

Hepatitis B Virus X Protein Shifts Human Hepatic Transforming Growth Factor (TGF)- β Signaling from Tumor Suppression to Oncogenesis in Early Chronic Hepatitis B

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Hepatitis B virus X (HBx) protein is suspected to participate in oncogenesis during chronic hepatitis B progression. Transforming growth factor β (TGF- β) signaling involves both tumor suppression and oncogenesis. TGF- β activates TGF- β type I receptor (T β RI) and c-Jun N-terminal kinase (JNK), which differentially phosphorylate the mediator Smad3 to become C-terminally phosphorylated Smad3 (pSmad3C) and linker-phosphorylated Smad3 (pSmad3L). Reversible shifting of Smad3-mediated signaling between tumor suppression and oncogenesis in HBx-expressing hepatocytes indicated that T β RI-dependent pSmad3C transmitted a tumor-suppressive TGF- β signal, while JNK-dependent pSmad3L promoted cell growth. We used immunostaining, immunoblotting, and *in vitro* kinase assay to compare pSmad3L- and pSmad3C-mediated signaling in biopsy specimens representing chronic hepatitis, cirrhosis, or hepatocellular carcinoma (HCC) from 90 patients chronically infected with hepatitis B virus (HBV) with signaling in liver specimens from HBx transgenic mice. In proportion to plasma HBV DNA levels, early chronic hepatitis B specimens showed prominence of pSmad3L in hepatocytic nuclei. HBx-activated JNK/pSmad3L/c-Myc oncogenic pathway was enhanced, while the T β RI/pSmad3C/p21^{WAF1} tumor-suppressive pathway was impaired as human and mouse HBx-associated hepatocarcinogenesis progressed. Of 28 patients with chronic hepatitis B who showed strong oncogenic pSmad3L signaling, six developed HCC within 12 years; only one of 32 patients showing little pSmad3L developed HCC. In contrast, seven of 30 patients with little Smad3C phosphorylation developed HCC, while no patient who retained hepatocytic tumor-suppressive pSmad3C developed HCC within 12 years. **Conclusion:** HBx shifts hepatocytic TGF- β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in early carcinogenic process. Hepatocytic pSmad3L and pSmad3C assessment in HBV-infected liver specimens should prove clinically useful for predicting risk of HCC. (HEPATOLOGY 2009;49:1203-1217.)

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and one of the most deadly, causing approximately 600,000 deaths yearly.¹ The overall incidence of HCC continues to rise, especially in western Europe and the United States.² During the past 20 years, striking advances have enhanced our understanding of HCC. More than 85% of HCC cases are related to known hepatitis B virus (HBV) and hepatitis C virus (HCV).

Abbreviations: Ab, antibody; HBV, hepatitis B virus; HBx, hepatitis B virus X; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; IgG, immunoglobulin G; JNK, c-Jun N-terminal kinase; PPM1A, protein phosphatase magnesium 1A; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3; SCP1-3, small C-terminal domain phosphatase 1-3; TGF- β , transforming growth factor β ; T β RI, TGF- β type I receptor; T β RII, TGF- β type II receptor.

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Received March 19, 2008; accepted November 25, 2008.

Supported by the Ministry of Education, Science, and Culture of Japan (K. M.).

A strong correlation between chronic HBV infection and HCC occurrence has long been apparent according to epidemiologic evidence and the finding of integrated HBV DNA sequences in virtually all HBV-related HCC.³ Hepatitis B virus X (HBx) oncoprotein has been implicated in HBV-mediated hepatocarcinogenesis,^{4,5} and persistent high-level expression of HBx protein in transgenic mouse liver results in hyperplasia leading to HCC, with no preceding inflammation.⁶ Although HBx does not bind DNA directly, HBx activates Ras/mitogen-activated protein kinase pathways including extracellular signal-regulated kinase and c-Jun N-terminal kinase (JNK),⁷ resulting in tumor cell growth and survival.

Transforming growth factor β (TGF- β) can inhibit epithelial cell growth, acting as a tumor suppressor. During carcinogenesis, however, cancer cells gain advantage by selective reduction of the tumor-suppressive activity of TGF- β together with augmentation of its oncogenic activity.⁸ This led us to hypothesize that alterations in the TGF- β signal transduction pathway could be involved in the development of HCC in long-standing HBV infection.

