal cellular control mechanisms. Thus, development of HCC involves sequential alterations of physiological

mechanisms regulating hepatocytic growth. Before consideration of molecular mechanisms of hepatocarcinogenesis, examination of the physiologic role of phospho-Smad3 signaling in liver regeneration is instructive. A unique feature of adult mammalian liver is its ability to accurately regenerate lost mass, which occurs following surgical resection or diffuse liver injury [57]. Although precise identities of cytokines and molecular mechanisms involved in liver regeneration are largely unknown, TGF-β and tumor necrosis factor (TNF)-α apparently act as positive and negative regulators of hepatocytic growth, respectively (Figure 1 A and 1B).

Hepatocytes undergo transition from a resting to a proliferative state after acute liver injury or partial hepatectomy [57]. Loss of parenchyma rapidly induces a wave of hepatocytic proliferation capable of restoring the total mass of the liver to normal. Several converging lines of evidence have established that pro-inflammatory cytokines such as TNF-α and interleukin (IL)-6 are important components of the mitogenic pathways leading to regeneration after acute liver injury [58]. Treatment of hepatocytes with antibodies against TNF-α resulted in decreased DNA synthesis and JNK activity [38]. DNA synthesis during liver regeneration was severely impaired in mice with a TNF-α type I receptor deficiency [59]. After acute liver injury, TGF-β increases in damaged livers within a time frame similar to that of increases in pro-inflammatory cytokines [60-62]. This raises the problem of how hepatocytes manage to proliferate in response to a mitogenic pro-inflammatory cytokine signal despite elevated TGF-β concentration. During liver regeneration, hepatocytes acquire temporary resistance to cytostatic effect of TGF-β, allowing them to proliferate [61-63]. The phosphorylation pattern of Smad3 in regenerative hepatocytes after acute liver injury suggested important participation of phospho-Smad3 in hepatocytic growth regulation. In actively growing hepatocytes, intracellular phosphorylation at Smad3L was found to be high [54,56,64]. Translocated to the nucleus, inflammatory cytokine-induced pSmad3L stimulated c-Myc transcription [54,64,65], which increased proliferation of hepatocytes and opposed the cytostatic action of the pSmad3C/$p21^{WAF1}$ pathway (Figure 1B). Accordingly, pSmad3C/$p21^{WAF1}$ was undetectable in regenerative hepatocytic nuclei; escape from TGF-β-induced cytostasis was crucial in a subset of progenitor cells devoted to ensuring epithelial renewal. Thus, pSmad3L signaling can permit liver regeneration in response to mitogenic pro-inflammatory cytokines even though TGF-β concentration is elevated after acute liver injury.

Liver regeneration is tightly controlled by a delicate balance between hepatocytic growth and inhibition. Anti-mitotic effects of TGF-β contribute to the termination of hepatocyte proliferation observed following the wave of DNA synthesis in the regenerating liver. Post regeneration, return of TGF-β sensitivity thus limits hepatocyte proliferation and terminates liver regeneration [61,63]. After TNF-α and pSmad3L decreased, hepatocytic proliferation ceased, as decreases in pSmad3L allowed increased sensitivity to phosphorylation at Smad3C by TβRI (Figure 1C). TGF-β-dependent pSmad3C appears to limit the proliferative response of regenerating hepatocytes through inhibition of the G1 to S phase transition in the cell-cycle. Such signaling represents a highly effective defense mechanism against development of HCC, since nonproliferating hepatocytes containing pSmad3C that might have sustained any mutations are destined to die [66].

http://dx.doi.org/10.5772/56739

7. Liver fibrosis as the largest single risk factor for HCC occurrence

Liver fibrosis usually precedes the multistage process of HCC development. Liver fibrosis is strongly associated with HCC, with 80 to 90% of HCCs arising in cirrhotic livers [67]. In hepatitis B infection is a risk factor for HCC, along with age, gender, viral DNA load, and viral core promoter mutation [68]. Fibrosis has also been identified as risk factor in hepatitis C infection, where cancer risk is directly related to fibrosis severity [69]. Similarly, HCC development is linked to alcoholic cirrhosis [70], nonalcoholic steatohepatitis (NASH) [70], and hemochromatosis [71], with a yearly HCC incidence of 1.7% in alcoholic cirrhosis [70] and 2.6% in NASH cirrhosis [72].

8. Involvement of both myofibroblasts and hepatocytes in liver fibrosis

Hepatic fibrosis is characterized by accumulation of excess ECM proteins, regardless of underlying etiology. Amount of matrix deposition reflects a balance between matrix synthesis and degradation [73,74]. When synthesis of ECM exceeds degradation, pathologic accumulation of ECM leads to liver fibrosis. Reversibility of experimental hepatic fibrosis and a striking decrease in collagenolytic activity observed in liver fibrosis models suggest crucial importance of impaired matrix degradation in hepatic fibrogenesis [75]. The plasminogen activator/plasmin system, which is situated upstream of the fibrolysis system, can directly degrade matrix components, and indirectly inhibit ECM deposition [76]. Plasminogen activator inhibitor-1 (PAI-1), the major physiologic inhibitor of plasminogen activator, is a potent promoter of fibrosis. Introduction of a PAI-1 small interfering RNA attenuates deposition of ECM and hydroxyproline content in experimental hepatic fibrosis [77].

Liver fibrosis is one of the most common pathologic processes occurring in response to increased inflammatry factors. A complex interplay among different hepatic cell types takes place in injured livers. Hepatocytes are the targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and chemical toxins [78]. Damaged hepatocytes induce recruitment of white blood cells by local inflammatory cells. Apoptosis of damaged hepatocytes stimulates fibrogenesis by Kupffer cells. Activated Kupffer cells secrete pro-inflammatory cytokines including TNF-α and IL, as well as TGF-β. Intensive studies have shown that hepatic stellate cells (HSC) are the major cell type responsible for matrix production in damaged liver tissues [75]. HSC, characterized by retinoid droplets in the cytoplasm, are present in the space of Disse [79].

Standardized methods of obtaining HSC from livers have been developed [80]. Long-term culture of HSC on plastic substrates is widely accepted as a model of liver fibrosis [79]. HSC spontaneously transdifferentiate to a myofibroblast (MFB) phenotype on plastic dishes, and this response reproduces the features of activation *in vivo*. MFB usually retain fibrogenic TGF-β signaling component, but have lost the capacity to respond to TGF-β with growth arrest [81]. Such a state of altered TGF-β responsiveness is also observed in pre-neoplastic hepatocytes,

which typically exhibit a limited growth inhibitory response to TGF-β, instead responding to TGF-β with pro-fibrogenic behavior [9].

Hepatic fibrosis results from a wound-healing response to repeated injury in chronic liver diseases [82], in which HSC undergo dramatic phenotypic activation, with acquisition of fibrogenic properties. Patients develop liver fibrosis as a result of chronic liver damage, characterized by ECM accumulation that distorts hepatic architecture by forming a fibrous scar [79]. Ultimately, nodules of regenerating hepatocytes become enclosed by scar tissue, an event defining cirrhosis. Excess deposition of ECM of which type I collagen predominates disrupts the normal architecture of the liver, resulting in pathologic damage with pathophysiologic consequences.

A new concept has been proposed that epithelial cells undergo a phenotypical change termed epithelial-mesenchymal transition (EMT), acquiring a fibroblastic phenotype. EMT facilitates metastasis and cancer development [83]. Pioneering studies on EMT in organ fibrosis were carried out in kidney, ocular lens, and lung [84,85]. Involvement of EMT also has been proposed in liver fibrosis. Zeisberg et al. demonstrated that hepatocytes acquire expression of fibroblast-specific protein 1 in response to CCl_4 injury *in vivo* or TGF-β *in vitro* [86].