Smads are central mediators of signals from the receptors for TGF- β superfamily members to the nucleus.⁹ Smads are modular proteins with conserved Mad-homology 1, intermediate linker, and Mad-homology 2 domains.¹⁰ The catalytically active TGF- β type I receptor (T β RI) phosphorylates the C-terminal serine residues of receptor-activated Smads, which include Smad2 and the highly related protein Smad3. The linker domain can undergo regulatory phosphorylation by other kinases including mitogen-activated protein kinases and cyclin-dependent kinases.¹¹⁻¹⁴ In contrast to the clearly activating role of the C-terminal phosphorylation events, the regulation of Smad activity by phosphorylation of the linker region is complex. Linker phosphorylation of Smad2 during human colorectal carcinogenesis results in cytoplasmic retention of Smad2 and inhibition of tumor-suppressive TGF- β signaling.^{11,15} However, Smad3 phosphorylated at the linker region (pSmad3L) is localized predominantly to cell nuclei in actively growing Ki-67-immunoreactive colon cancer with distant metastasis.¹⁵ Reversible shifting of Smad-dependent signaling between tumor suppression and oncogenesis in hyperactive Ras-expressing cells indicates that Smad3 phosphor-

ylated at the C-terminal region (pSmad3C) transmits a tumor-suppressive TGF- β signal, whereas oncogenic activities such as cell proliferation and invasion are promoted by the pSmad3L pathway.¹⁶ In addition, Roberts' group¹⁷ has recently reported that Smad3 is critical for Ras/JNK-mediated transformation. Taken together, these findings indicate that oncogenic TGF- β signaling results from the functional collaboration of Ras and Smad3 rather than from Ras-mediated inhibition of the Smad3 pathway. Linker phosphorylation of Smad3 indirectly inhibits C-terminal phosphorylation, minimizing tumor-suppressive pSmad3C signaling.¹⁶ Notably, pSmad3L-mediated signaling in activated hepatic stellate cells (HSCs) promotes liver fibrosis by stimulating extracellular matrix deposition.^{13,18}

The role of HBV and HCV in tumor formation appears to be complex and may involve both direct and indirect mechanisms.¹⁹ Integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. Alternatively, chronic liver inflammation and hepatic regeneration induced by host cellular immune responses can increase the risk of HCC development. During progression of HCV-related chronic liver disorders, hepatocytes affected by chronic inflammation undergo a transition from the tumor-suppressive pSmad3C pathway to the JNK/pSmad3L pathway.²⁰ Our present studies extend the previous observations to HBV-related hepatocarcinogenesis. We study Smad3 phosphorylation profiles in HBV-infected human liver and HBx transgenic mouse liver, concluding that HBx oncoprotein in early stages of chronic hepatitis B contributes directly to hepatocarcinogenesis by shifting hepatocytic Smad3-mediated signaling from tumor suppression to oncogenesis.

Patients and Methods

Patients, Follow-up, and Detection of HCC.

Ninety patients with HBV-related chronic liver disease underwent liver biopsy at the Department of Gastroenterology and Hepatology of Kansai Medical University Hospital between 1992 and 1994. All patients were seropositive for hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL) and were seronegative for anti-HCV antibody (Ortho Diagnostics, Tokyo, Japan). Patients included 70 with chronic hepatitis, 10 with cir-

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22765

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found on the online version of this article.

rhosis, and 10 with HCC. Sixty of the chronic hepatitis patients were enrolled in a program for early diagnosis of HCC; the other 10 were lost to follow-up. HBV DNA (Roche Diagnostics, Tokyo, Japan) and hepatitis B envelope antigen (Abbott Laboratories) were measured at the time of liver biopsy. During the surveillance period, patients were followed up with abdominal ultrasonography and plasma alpha-fetoprotein determinations every 3 to 6 months. We also made a random choice of 20 chronic hepatitis B specimens with little fibrosis (F1) and little inflammation (A1) from the liver biopsy specimens of the patients showing high plasma HBV DNA levels.