9. Fibrogenic pSmad3L signaling shared between MFB and pre-neoplastic hepatocytes

As a result of chronic liver damage, HSC undergo progressive activation to become MFB-like cells. During transdifferentiation in culture, pSmad3C-mediated signal decreases while the pSmad3L pathway predominates [23]. These observations complement the finding of pSmad3L rather than pSmad3C in nuclei of α-smooth muscle actin (SMA)-immunoreactive MFB in portal tracts of chronically HCV-infected liver specimens [64]. The presence of α-SMA is associated with transdifferentiation of HSC into scar-forming MFB, an event considered pivotal in the fibrogenic response [75].

Plasma TGF-β, TNF-α, and PAI-1 concentrations are usually elevated in patients with chronic liver diseases [87-89]. Since pSmad3L can transmit a fibrogenic signal by stimulating PAI-1 transcription (Figure 1B) [23], we investigated the pSmad3L pathway in human chronic liver disease. The results indicated nuclear localization of pSmad3L in PAI-1-immunoreactive MFBs and hepatocytes in chronic hepatitis specimens [64]. Thus, hepatocytes are regulated by the same pSmad3L pathway as are MFBs. Hepatocytes in HCV-infected livers, particularly those adjacent to inflamed portal tracts, exhibited phosphorylation at Smad3L [64]. Extent of phosphorylation at Smad3L was less in hepatocytes distant from portal tracts, in sharp contrast to pSmad3C, which was predominantly located in hepatocytic nuclei distant from portal tracts [64]. Extent of hepatocytic pSmad3L/PAI-1 increased in proportion to fibrotic stage in chronic liver diseases [56,74]. TGF-β and pro-inflammatory cytokines are released from infiltrating Kupffer cells in portal tracts to activate JNK [90,91]. Considering these findings together with a previous observation showing transcriptional activation of the *PAI-1* gene by JNK [92], TGF-β and TNF-α can mediate JNK/pSmad3L signaling that in turn induces PAI-1 expression and

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promotes ECM deposition in both hepatocytes and MFB. Thus, hepatocytes affected by chronic inflammation undergo transition from the tumor-suppressive pSmad3C pathway, characteristic of mature hepatocytes, to the JNK/pSmad3L/PAI-1 pathway, which favors a state of flux characterized by MFB.

Our findings support many important papers reporting that hepatocytes can promote fibrogenesis via TGF-β/Smad signaling. Dooley et al. reported that overexpression of inhibitory Smad7 in hepatocytes attenuated TGF-β-mediated fibrogenesis by blocking Smad signaling [93]. Since the large latent TGF-β complex consisting of TGF-β, the N-terminal part of its precursor, and the latent TGF-β binding protein exists in not only HSC but also hepatocytes, the complex can transmit a pro-fibrogenic signal [94], although intracellular functions of the TGF-β complex are poorly understood. TGF-β down-stream mediator connective tissue growth factor (CTGF) also involves hepatic fibro-carcinogenesis [95]. CTGF expression increases in fibrotic livers and various tumor tissues [96]. More importantly, *in vivo* knockdown of CTGF by small interfering RNA leads to substantial attenuation of experimental liver fibrosis. Differential regulation of CTGF expression in hepatocytes and HSC by Smad2 signaling may contribute to hepatic fibro-carcinogenesis [97]. Interestingly, a methylxanthine, caffeine, inhibits synthesis of CTGF in hepatocytes and HSC, primarily by inducing degradation of Smad2 [96].

10. Additive promotion of human carcinogenesis by persistent hepatitis viral infection and chronic inflammation

Various experiments support the notion that a single promoting agent is insufficient for development of cancer. Hepatocarcinogenesis is multi-factorial, involving collaboration between 2 or more promoting agents in HCC occurrence [98]. Among tumor-promoting agents, hepatitis viruses and chronic inflammation directly participate in HCC pathogenesis, which frequently occurs during long-standing hepatitis viral infection.

Many clinical observations suggest that persistent hepatitis viral infection and chronic inflammation additively influence development of human HCC. For example, alcohol consumption is a recognized major cause of liver disease, and plays an important role in progression to HCC. However, alcoholic hepatitis progresses less frequently to HCC than HBV- or HCV- related hepatitis. In addition, patients with both viral infection and alcohol consumption have a higher risk of developing HCC than those with alcohol consumption alone [3,99,100]. Autoimmune hepatitis (AIH) and primary billiary cirrhosis (PBC) are chronic inflammatory disorders that proceed to cirrhosis. However, HCC only rarely arises from AIH or PBC, particularly in the absence of HBV or HCV infection [101,102]. Conversely, asymptomatic HBV or HCV carriers maintaining normal alanine aminotransferase (ALT) levels despite intensive viral replication less frequently develop HCC than patients with chronic hepatitis B. The annual risk of HCC occurrence in HBV healthy carriers is 0.26% to 0.6%, while risk increases to 1% in patients with chronic active hepatitis B [103]. Moreover, HBV can act synergistically with HCV. Patients co-infected with HBV and HCV have a 2- to 6-fold higher

risk of HCC occurrence than those with either infection alone [104,105]. Accordingly, we will consider how the oncogenic JNK/pSmad3L pathway induces development of HCC, with particular attention to potential synergy between hepatitis viruses and inflammation in formation of pre-neoplastic hepatocytes.

11. Hepatitis virus components can activate oncogenic JNK/pSmad3L pathway

One of the earliest evidence linking HBV to development of HCC was obtained in the woodchuck hepatitis virus model, in which 100% of rodents infected with woodchuck hepatitis virus developed HCC [106]. Because 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 HBV-related HCC, usually even before development of HCC [107]. Integration of HBV DNA is not restricted to HCC but also is found in non-tumor tissue in patients with chronically HBV infection [108,109]. 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 [110,111]. Many integration events occur near or within fragile sites or other cancer-associated regions of the human genome that are prone to instability in tumor development and progression. Genetic instability associated with integration may alter expression of oncogenes, tumor suppressor genes, and microRNAs [111]. A recent large-scale analysis of HBV DNA integration sites in cellular DNA found a preference for sites regulating cell signaling, proliferation, and viability [112]. A large proportion of HCC have integrated HBV sequences encoding HBV X (HBx) and/or truncated envelope pre-S2/S proteins.

The HBx protein encoded by the X gene has been long suspected as a viral oncoprotein participating in hepatocarcinogenesis. This protein is involved in liver cell transformation because of its pleiotropic activities on cell cycle regulation, cell signaling pathways and DNA repair [113-115]. Numerous attempts have been made to examine the oncogenic potential of HBx in cell culture. However, its transforming ability was barely measurable evident only when cells were immortalized by other oncogenes, such as SV40 T-antigen [116,117] or TGF-α [118]. Furthermore, most transgenic mice harboring the HBx gene did not develop serious liver diseases or tumors [119]. Only in a certain transgenic lineage of CD-1 strain, HBx weakly promoted carcinogenesis, where HBx was highly expressed [120]. A second mouse lineage with lower HBx expression developed liver tumors at the same rate as normal CD-1 mice [121]. HBx was shown to potentiate c-Myc-induced liver carcinogenesis in transgenic mice [122]. Thus, HBx does not have strong transforming activity, but HBx overexpression in a certain genetic background might induce tumor formation in a multistage transformation, most likely in collaboration with other cellular oncogenic pathways.

HBx is mainly located in the cytoplasm and exhibits pleiotropic effects that modulate cell responses to oncogenic signaling pathways [114]. HBx protein do not bind directly to DNA, but rather acts on cellular promoters. Such protein-protein interaction can modulate cytoplas-

mic pathways [113,114,123]. For example, HBx protein was found to activate the JNK-dependent pathway and up-regulate oncogenic c-Myc gene expression [124].

To investigate whether HBx alters phospho-Smad3 signaling in hepatocytes, we stably transfected immortalized rat hepatocytes using a construct of HBx with a mammalian expression vector, resulting in high HBx-expressing cells [56]. High expression of HBx protein in hepatocytes tended to shut down pSmad3C-mediated signaling and favored acquisition of constitutively active JNK-mediated pSmad3L signaling, which fostered hepatocytic growth by up-regulating c-Myc (Figure 1B).

In transgenic models, HBx played an important role in hepatocarcinogenesis via the pSmad3L/c-Myc pathway [56]. HBx transgenic mouse livers progressed through hyperplasia to HCC. HBx, pSmad3L, and c-Myc were not detected in normal mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding central veins where Bromodeoxyuridine (BrdU) was uptaken into the hepatocytes [121]. Smad3L was phosphorylated in hepatocytic nuclei of the centrilobular region, where HBx and c-Myc were expressed. 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 amount of HBV-DNA [56]. Taken together with results of *in vitro* experiments using HBx-expressing hepatocytes and HBx transgenic livers, these human findings indicate that HBx oncoprotein participates directly in hepatocarcinogenesis by shifting hepatocytic phospho-Smad3 signaling from the tumor-suppressive pSmad3C/$p21^{WAF1}$ pathway to the oncogenic JNK/pSmad3L/c-Myc pathway (Figure 1B), [56].

Unlike HBV, HCV is a positive-single-strand RNA virus, apparently incapable of integration into the host's genome. The HCV genome has a long open reading frame, which encodes a polyprotein precursor [125,126]. This polyprotein is cleaved by both 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) [127,128]. The HCV components modulate a number of cellular regulatory functions by targeting a wide spectrum of cellular signaling pathways [129-136]. HCV core expression has been shown to induce activation of the JNK pathway in regulation of vascular endothelial growth factor [136]. NS5A acts as a positive regulator of the JNK signaling pathway by interacting with tumor necrosis factor receptor-associated factor 2, which may play a key role in HCV pathogenesis [137]. In an HCV infection model, Lin *et al.* demonstrated that HCV directly induced TGF-β release from hepatocytes in reactive oxygen species (ROS)-dependent and JNK-dependent manner [138]. Moreover, recent studies using transgenic mouse models indicate that HCV directly involves hepatocarcinogenesis. Three different HCV core transgenic lines develop liver steatosis and HCC [139-141]. Accordingly, future studies are expected to prove that the HCV components can activate the oncogenic JNK/pSmad3L pathway.

12. Activation of the oncogenic JNK/pSmad3L pathway by chronic inflammation

Inflammatory microenvironments are present in human hepatocarcinogenesis before malignant change occurs. A hepatitis virus infection triggers chronic inflammation, increasing the risk of HCC development. Several studies have discussed how chronic inflammation affects the proliferation and survival of hepatocytes [142,143]. TNF-α, IL-1β and IL-6 are multifunctional pro-inflammatory cytokines largely responsible for the hepatic response to chronic inflammation [144-146]. Serum concentrations of these cytokines are increased in chronic liver inflammation including hepatitis viral infection and steatohepatitis [147]. JNK is a key signal transducer for inflammatory cytokines and has emerged as an important endogenous tumor promoter [148,149].

TGF-β is also released by infiltrating Kupffer cells, the liver's resident macrophages, in portal tracts during chronic inflammation [150]. These findings suggest that elevated pro-inflammatory cytokines might alter hepatocytic TGF-β signaling in inflammatory microenvironments. We investigated this hypothesis using rat cultured hepatocytes [64]. Pretreatment of hepatocytes with SP600125, a JNK inhibitor, reduced the subsequent increase in pSmad3L, c-Myc transcription, and hepatocytic growth triggered by pro-inflammatory cytokine stimulation (Figure 1C), suggesting a direct role of the JNK/pSmad3L/c-Myc pathway in facilitating hepatocytic growth in response to cytokine stimulation (Figure 1B).

Experimental models of HCC including inflammation can elucidate how chronic inflammation contributes to hepatocarcinogenesis. In a rat model involving diethylnitrosamine (DEN)-induced carcinogenesis, chronic inflammation liver accompanies abnormalities that progress to HCC [151]. This DEN-induced rat HCC is histologically and genetically similar to human HCC, and also is associated with chronic inflammation [152]. In this chemical model, JNK act participates importantly in hepatocarcinogenesis via pSmad3L/c-Myc signaling. In DEN-treated livers, the JNK/pSmad3L/c-Myc pathway was activated in early pre-neoplastic hepatocytes (Figure 1B), [54]. Moreover, a JNK inhibitor SP600125 suppressed HCC development in DEN-treated rat livers by restoring carcinogenic pSmad3L/c-Myc to the basal pSmad3C/$p21^{WAF1}$ pathway in the pre-neoplastic hepatocytes (Figure 1C), [54].

In human chronic hepatitis C specimens, mainly in groups of hepatocytes adjoining inflammatory cells in portal tracts, Smad3 was found to be phosphorylated at the linker region [64]. Furthermore, positivity of hepatocytic nuclei for pSmad3L/c-Myc in chronic hepatitis C specimens showed a significant relationship with necrosis and inflammatory activity [64]. Taken together with the results of *in vitro* experiments and DEN-treated rat livers, the human findings indicate that chronic inflammation directly participates in hepatocarcinogenesis by shifting hepatocytic phospho-Smad3 signaling from the tumor-suppressive pSmad3C/$p21^{WAF1}$ pathway to the oncogenic JNK/pSmad3L/c-Myc pathway [54,64].

Many tumor-enhancing effects of pro-inflammatory cytokines on hepatocytes are exerted at the level of tumor promotion [58]. TNF-α promotes HCC occurrence in mice lacking the P-

glycoprotein Mdr2 [153]. HCC follows cholestatic inflammation in these mice. Incidence of HCC can be enhanced by another member of the TNF family, lymphotoxin β [154]. Tumor-promoting cytokines produced by Kupffer cells activate several transcription factors, including NF-kB, STAT3, and AP-1, in pre-malignant hepatocytes [155]. The activated transcription factors stimulate transcription of their target genes involved in hepatocytic proliferation and survival, representing a major tumor-promoting mechanism. Similarly to these transcription factors, tumor-promoting actions of hepatocytic Smad3 in human chronic liver disease rarely result from direct mutations [156]. Instead, pSmad3L depends on mitogenic pro-inflammatory cytokine signals produced by neighboring Kupffer cells.

13. Constitutive phosphorylation at Smad3L in pre-neoplastic hepatocytes in cirrhotic human liver

The mechanism regulating regeneration, which avoids accumulation of deleterious mutations in genes that promote cell growth and division, must be disrupted before hepatocytes can throw off normal restraints and behave as an asocial HCC. Constitutive phosphorylation at Smad3L is observed in pre-malignant hepatocytes in cirrhosis [56,64]. Constitutively active pSmad3L stimulates hepatocytes to proliferate continuously in human livers that normally experience little proliferation because hepatocytic regeneration is tightly regulated by cytostatic pSmad3C signaling. Since JNK is constitutively activated in pre-neoplastic hepatocytes in cirrhotic human liver [157], constitutive Smad3L phosphorylation in pre-malignant lesions can be a direct consequence of proto-oncogene-mediated JNK signaling. Somatic mutations in pre-neoplastic hepatocytes include changes in the *Ras* pathway that favor progression from cirrhosis toward HCC [158]. In pre-neoplastic hepatocyte nuclei, pSmad3L/c-Myc can accumulate when somatic mutations constitutively activate the JNK pathway to phosphorylate Smad3 at the linker region (Figure 1B). Then, the proliferative effect mediated via the pSmad3L/c-Myc pathway constitutively keeps on suppresses the growth-inhibitory pSmad3C/$p21^{WAF1}$ pathway in the nuclei of pre-neoplastic hepatocytes.