Necroinflammatory activity and fibrotic stage were graded histologically according to the classification of Desmet and colleagues.²¹ We counted and scored pSmad3, HBx, and c-Myc positivity in hepatocytes as follows: 0, no positivity; 1, <25%; 2, 25% to 50%; 3, 50% to 75%; 4, >75%.²⁰ Written informed consent was obtained from each patient according to the Helsinki Declaration. We also obtained approval for this study from our institutional ethics committee.

Reverse-Transcription Polymerase Chain Reaction.

Reverse-transcription polymerase chain reaction of TGF- β type II receptor (T β RII), Smad2, and Smad4 genes was performed as described.¹⁵

Domain-Specific Antibodies Against the Phosphorylated Smad3. Two polyclonal anti-phospho-Smad3 sera— α pSmad3L (Ser 208/213) and α pSmad3C (Ser 423/425)—were raised against the phosphorylated linker and C-terminal regions of Smad3 by immunization of rabbits with synthetic peptides. Relevant antisera were affinity-purified using phosphorylated peptides as described.¹³

Transgenic Animals. HBx transgenic mice were derived by microinjection of a 1151-bp HBV DNA fragment containing the HBx gene with its own regulatory elements and polyadenylation signal into fertilized eggs of CD-1 mice. An independent line (H9) was derived from founders.⁶

Immunohistochemical and Immunofluorescence Analyses. Immunohistochemical and immunofluorescence analyses were performed as described.¹⁸ Primary antibodies (Abs) used in this study included mouse monoclonal anti-HBx Ab (2 μ g/mL; Abcam, Cambridge, UK), mouse monoclonal anti-c-Myc Ab (10 μ g/mL; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-p21^{WAF1} Ab (0.5 μ g/mL; DAKO, Glostrup, Denmark), in addition to the affinity-purified rabbit polyclonal anti-pSmad3L (2 μ g/mL) and anti-pSmad3C (0.5 μ g/mL) as described above. Anti-pSmad3C Ab cross-reacted weakly with C-terminally phosphorylated Smad2: to block binding of anti-

pSmad3C Ab to phosphorylated domains in Smad2, anti-pSmad3C Ab was adsorbed with 1 μ g/mL C-terminally phosphorylated Smad2 peptide.

For immunohistochemical analyses, sections exposed to primary Abs were then incubated with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulin G (IgG) (DAKO). Finally, sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and mounted under coverslips.

For double-labeling immunofluorescence analyses, sections exposed to a pair of primary Abs (rabbit plus mouse) were then incubated in a 1:500 dilution of goat anti-rabbit IgG conjugated with a red fluorophore (Alexa Fluor 594; Molecular Probes, Eugene, OR) and goat anti-mouse IgG conjugated with a green fluorophore (Alexa Fluor 488; Molecular Probes). Images were obtained with a fluorescence microscope (Carl Zeiss Microimaging, Oberkochen, Germany).

Immunoprecipitation and Immunoblotting. pSmad3L and pSmad3C immunoblots on Smad3 immunoprecipitates of cell extracts from frozen tissues representing either HCC or underlying liver diseases were performed as described.²⁰

In Vitro Kinase Assay. *In vitro* kinase assay was performed as described.¹²

Statistical Analyses. The Kaplan-Meier method was used to determine the cumulative probability of appearance of HCC during the 12-year follow-up period. HCC occurrence curves were compared between patients with abundant (scores 3 to 4) and those with sparse (scores 0 to 2) Smad3L/C phosphorylation, by means of the log-rank test. For continuous variables, the optimal cutoff threshold for defining groups was established using receiver operating characteristics curves. All parameters with *P* values less than 0.10 in the univariate analysis were selected for multivariate analysis, which was performed using the Cox proportional hazards model.²² *P* values less than 0.05 were considered significant. The Mann-Whitney U test was used to identify significant differences in hepatocytic pSmad3L and pSmad3C positivity among fibrotic stages.

Results

Two Distinct Hepatocytic Smad3 Signaling Pathways in Human Chronic Hepatitis B: pSmad3L- and pSmad3C-Dominant Types. We initially analyzed mutations of T β RII, Smad2, and Smad4 genes in 10 HCC and six cirrhotic liver samples, finding no mutations in

Table 1. Clinicopathologic Features, Smad3L/C Phosphorylation, and HBx and c-Myc Positivities in Specimens from Patients with HBV-Related Chronic Liver Disease

	Fibrotic Stage*					
	Normal	F1	F2	F3	F4	HCC
Patients, n	2	20	27	23	10	10
Sex (male/female), n	2/0	13/7	19/8	17/6	5/5	10/0
Age (years), mean \pm SD	57.0 \pm 9.9	35.5 \pm 14.3	34.3 \pm 13.9	43.1 \pm 13.7	59.6 \pm 7.6	54.0 \pm 15.1
pSmad3L staining, n [†]						
0	2	0	0	0	0	0
1	0	8	6	2	1	0
2	0	6	6	7	1	0
3	0	3	11	11	2	5
4	0	3	4	3	6	5
pSmad3C staining, n [†]						
0	0	0	0	0	0	0
1	0	0	4	4	2	4
2	0	4	9	12	7	3
3	2	11	7	5	1	3
4	0	5	7	2	0	0
Activity, n*						
A0	2	1	0	0	0	0
A1	0	17	6	1	0	1
A2	0	2	19	11	3	7
A3	0	0	2	11	7	2
HBx staining, n [†]						
0	2	0	0	0	1	1
1	0	6	5	3	2	3
2	0	7	11	8	3	3
3	0	3	7	6	2	2
4	0	4	4	6	2	1
c-Myc staining, n [†]						
0	2	0	0	0	0	0
1	0	2	4	1	1	0
2	0	9	10	8	3	1
3	0	6	8	8	3	3
4	0	3	5	6	3	6
Histology of HCC (well/moderate) [‡]						6/4
TNM stage (I/II/III/IV) [‡]						4/4/2/0
Size of tumor (cm), mean \pm SD						2.2 \pm 0.3
AST (IU/L), mean \pm SD	22.5 \pm 3.5	68.6 \pm 56.1	92.8 \pm 65.8	79.7 \pm 51.8	82.0 \pm 53.1	71.0 \pm 36.4
ALT (IU/L), mean \pm SD	24.0 \pm 2.8	104 \pm 83.5	141 \pm 97.5	84.5 \pm 83.1	68.2 \pm 52.3	59.1 \pm 32.2
Platelet count ($\times 10^9/L$), mean \pm SD	25.0 \pm 4.2	17.1 \pm 3.6	15.8 \pm 4.9	14.1 \pm 7.1	9.7 \pm 6.7	9.0 \pm 3.7
AFP (ng/mL), mean \pm SD	2.1 \pm 1.3	6.8 \pm 4.6	14.8 \pm 12.2	66.2 \pm 138	132 \pm 208	164 \pm 184

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; pSmad3L, linker-phosphorylated Smad3; pSmad3C, C-terminally phosphorylated Smad3; SD, standard deviation; TNM, tumor-node-metastasis.

*Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.

[†]Hepatocytic Smad3 phosphorylation is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation. Extent of HBx and c-Myc expression is indicated as that of pSmad3L positivity.

[‡]Histological grading of HCC is classified according to the criteria of the International Working Party.

[§]TNM is classified by the International Union Against Cancer and American joint Committee on Cancer.

any sample. This confirms the low probability of mutations in HCC tissues, which has been reported recently.²³

To investigate domain-specific phosphorylation mediating Smad3 signaling *in vivo*, we generated two Abs specific to each phosphorylation site, and determined the distribution of pSmad3L and pSmad3C in chronic hepatitis B and C specimens. Table 1 shows clinical background and positivity for pSmad3L and pSmad3C in 90

patients with HBV-related chronic liver diseases. We also studied HCC occurrence over 12 years in 60 patients with chronic hepatitis B who were enrolled in a program for early diagnosis of HCC (Table 2). We recently reported that Smad3 was phosphorylated at the linker region, particularly in groups of hepatocytes adjoining collagen fibers in portal tracts in chronic hepatitis C.²⁰ In contrast, the distribution of pSmad3L and pSmad3C in chronic

Table 2. Clinicopathologic Features, Smad3L/C Phosphorylation, and HCC Incidence in Specimens from Patients with HBV-Related Chronic Hepatitis