Pre-neoplastic hepatocytes and HCC show reduction of anti-mitogenic responses to TGF-β [20,37]. 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 [39,159,160]. The TGF-β/pSmad3C pathway is required for maintenance of genomic stability, induction of replicative senescence, and suppression of telomerase [161-163]. Selection for genetic instability occurs in clones of aberrant cells able to produce tumors, since genetic instability greatly accelerates accumulation of further genetic and epigenetic changes required for tumor progression. In this regard, the TGF-β/pSmad3C pathway contributes to tumor suppression along with its cytostatic effect.

14. Chronic inflammation together with hepatitis virus effects in shifting phospho-Smad3 signaling into oncogene-dependent fibro-carcinogenic signaling

In the pathogenesis of HCC, continuous viral infection and chronic inflammation have a prominent role. On the other hand, detailed analysis of HCC development in experimental animals and correlation of these results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC [164]and to a lesser extent in morphologically defined pre-neoplastic precursor lesions [165]. Thus, a series of mutations may accumulate in individual hepatocytes over time. Finally, hepatocytes come to carry somatic mutations that lead to focal uncontrolled hepatocytic growth and eventual malignant cell transformation, in some cases, HCC [166].

Chronic inflammation associated with hepatitis virus infection may be the primary initial requirement in multistep hepatocarcinogenesis. If pSmad3L-positive and pSmad3C-negative hepatocytes survive in the course of chronic hepatitis, such hepatocytes and their descendants can accumulate, and acquire various mutated alleles. Mutations may involve genes in *Ras* pathway [158]that impel pre-neoplastic hepatocytes with constitutive phosphorylation at Smad3L toward a neoplastic growth [8]. Tumor promotion results in further selective clonal expansion of initiated cells, thereby enhancing the likelihood of additional genetic damage as a consequence of endogenous mutations. During tumor progression, premalignant cells continue to develop progressive phenotypic changes and genomic instability, developing into overt HCC.

15. The JNK/pSmad3L pathway as a therapeutic target to avert HCC development

Clinical analyses of pSmad3L and pSmad3C in human tumor formation have provided substantial mechanistic insights. For example, specimens from patients with chronic hepatitis B who develop HCC show abundant Smad3L but limited Smad3C phosphorylation in hepatocytic nuclei, while other patients with abundant hepatocytic pSmad3C but limited pSmad3L do not develop HCC [56]. The same relationships are observed in human HCV-related hepatocarcinogenesis [64]. These clinical observations support roles for pSmad3C as a tumor-suppressor and pSmad3L as a promoter during human carcinogenesis.

HCC is a highly chemoresistant cancer with no effective systemic cytotoxic chemotherapy [167]. Despite surgical or locoregional therapies, the prognosis remains poor because of high likelihood of tumor recurrence or progression and there are no well-established effective adjuvant therapies [168]. Molecular events that affect carcinogenesis need to be identified and targeted to validate new treatment approaches and expand available therapeutics to include chemoprevention to other therapeutics. Since JNK acts as an important regulator of Smad3 signaling that increases the basal amount of hepatocytic pSmad3L available for cell growth

while inactivating the TGF-β-dependent cytostatic actions of pSmad3C (Figure 1B), pharmacologic interference with JNK/pSmad3L signaling could interrupt carcinogenesis. A key therapeutic aim in chronic liver disorders is restoration of lost tumor-suppressive function observed in normal hepatocytes, at the expense of effects promoting hepatic carcinogenesis [169]. To accomplish this difficult aim, Nagata *et al*. (2009) administered a JNK inhibitor SP600125 to rats and were able to suppress chemical carcinogenesis by shifting hepatocytic Smad3 signaling from the carcinogenic pSmad3L pathway to the tumor-suppressive pSmad3C pathway (Figure 1C), [54]. These studies provide evidence that JNK/pSmad3L is an important target for development of chemopreventive and therapeutic measures to reduce emergence of HCC in the context of chronic liver injury and to slow progression of existing tumors. We must also consider whether long-term use of any drug inhibiting C-terminal phosphorylation of R-Smads might cause cancer development [7].

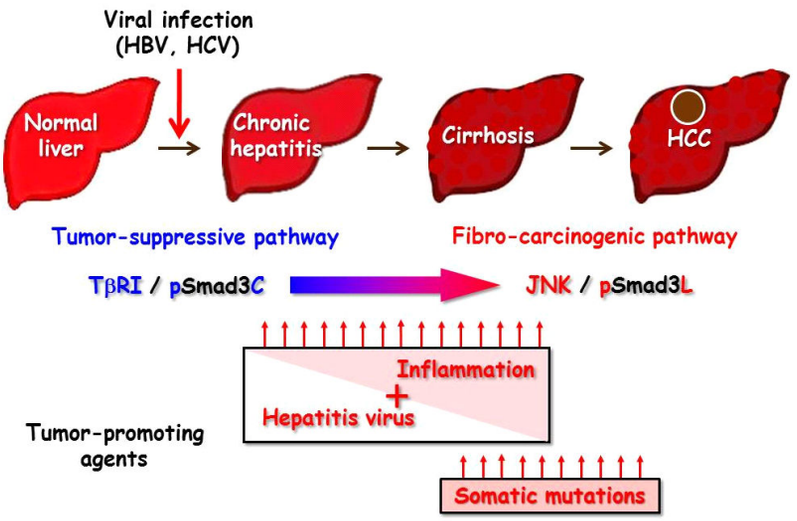

Figure 2. After hepatitis virus infection, early chronic inflammation and subsequent somatic mutations shift hepatocytic phospho-Smad3 signaling from the tumor-suppressive TβRI/pSmad3C made to the fibro-carcinogenic JNK/pSmad3L mode characteristic of MFB, accelerating liver fibrosis while increasing risk of HCC. Both hepatitis virus infection and chronic inflammation represent early fibro-carcinogenic steps representing non-mutagenic tumor-promoting stimuli. In advanced liver fibrosis, mitogenic genetic or epigenetic alterations can drive multistep 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.

16. Conclusion and perspectives

Human fibro-carcinogenesis is a complex multistep process, which involves dysregulation of physiological signal transduction pathways. To maintain hepatic homeostasis, hepatocytic TβRI/pSmad3C/p21^{WAF1} terminates mitogenic JNK/pSmad3L/c-Myc signaling after liver regeneration. During progression of chronic liver diseases, however, early pro-inflammatory cytokines together with hepatitis viruses and subsequent somatic mutations switch hepatocytic phospho-Smad3 signaling from the tumor-suppressive TβRI/pSmad3C to the

fibro-carcinogenic JNK/pSmad3L mode characteristic of MFB, which accelerates liver fibrosis while increasing risk of HCC (Figure 2). Our model is likely to represent a crucial molecular mechanism by which most HCCs arise in from fibrosis or cirrhosis caused by chronic inflammation associated with persistent hepatitis virus infection [164]. Thus, Smad phosphoisoforms function as an important orchestrator of a human chronic inflammation-fibrosis-HCC axis [9,170].