Patient No.	Sex	Age	Incidence of HCC	pSmad3L Staining*	pSmad3C Staining*	Fibrotic Stage†	Inflammatory Activity†	HBV DNA (log copies/mL)	HBeAg
1	M	62	○	4	2	3	3	5.4	+
2	F	44	○	4	2	2	2	5.5	-
3	M	22	○	4	2	2	2	5.2	-
4	F	20		4	4	3	3	3.0	-
5	M	43		4	4	2	2	4.5	-
6	M	30		4	2	2	3	4.0	-
7	M	30		4	2	2	3	5.6	-
8	M	65	○	3	2	3	3	4.0	+
9	F	56	○	3	2	3	2	3.7	+
10	M	52	○	3	1	1	1	6.4	-
11	F	40		3	1	1	1	6.9	-
12	M	44		3	1	3	3	5.1	-
13	M	45		3	1	3	3	3.8	-
14	M	28		3	2	3	1	3.0	-
15	M	60		3	2	3	2	2.8	-
16	M	44		3	2	3	3	5.2	+
17	M	44		3	2	3	3	3.2	+
18	M	44		3	2	3	3	4.4	-
19	F	26		3	2	2	1	4.9	-
20	M	20		3	2	2	1	2.9	+
21	M	59		3	2	2	2	4.4	-
22	M	43		3	3	2	2	3.2	+
23	M	29		3	3	3	2	6.2	-
24	M	29		3	3	3	2	3.0	-
25	M	25		3	4	1	1	3.5	-
26	F	33		3	4	2	2	4.6	+
27	M	19		3	3	3	2	5.6	-
28	M	63		3	4	2	2	5.1	-
29	M	52	○	2	1	3	2	3.7	-
30	M	44		2	2	3	3	5.2	-
31	M	29		2	2	3	2	3.9	+
32	F	46		2	4	3	3	3.2	-
33	M	25		2	2	1	2	5.0	-
34	F	23		2	3	1	1	2.1	-
35	F	31		2	3	2	2	3.9	+
36	F	26		2	3	1	1	2.4	-
37	M	35		2	3	1	1	5.6	-
38	M	20		2	3	2	1	3.2	+
39	F	56		2	3	3	2	5.1	+
40	F	36		2	3	3	2	2.6	-
41	F	25		2	3	2	1	5.1	-
42	F	23		2	4	2	1	3.5	-
43	F	41		2	4	1	1	2.0	+
44	M	29		2	4	2	2	4.5	-
45	M	31		2	4	1	1	5.9	+
46	M	42		2	1	2	2	3.7	-
47	M	24		1	1	2	2	3.9	+
48	M	28		1	2	3	2	3.8	-
49	F	11		1	2	2	2	3.0	+
50	M	40		1	1	2	2	3.2	-
51	M	37		1	2	2	2	3.0	-
52	F	10		1	2	2	2	2.3	-
53	M	16		1	3	1	1	5.1	-
54	M	41		1	3	1	1	4.3	-
55	M	40		1	3	1	1	2.2	-
56	M	53		1	3	1	1	2.7	-
57	M	27		1	3	1	1	4.6	-
58	M	53		1	4	1	1	3.3	-
59	M	30		1	4	2	2	2.1	-
60	F	22		1	4	1	0	3.7	-

Abbreviations: F, female; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; M, male; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3.

*Hepatocytic Smad3 phosphorylation is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation.

†Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.

hepatitis B specimens was divided into two distinct patterns. In one liver specimen with moderate fibrosis and inflammation from patient 2 in Table 2 who was diagnosed with HCC 9 years later, intense pSmad3L immunostaining was present in the nuclei of all hepatocytes throughout the liver lobules; C-terminal phosphorylation of Smad3 was strongly suppressed in hepatocytic nuclei (Fig. 1A and Supplementary Fig. 1). In another specimen with similar fibrotic stage and necroinflammatory activity from patient 44 in Table 2 who had not developed HCC, many hepatocytes retained phosphorylation at Smad3C but showed scarce phosphorylation at Smad3L (Fig. 1B). Among 37 patients with chronic hepatitis B who had strong pSmad3L positivity, 24 patients showed little Smad3C phosphorylation, and only 13 patients

Table 3. Correlation Between pSmad3L and pSmad3C in Chronic Hepatitis B Specimens

	pSmad3C Positivity *		Total
	Low (1 and 2)	High (3 and 4)	
pSmad3L positivity *			
Low (1 and 2)	11	22	33
High (3 and 4)	24	13	37
Total	35	35	70

Abbreviations: pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3.

*Hepatocytic Smad3 phosphorylation is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation.

showed abundant Smad3C phosphorylation (64.9% versus 35.1% [$P = 0.03$]) (Table 3). In contrast, 22 patients with little Smad3L phosphorylation (scores 0 to 2) versus only 13 patients with abundant Smad3L phosphorylation (scores 3 to 4) showed strong pSmad3C positivity (62.9% versus 37.1% [$P = 0.04$]). Because the extent of Smad3L phosphorylation increased as fibrotic stage and necroinflammatory activity progressed in chronic hepatitis C, Smad3L showed little phosphorylation in early chronic hepatitis C, Smad3 in hepatocytic nuclei remained high (staining scored as 3 or 4) in 21 of 47 patients with chronic hepatitis B (F1 to F2) (Fig. 1C). These results indicate differential mechanisms of HBV- and HCV-

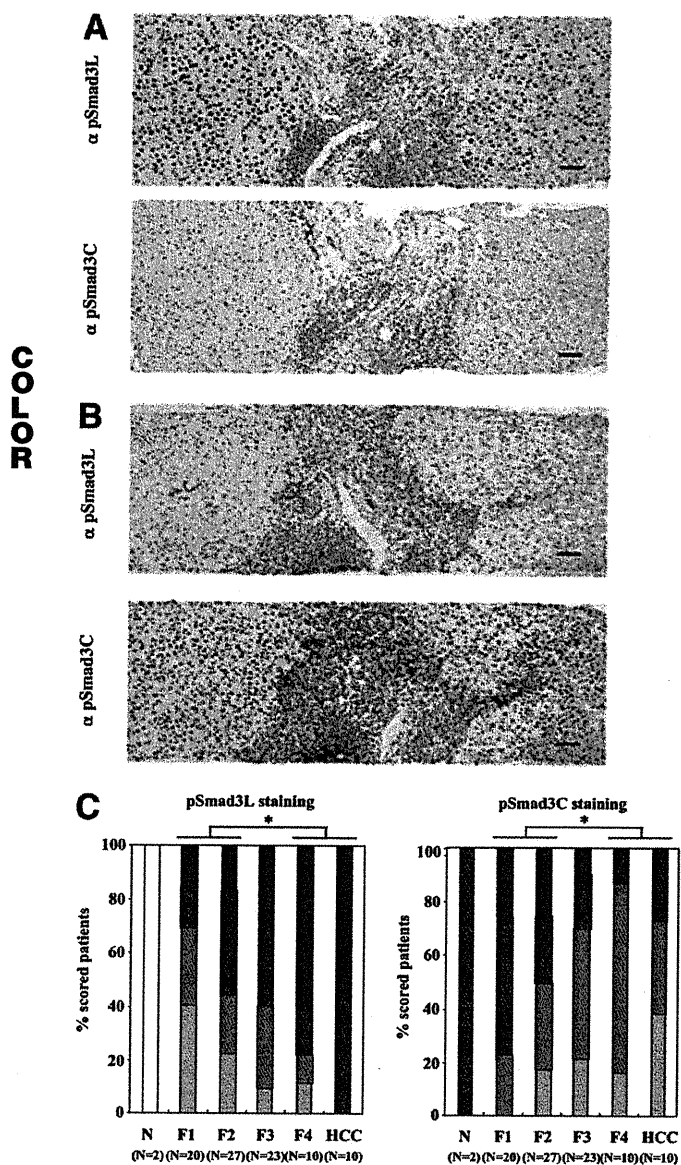
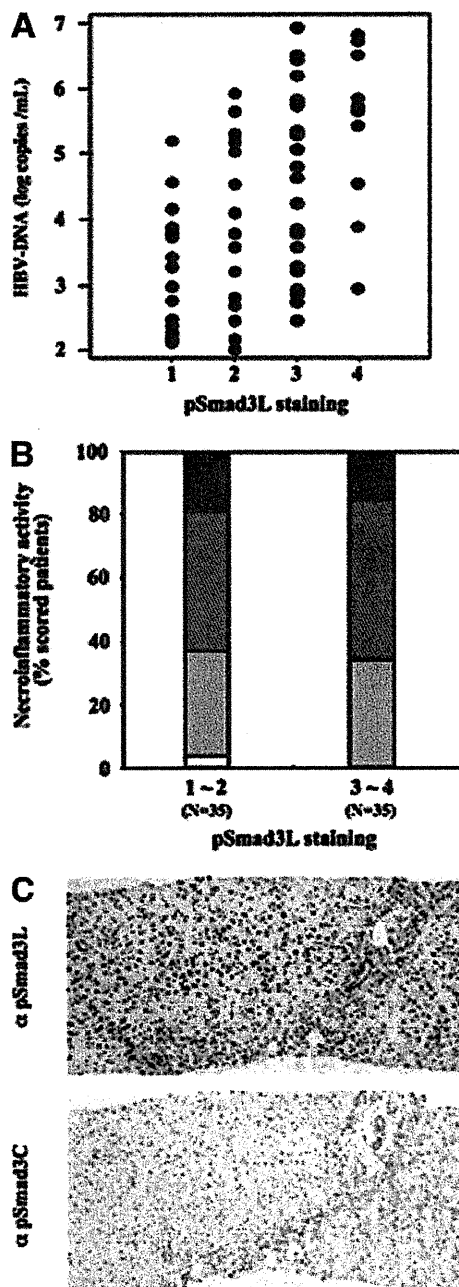