Recent studies in animal models using conditional transgenic expression have suggested an intriguing reversibility of malignant transformation at specific time points if the primary inciting cause of the neoplasia is eliminated [171,172]. However, the fibro-carcinogenic stage in human chronic liver at which the process becomes irreversible. Chronic hepatitis B and C can be cured if patients are treated with antiviral therapy that arrests chronic inflammation by eradicating hepatic HBV and HCV populations. Continued histologic improvement and reversal of fibrosis by antiviral therapy can lead to reduction of HCC development [173,174], but prevention appears most effective when therapy is given before development of cirrhosis. Chronic hepatitis is clearly dependent on continued promoter stimulation - involving in this case the presence of hepatitis viruses and chronic inflammation. However, many patients with cirrhosis have evolved beyond dependence on inflammation because hepatocytes have acquired genetic and epigenetic carcinogenic properties. We are carrying out several trials to determine whether or not antiviral therapy can decrease liver fibrosis and lower HCC incidence. The trials will bear upon important questions regarding relative participation in fibro-carcinogenesis of inflammation-dependent and oncogene-dependent Smad3 phosphoisoform signaling in HBV- and HCV-related chronic liver disorders. In the trials, pathologic analyses using domain-specific phospho-Smad3 Abs, together with clinical data, will be used to evaluate the benefit from antiviral therapy, which decreases stimulation of the inflammation-dependent Smad phosphorisoform pathway. After antiviral therapy, hepatocytic pSmad3L and pSmad3C assessment in liver specimens should prove clinically useful for predicting progression of fibrosis and risk of HCC.

Author details

Miki Murata*, Katsunori Yoshida and Koichi Matsuzaki

*Address all correspondence to: muratami@takii.kmu.ac.jp

Departments of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan

References

[1] El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132(7) 2557-2576.

[2] McGlynn KA, London WT. Epidemiology and natural history of hepatocellular carcinoma. Best Pract Res Clin Gastroenterol 2005;19(1) 3-23.

[3] Donato F, Tagger A, Gelatti U, Parrinello G, Boffetta P, Albertini A, Decarli A, Trevisi P, Ribero ML, Martelli C, Porru S, Nardi G. Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women. Am J Epidemiol 2002;155(4) 323-331.

[4] Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H, Kawashima T. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328(25) 1797-1801.

[5] Moses HL, Yang EY, and Pietenpol JA. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 1990;63(2) 245-247.

[6] Roberts AB, Sporn MB. The transforming growth factor-βs. In: Sporn MB, Roberts AB. (eds.) Peptide Growth Factors and Their Receptors. Springer, Berlin; 1990. p 419-472.

[7] Bellam N, Pasche B. TGF-β signaling alterations and colon cancer. Cancer Treat Res 2010;155:85-103.

[8] Matsuzaki K. Smad phosphoisoform signaling specificity: the right place at the right time. Carcinogenesis 2011;32(11) 1578-1588.

[9] Matsuzaki K. Smad phosphoisoform signals in acute and chronic liver injury: similarities and differences between epithelial and mesenchymal cells. Cell Tissue Res 2012;347(1) 225-243.

[10] Derynck R, Miyazono K. The TGF-β signaling. Cold Spring Harbor Laboratory Press, NewYork; 2008.

[11] Heldin CH, Miyazono K, ten Dijke P. TGF-β signaling from cell membrane to nucleus through SMAD proteins. Nature 1997;390(6659) 465-471.

[12] Wrana JL. Regulation of Smad activity. Cell 2000;100(2) 189-192.

[13] Shi Y, Massagué J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 2003;113(6) 685-700.

[14] Guo X, Wang XF. Signaling cross-talk between TGF-β/BMP and other pathways. Cell Res 2009;19(1) 71-88.

[15] Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGF-β/Smad signaling by oncogenic Ras. Genes Dev 1999;13(7) 804-816.

[16] Mori S, Matsuzaki K, Yoshida K, Furukawa F, Tahashi Y, Yamagata H, Sekimoto G, Seki T, Matsui H, Nishizawa M, Fujisawa J, Okazaki K. TGF-β and HGF transmit the

signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. Oncogene 2004;23(44) 7416-7429.

[17] Matsuura I, Denissova NG, Wang G, He D, Long J, Liu F. Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature 2004;430(6996) 226-231.

[18] Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-β-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem 2005;280(2) 1024-1036.

[19] Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, Seki T, Matsui H, Fujisawa J, Okazaki K. Reversible Smad-dependent signaling between tumor suppression and oncogenesis. Reversible Smad-dependent signaling between tumor suppression and oncogenesis. Cancer Res 2007;67(11) 5090-5096.

[20] Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, Seki T, Taketani S, Fujisawa J, Okazaki K. Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-β signal in later stages of human colorectal cancer. Cancer Res 2009;69(13) 5321-5330.

[21] Liu F. Smad3 phosphorylation by cyclin-dependent kinases. Cytokine Growth Factor Rev 2006;17(1-2) 9-17.

[22] Wicks SJ, Lui S, Abdel-Wahab N, Mason RM, Chantry A. Inactivation of Smad-transforming growth factor β signaling by Ca(2+)-calmodulin-dependent protein kinase II. Mol Cell Biol 2000;20(21) 8103-8111.

[23] Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J, Inoue K. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. Hepatology 2003;38(4) 879-889.

[24] Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA, Lebrun JJ. The G protein-coupled receptor kinase-2 is a TGF-β-inducible antagonist of TGF-β signal transduction. EMBO J 2005;24(18) 3247-3258.

[25] Millet C, Yamashita M, Heller M, Yu LR, Veenstra TD, Zhang YE. A negative feedback control of transforming growth factor-β signaling by glycogen synthase kinase 3-mediated Smad3 linker phosphorylation at Ser-204. J Biol Chem 2009;284(30) 19808-19816.

[26] Alarcón C, Zaromytidou AI, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D, Massagué J. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 2009;139(4) 757-769.

[27] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4(7) 499-511.

[28] Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M,Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S. TAK1, but not TAB1 or

TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 2005;19(22) 2668-2681.

[29] Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res 2009;19(2) 156-172.

[30] Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-β family proteins in development and disease. Nat Cell Biol 2007;9(9) 1000-1004.

[31] Schnabl B, Kweon YO, Frederick JP, Wang XF, Rippe RA, Brenner DA. The role of Smad3 in mediating mouse hepatic stellate cell activation. Hepatology 2001;34(1) 89-100.

[32] Kodama Y, Kisseleva T, Iwaisako K, Miura K, Taura K, De Minicis S, Osterreicher CH, Schnabl B, Seki E, Brenner DA. c-Jun N-terminal kinase-1 from hematopoietic cells mediates progression from hepatic steatosis to steatohepatitis and fibrosis in mice. Gastroenterology 2009;137(4) 1467-1477.

[33] Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest 2008;118(12) 3943-3953.

[34] Matsuzaki K. Smad3 phosphoisoform-mediated signaling during sporadic human colorectal carcinogenesis. Histol Histopathol 2006;21(6) 645-662.

[35] Sapkota G, Knockaert M, Alarcón C, Montalvo E, Brivanlou AH, Massagué J. Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-β pathways. J Biol Chem 2006;281(52) 40412-40419.

[36] Wrighton KH, Lin X, Feng X-H. Phospho-control of TGF-β superfamily signaling. Cell Res 2009;19(1) 8-20.

[37] Matsuzaki K. Modulation of TGF-β signaling during progression of chronic liver diseases. Front Biosci 2009;14: 2923-2934.

[38] Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology 2006;43(2 Suppl 1) S45-53.

[39] Massagué J. TGF-β in Cancer. Cell 2008;134(2) 215-230.