Fig. 1. Two distinct hepatocytic Smad3 signaling pathways in early chronic hepatitis B: pSmad3L- and pSmad3C-dominant types. (A) Smad3 in the nuclei of hepatocytes was phosphorylated sparsely at the C-terminal region (α pSmad3C column) but intensely at the linker region (α pSmad3L column). The liver specimen showing moderate fibrosis and inflammation was obtained from patient 2 in Table 2 diagnosed with HCC 9 years later. Bar = 50 μ m. (B) In patient 44 in Table 2 who had not developed HCC, hepatocytes retained phosphorylation at Smad3C (α pSmad3C column) but showed little phosphorylation at Smad3L (α pSmad3L column). The specimen showed degrees of fibrosis and necroinflammatory activity similar to those in (A). Formalin-fixed, paraffin-embedded liver sections were stained with anti-pSmad3L Ab (α pSmad3L column) and anti-pSmad3C Ab (α pSmad3C column). The pSmad3C section was paired with an adjacent section stained using anti-pSmad3L Ab. Abs were then bound by goat anti-rabbit IgG conjugated with peroxidase-labeled polymer. Peroxidase activity was detected with 3,3'-diaminobenzidine tetrahydrochloride. All sections were counterstained with hematoxylin (blue). Brown color indicates specific Ab reactivity. Bar = 50 μ m. (C) Degrees of Smad3 phosphorylation were stable in hepatocytic nuclei in early chronic hepatitis B specimens (F1 to F2), whereas pSmad3L increased and pSmad3C decreased as chronic hepatitis B (F3) progressed through cirrhosis to HCC. Smad3 phosphorylation in hepatocytes did not change between F1 and F2 stages. Phosphorylation of Smad3L and Smad3C in hepatocytes of cirrhotic liver (F4) and HCC was greater and less than that in livers with grade F1 and F2 fibrosis, respectively. Extent of Smad3 phosphorylation: \square , 0; \square , 1; \square , 2; \square , 3; \blacksquare , 4. * $P < 0.05$.

associated carcinogenesis, especially in the early stages of chronic hepatitis.

pSmad3L Prominence in Hepatocytic Nuclei in Proportion to Plasma HBV DNA Levels. Because HCC risk is related to plasma HBV DNA levels and chronic inflammation,²⁴ we next investigated the correlation of hepatocytic pSmad3L positivity with plasma HBV DNA levels and necroinflammatory activity in chronic hepatitis B patients (Fig. 2). Positivity of hepatocytic nuclei for pSmad3L in chronic hepatitis B specimens gradually increased in proportion to amounts of HBV DNA.