[40] Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-β family signaling. Nature 2003;425(6958) 577-584.

[41] Caestecker MP de, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ. Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. Genes Dev 1998;12(11) 1587-1592.

[42] Lehmann K, Janda E, Pierreux CE, Rytomaa M, Schulze A, McMahon M, Hill CS, Beug H, Downward J. Raf induces TGF-β production while blocking its apoptotic but

not invasive responses: a mechanism leading to increased malignancy in epithelial cells. Genes Dev 2000;14(20) 2610-2622.

[43] Yue J, Mulder KM. Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a Smad-dependent pathway. J Biol Chem 2000;275(40) 30765-30773.

[44] Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol 2002;4(7) 487-494.

[45] Funaba M, Zimmerman CM, Mathews LS. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J Biol Chem 2002;277(44) 41361-41368.

[46] Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Günter S. Ras and TGF-β cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 2002;156(2) 299-313.

[47] Suzuki K, Wilkes MC, Garamszegi N, Edens M, Leof EB. Transforming growth factor β signaling via Ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses. Cancer Res 2007;67(8) 3673-3682.

[48] Li F, Zeng B, Chai Y, Cai P, Fan C, Cheng T. The linker region of Smad2 mediates TGF-beta-dependent ERK2-induced collagen synthesis. Biochem Biophys Res Commun 2009;386(2) 289-293.

[49] Burch ML, Yang SN, Ballinger ML, Getachew R, Osman N, Little PJ. TGF-β stimulates biglycan synthesis via p38 and ERK phosphorylation of the linker region of Smad2. Cell Mol Life Sci 2010;67(12) 2077-2090.

[50] Jiang W, Zhang Y, Wu H, Zhang X, Gan H, Sun J, Chen Q, Guo M, Zhang Z. Role of cross-talk between the Smad2 and MAPK pathways in TGF-β1-induced collagen IV expression in mesangial cells. Int J Mol Med 2010;26(4) 571-576.

[51] Wang G, Long J, Matsuura I, He D, Liu F. The Smad3 linker region contains a transcriptional activation domain. Biochem J 2005;386(Pt 1) 29-34.

[52] Prokova V, Mavridou S, Papakosta P, Kardassis D. Characterization of a novel transcriptionally active domain in the transforming growth factor beta-regulated Smad3 protein. Nucleic Acids Res 2005;33(12) 3708-3721.

[53] Vasilaki E, Siderakis M, Papakosta P, Skourti-Stathaki K, Mavridou S, Kardassis D. Novel regulation of Smad3 oligomerization and DNA binding by its linker domain. Biochemistry 2009;48(35) 8366-8378.

[54] Nagata H, Hatano E, Tada M, Murata M, Kitamura K, Asechi H, Narita M, Yanagida A, Tamaki N, Yagi S, Ikai I, 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(6) 1944-1953.

[55] Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J Biol Chem 1999;274(52) 37413-37420.

[56] 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(4) 1203-1217.

[57] Michalopoulos GK. Liver regeneration. J Cell Physiol 2007;213(2) 286-300.

[58] Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140(6) 883-899.

[59] Yamada Y, Kirillova I, Peschon JJ, Fausto N. Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc Natl Acad Sci U S A 1997;94(4) 1441-1446.

[60] Tilg H, Kaser A, Moschen AR. How to modulate inflammatory cytokines in liver diseases. Liver Int 2006;26(9) 1029-1039.

[61] Date M, Matsuzaki K, Matsushita M, Sakitani K, Shibano K, Okajima A, Yamamoto C, Ogata N,Okumura T, Seki T, Kubota Y, Kan M, McKeehan WL, Inoue K. Differential expression of transforming growth factor-β and its receptors in hepatocytes and nonparenchymal cells of rat liver after CCl4 administration. J Hepatol 1998;28(4) 572-581.

[62] Date M, Matsuzaki K, Matsushita M, Tahashi Y, Sakitani K, Inoue K. Differential regulation of activin A for hepatocyte growth and fibronectin synthesis in rat liver injury. J Hepatol 2000;32(2) 251-260.

[63] Date M, Matsuzaki K, Matsushita M, Tahashi Y, Furukawa F, Inoue K. Modulation of transforming growth factor β function in hepatocytes and hepatic stellate cells in rat liver injury. Gut 2000;46(5) 719-724.

[64] Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, Kaibori M, Kamiyama Y, Nishizawa M, Fujisawa J, Okazaki K, Seki T. Chronic inflammation associated with hepatitis C viral infection perturbs hepatic TGF-β signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology 2007;46(1) 48-57.

[65] Kawamata S, Matsuzaki K, Murata M, Seki T, Matsuoka K, Iwao Y, Hibi T, Okazaki K. Oncogenic Smad3 signaling induced by chronic inflammation is an early event in ulcerative colitis-associated carcinogenesis. Inflamm Bowel Dis 2011;17(3) 683-695.

[66] Yang YA, Zhang GM, Feigenbaum L, Zhang YE. Smad3 reduces susceptibility to hepatocarcinoma by sensitizing hepatocytes to apotosis thorough downregulation of Bcl-2. Cancer Cell 2006;9(6) 445-457.

[67] Caldwell S, Park SH. The epidemiology of hepatocellular cancer: from the perspectives of public health problem to tumor biology. J Gastroenterol 2009;44 (Suppl 19) S96-101.

[68] Seitz and Stickel. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. Biol Chem 2006;387(4) 349-360.

[69] Yuen MF, Tanaka Y, Fong DY, Fung J, Wong DK, Yuen JC, But DY, Chan AO, Wong BC, Mizokami M, Lai CL. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. J Hepatol 2009;50(1) 80-88.

[70] Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127(Suppl 1) S35-50.

[71] Kris VK. Iron, hemochromatosis and hepatocellular carcinoma. Gastroenterology 2004;127(5 Suppl 1) S79-86.

[72] Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology 2010;51(6) 1972-1978.

[73] Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2000;279(2) G245-249.

[74] Popov Y, Schuppan D. Targeting liver fibrosis: strategies for development and validation of antifibrotic therapies. Hepatology 2009;50(4) 1294-1306.

[75] Pinzani M, Macias-Barragan J. Update on the pathophysiology of liver fibrosis. Expert Rev Gastroenterol Hepatol 2010;4(4) 459-472.

[76] Eddy AA. Serine proteases, inhibitors and receptors in renal fibrosis. Thromb Haemost 2009;101(4) 656-664.

[77] Hu PF, Chen H, Zhong W, Lin Y, Zhang X, Chen YX, Xie WF. Adenovirus-mediated transfer of siRNA against PAI-1 mRNA ameliorates hepatic fibrosis in rats. J Hepatol 2009;51(1) 102-113.

[78] Taub R. Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 2004;5(10) 836-847.

[79] Friedman SL. Evolving challenges in hepatic fibrosis. Nat Rev Gastroenterol Hepatol 2010;7(8) 425-436.

[80] Kawada N. The hepatic perisinusoidal stellate cell. Histol Histopathol 1997;12(4) 1069-1080.

[81] Inagaki Y, Okazaki I. Emerging insights into transforming growth factor β Smad signal in hepatic fibrogenesis. Gut 2007;56(2) 284-292.

[82] Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115(2) 209-218.

[83] Weinberg RA.The Biology of Cancer. Garlang Science, New York; 2007

[84] Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002;110(3) 341-350.

[85] Saika S, Kono-Saika S, Ohnishi Y, Sato M, Muragaki Y, Ooshima A, Flanders KC, Yoo J, Anzano M, Liu CY, Kao WW, Roberts AB. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am J Pathol 2004;164(2) 651-663.

[86] Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007;282(32) 23337-23347.

[87] Shirai Y, Kawata S, Tamura S, Ito N, Tsushima H, Takaishi K, Kiso S, Matsuzawa Y. Plasma transforming growth factor-β1 in patients with hepatocellular carcinoma: comparison with chronic liver diseases. Cancer 1994;73(9) 2275-2279.

[88] Tilg H. Cytokines and liver diseases. Can J Gastroenterol 2001;15(10) 661-668.

[89] Sugano Y, Matsuzaki K, Tahashi Y, Furukawa F, Mori S, Yamagata H, Yoshida K, Matsushita M, Nishizawa M, Fujisawa J, Inoue K. Distortion of autocrine transforming growth factor β signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. Oncogene 2003;22(15) 2309-2321.

[90] Yoshida K, Matsuzaki K, Mori S, Tahashi Y, Yamagata H, Furukawa F, Seki T, Nishizawa M, Fujisawa J, Okazaki K. Transforming growth factor-β and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. Am J Pathol 2005;166(4) 1029-1039.

[91] Kluwe J, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, Colmenero J, Bataller R, Schwabe RF. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology 2010;138(1) 347-359.

[92] Nagamine Y. Transcriptional regulation of the plasminogen activator inhibitor type 1-with an emphasis on negative regulation. Thromb Haemost 2008;100(6) 1007-1013.

[93] Dooley S, Hamzavi J, Ciuclan L, Godoy P, Ilkavets I, Ehnert S, Ueberham E, Gebhardt R, Kanzler S, Geier A, Breitkopf K, Weng H, Mertens PR. Hepatocyte-specific Smad7 expression attenuates TGF-β-mediated fibrogenesis and protects against liver damage. Gastroenterology 2008;135(2) 642-659.

[94] Roth-Eichhorn S, Kühl K, Gressner AM. Subcellular localization of (latent) transforming growth factor beta and the latent TGF-beta binding protein in rat hepatocytes and hepatic stellate cells. Hepatology 1998;28(6) 1588-1596.

[95] Weng HL, Ciuclan L, Liu Y, Hamzavi J, Godoy P, Gaitantzi H, Kanzler S, Heuchel R, Ueberham U, Gebhardt R, Breitkopf K, Dooley S. Profibrogenic transforming growth

factor-beta/activin receptor-like kinase 5 signaling via connective tissue growth factor expression in hepatocytes. Hepatology 2007;46(4) 1257-1270.

[96] Gressner OA. Less Smad2 is good for you! A scientific update on coffee's liver benefits. Hepatology 2009;50(3) 970-978.

[97] Gressner OA, Lahme B, Demirci I, Gressner AM, Weiskirchen R. Differential effects of TGF-beta on connective tissue growth factor (CTGF/CCN2) expression in hepatic stellate cells and hepatocytes. J Hepatol 2007;47(5) 699-710.

[98] Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48(6) 2047-2063.

[99] Bréchot C, Nalpas B, Couroucé AM, Duhamel G, Callard P, Carnot F, Tiollais P, Berthelot P. Evidence that hepatitis B virus has a role in liver-cell carcinoma in alcoholic liver disease. N Engl J Med 1982;306(23) 1384-1387.

[100] Hassan MM, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. Hepatology 2002;36(5) 1206-1213.

[101] Park SZ, Nagorney DM, Czaja AJ. Hepatocellular carcinoma in autoimmune hepatitis. Dig Dis Sci 2000;45(10) 1944-1948.

[102] Shibuya A, Tanaka K, Miyakawa H, Shibata M, Takatori M, Sekiyama K, Hashimoto N, Amaki S, Komatsu T, Morizane T. Hepatocellular carcinoma and survival in patients with primary biliary cirrhosis. Hepatology 2002;35(5) 1172-1178.

[103] But DY, Lai CL, Yuen MF. Natural history of hepatitis-related hepatocellular carcinoma. World J Gastroenterol 2008;14(11) 1652-1656.

[104] Tsai JF, Jeng JE, Ho MS, Chang WY, Hsieh MY, Lin ZY, Tsai JH. Effect of hepatitis C and B virus infection on risk of hepatocellular carcinoma: a prospective study. Br J Cancer 1997;76(7) 968-974.

[105] Chiaramonte M, Stroffolini T, Vian A, Stazi MA, Floreani A, Lorenzoni U, Lobello S, Farinati F, Naccarato R. Rate of incidence of hepatocellular carcinoma in patients with compensated viral cirrhosis. Cancer 1999;85(10) 2132-2137.

[106] Buendia MA. Mammalian hepatitis B viruses and primary liver cancer. Semin Cancer Biol 1992;3(5) 309-320.

[107] Jiang Z, Jhunjhunwala S, Liu J, Haverty PM, Kennemer MI, Guan Y, Lee W, Carnevali P, Stinson J, Johnson S, Diao J, Yeung S, Jubb A, Ye W, Wu TD, Kapadia SB, de Sauvage FJ, Gentleman RC, Stern HM, Seshagiri S, Pant KP, Modrusan Z, Ballinger DG, Zhang Z. The effects of hepatitis B virus integration into the genomes of hepatocellular carcinoma patients. Genome research 2010;22(4) 593-601.

[108] Bréchot C, Pourcel C, Louise A, Rain B, Tiollais P. Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 1980;286(5772) 533-535.

[109] Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma studies in percutaneous liver biopsies and post-mortem tissue specimens. N Engl J Med 1981;305(18) 1067-1073.

[110] Guerrero RB, Roberts LR. The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. J Hepatol 2005;42(5) 760-777.

[111] Feitelson MA, Lee J. Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. Cancer Lett 2007;252(2) 157-170.

[112] Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Brećhot C. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. Gut 2005;54(8) 1162-1168.

[113] Andrisani OM, Barnabas S. The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis. Int J Oncol 1999;15(2) 373-379.

[114] Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol 2004;78(23) 12725-12734.

[115] Tang H, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. Cancer Sci 2006;97(10) 977-983.

[116] Seifer M, Höhne M, Schaefer S, Gerlich WH. In vitro tumorigenicity of hepatitis B virus DNA and HBx protein. J Hepatol 1991;13(Suppl 4) S61-65.

[117] Gottlob K, Pagano S, Levrero M, Graessmann A. Hepatitis B virus X protein transcription activation domains are neither required nor sufficient for cell transformation. Cancer Res 1998;58(16) 3566-3570.

[118] Oguey D, Dumenco LL, Pierce RH, Fausto N. Analysis of the tumorigenicity of the X gene of hepatitis B virus in a nontransformed hepatocyte cell line and the effects of cotransfection with a murine p53 mutant equivalent to human codon 249. Hepatology 1996;24(5) 1024-1033.

[119] Reifenberg K, Löhler J, Pudollek HP, Schmitteckert E, Spindler G, Köck J, Schlicht HJ. Long-term expression of the hepatitis B virus core-e- and X-proteins does not cause pathologic changes in transgenic mice. J Hepatol 1997;26(1) 119-130.

[120] Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351(6324) 317-320.

[121] Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology 1994;19(4) 810-819.

[122] Terradillos O, Billet O, Renard CA, Levy R, Molina T, Briand P, Buendia MA. The hepatitis B virus X gene potentiates c-myc-induced liver oncogenesis in transgenic mice. Oncogene 1997;14(4) 395-404.

[123] Pang R, Tse E, Poon RT. Molecular pathways in hepatocellular carcinoma. Cancer Lett 2006;240(2) 157-169.

[124] Feitelson MA. c-myc overexpression in hepatocarcinogenesis. Hum Pathol 2004;35(11) 1299-1302.

[125] Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci U S A 1991;88(6) 2451-2455.

[126] Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I & Okayama H. Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 1991;65(3) 1105-1113.

[127] Lin C, Lindenbach BD, Pragai BM, McCourt DW, Rice CM. Processing in the hepatitis C virus E2-xlink region: identification of p7 and two distinct E2-specific products with different C termini. J Virol 1994;68(8) 5063-5073.

[128] Reed KE, Rice CM. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr Top Microbiol Immunol 2000;242:55-84.

[129] Hayashi J, Aoki H, Kajino K, Moriyama M, Arakawa Y, Hino O. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha. Hepatology 2000;32(5) 958-961.

[130] Erhardt A, Hassan M, Heintges T, Haussinger D. Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line. Virology 2002;292(2) 272-284.

[131] He Y, Nakao H, Tan SL, Polyak SJ, Neddermann P, Vijaysri S, Jacobs BL, Katze MG. Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. J Virol 2002;76(18) 9207-9217.

[132] Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, Simon FR. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem J 2004;378(Pt 3) 919-928.

[133] Zhao LJ, Wang L, Ren H, Cao J, Li L, Ke JS, Qi ZT. Hepatitis C virus E2 protein promotes human hepatoma cell proliferation through the MAPK/ERK signaling pathway via cellular receptors. Exp Cell Res 2005;305(1) 23-32.

[134] Hassan M, Ghozlan H, Abdel-Kader O. Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) nonstructural protein 3 (NS3)- mediated cell growth. Virology 2005;333(2) 324-336.

[135] Choi SH, Hwang SB. Modulation of the transforming growth factor-beta signal transduction pathway by hepatitis C virus nonstructural 5A protein. J Biol Chem 2006;281(11) 7468-7478.

[136] Hassan M, Selimovic D, Ghozlan H, Abdel-kader O. Hepatitis C virus core protein triggers hepatic angiogenesis by a mechanism including multiple pathways. Hepatology 2009;49(5) 1469-1482.

[137] Park KJ, Choi SH, Choi DH, Park JM, Yie SW, Lee SY, Hwang SB. 1Hepatitis C virus NS5A protein modulates c-Jun N-terminal kinase through interaction with tumor necrosis factor receptor-associated factor 2. J Biol Chem 2003;278(33) 30711-30718.

[138] Lin W, Tsai WL, Shao RX, Wu G, Peng LF, Barlow LL, Chung WJ, Zhang L, Zhao H, Jang JY, Chung RT. Hepatitis C virus regulates transforming growth factor beta1 production through the generation of reactive oxygen species in a nuclear factor kappaB-dependent manner. Gastroenterology 2010;138(7) 2509-2518.

[139] Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78 (Pt 7) 1527-1531.

[140] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4(9) 1065-1067.

[141] Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA, Lemon SM. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002;122(2) 352-365.

[142] Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 2005;7(3) 211-217.

[143] Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420(6917) 860-867.

[144] Grivennikov S, Karin M. Autocrine IL-6 signaling: a key event in tumorigenesis? Cancer Cell 2008;13(1) 7-9.

[145] Szlosarek PW, Balkwill FR. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. Lancet Oncol 2003;4(9) 565-573.

[146] Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A 2003;100(5) 2645-2650.

[147] Jonsson JR, Barrie HD, O'Rourke P, Clouston AD, Powell EE. Obesity and steatosis influence serum and hepatic inflammatory markers in chronic hepatitis C. Hepatology 2008;48(1) 80-87.

[148] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270(28) 16483-16486.

[149] Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103(2) 239-252.

[150] Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454(7203) 436-444.

[151] Tada M, Hatano E, Taura K, Nitta T, Koizumi N, Ikai I, et al. High volume hydrodynamic injection of plasmid DNA via the hepatic artery results in a high level of gene expression in rat hepatocellular carcinoma induced by diethylnitrosamine. J Gene Med 2006;8(8) 1018-1026.

[152] Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, Clergue F, Poupon R, Barbu V, Rosmorduc O. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. Hepatology 2005;41(2) 307-314.

[153] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431(7007) 461-466.

[154] Haybaeck J, Zeller N, Wolf MJ, Weber A, Wagner U, Kurrer MO, Bremer J, Iezzi G, Graf R, Clavien PA, Thimme R, Blum H, Nedospasov SA, Zatloukal K, Ramzan M, Ciesek S, Pietschmann T, Marche PN, Karin M, Kopf M, Browning JL, Aguzzi A, Heikenwalder M. A lymphotoxin-driven pathway to hepatocellular carcinoma. Cancer Cell 2009;16(4) 295-308.

[155] He G, Karin M. NF-κB and STAT3 - key players in liver inflammation and cancer. Cell Res 2011;21(1) 159-168.

[156] Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, Flanders KC. Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. Cytokine Growth Factor Rev 2006;17(1-2) 19-27.

[157] Sugioka Y, Watanabe T, Inagaki Y, Kushida M, Niioka M, Endo H, Higashiyama R, Okazaki I. c-Jun NH2-terminal kinase pathway is involved in constitutive matrix metalloproteinase-1 expression in a hepatocellular carcinoma-derived cell line. Int J Cancer 2004; 109(6) 867-874.

[158] Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, Calderaro J, Bioulac-Sage P, Letexier M, Degos F, Clément B, Balabaud C, Chevet E, Laurent A, Couchy G, Letouzé E, Calvo F, Zucman-Rossi J. Integrated analysis of somatic muta-

tions and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44(6) 694-698.

[159] Wakefield LM, Roberts AB. TGF-β signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 2002;12(1) 22-29.

[160] Pardali K, Moustakas A. Actions of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta 2007;1775(1) 21-62.

[161] Glick AB, Weinberg WC, Wu IH, Quan W, Yuspa SH. Transforming growth factor β1 suppresses genomic instability independent of a G1 arrest, p53, and Rb. Cancer Res 1996;56(16) 3645-3650.

[162] Tremain R, Marko M, Kinnimulki V, Ueno H, Bottinger E, Glick A. Defects in TGF-β signaling overcome senescence of mouse keratinocytes expressing v-Ha-ras. Oncogene. 2000;19(13) 1698-1709.

[163] Yang H, Kyo S, Takatura M, Sun L. Autocrine transforming growth factor beta suppresses telomerase activity and transcription of human telomerase reverse transcriptase in human cancer cells. Cell Growth Differ 2001;12(2) 119-127.

[164] Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31(4) 339-346.

[165] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362(9399) 1907-1917.

[166] Kew MC, Yu MC, Kedda MA, Coppin A, Sarkin A, Hodkinson J. The relative roles of hepatitis B and C viruses in the etiology of hepatocellular carcinoma in Southern African Blacks. Gastroenterology 1997;112(1) 184-187.

[167] Lopez PM, Villanueva A, Llovet JM. Systematic review: evidence-based management of hepatocellular carcinoma–an updated analysis of randomized controlled trials. Aliment Pharmacol Ther 2006;23(11) 1535-1547.

[168] Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM, Wong J. Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann Surg 2001;234(1) 63-70.

[169] Felsher DW. Reversibility of oncogene-induced cancer. Curr Opin Genet Dev 2004;14(1) 37-42.

[170] Elsharkawy AM, Mann DA. Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. Hepatology 2007;46(2) 590-597.

[171] Ewald D, Li M, Efrat S, Auer G, Wall RJ, Furth PA, Hennighausen. Time-sensitive reversal of hyperplasia in transgenic mice expressing SV40 T antigen. Science 1996;273(5280) 1384-1386.

[172] Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell 1999;4(2) 199-207.

[173] 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(3) 174-181.

[174] 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(7) 517-524.