Sixteen sera samples of 35 patients with abundant Smad3L phosphorylation (scores 3 to 4) but only seven sera samples of 35 patients with little Smad3L phosphorylation (scores 0 to 2) contained more than 5.0 log copies/mL (45.7% versus 20.0% [$P = 0.02$]) (Fig. 2A). However, 23 of 35 chronic hepatitis B patients with abundant Smad3L phosphorylation and 22 of 35 patients with little Smad3L phosphorylation showed a high level of inflammatory activity (A 2 to 3) (65.7% versus 62.9% [$P = 0.80$]) (Fig. 2B). These results indicated that HBV itself could up-regulate the hepatocytic phosphorylation at Smad3L, but inflammation could not strongly affect linker phosphorylation.

To further confirm the direct effects of HBV, besides chronic inflammation, on phosphorylation at Smad3L in early chronic hepatitis B, we examined the degrees of pSmad3L and pSmad3C in a group of patients with little fibrosis (F1), little inflammation (A1), and high plasma HBV DNA. Smad3L was highly phosphorylated in hepatocytic nuclei, whereas the phosphorylation at Smad3C was suppressed (Fig. 2C). Of 20 chronic hepatitis B samples, 12 samples showed abundant Smad3L phosphorylation (scores 3 to 4), but only five samples had abundant Smad3C phosphorylation (60.0% versus 25.0% [$P = 0.03$]) (Table 4).

HBx Protein Involvement in c-Myc-Mediated Oncogenic Activity via the pSmad3L Pathway in Human Chronic Hepatitis B. Integrated viral sequences produce HBx protein, which brings about up-regulation of c-Myc oncoprotein.²⁵ We therefore investigated whether HBx protein affected Smad3L phosphorylation and expression of c-Myc in biopsy specimens from HBV-infected livers by immunostaining sections for

Fig. 2. In proportion to plasma HBV DNA levels, JNK-dependent pSmad3L became prominent in the nuclei of hepatocytes in human early chronic hepatitis B. (A) Positivity for pSmad3L in hepatocytic nuclei in chronic hepatitis B specimens was greater in proportion to plasma HBV DNA levels. Patients with strong pSmad3L positivity in hepatocytic nuclei (staining scored as 3 or 4) had more HBV DNA in plasma than patients with weak pSmad3L positivity (staining scored as 0 to 2). Hepatocytic Smad3 phosphorylation in chronic hepatitis B specimens is scored as follows: 0, no phosphorylation; 1, <25%; 2, 25% to 50%; 3, 50% to 75%; 4, >75%. (B) Degree of Smad3 phosphorylation at the linker region did not strongly correlate with necroinflammatory activity of chronic hepatitis B. Hepatocytic Smad3 phosphorylation at the linker region in livers with necroinflammatory activities of A2 to A3 was essentially similar to phosphorylation in those with activities of A0 to A1. Extent of necroinflammatory activity: □, 0; ▤, 1; ▥, 2; ▦, 3. (C) Smad3 in the nuclei of hepatocytes was phosphorylated intensely at linker region (α pSmad3L column) but sparsely at the C-terminal region (α pSmad3C column). The liver specimen showing minimal fibrosis (F1) and inflammation (A1) was obtained from patient 10 in Table 2 who showed high plasma HBV DNA and was diagnosed with HCC 4 years later.

Table 4. Clinicopathologic Features, Smad3L/C Phosphorylation, and Plasma HBV DNA Levels in Specimens from Patients with Early Chronic Hepatitis B

Patient No.	Sex	Age	pSmad3L staining*	pSmad3C staining*	Fibrotic Stage†	Inflammatory Activity†	HBV DNA (log copies/mL)
1	M	46	4	2	1	1	5.6
2	M	45	4	2	1	1	5.4
3	F	38	3	1	1	1	6.2
4	F	49	3	1	1	1	5.2
5	F	52	3	1	1	1	6.1
6	F	40	3	2	1	1	5.8
7	F	28	3	2	1	1	5.6
8	M	38	3	2	1	1	5.5
9	M	44	3	2	1	1	5.3
10	F	40	3	2	1	1	5.4
11	M	55	3	2	1	1	5.1
12	F	43	3	3	1	1	5.8
13	M	34	2	1	1	1	5.2
14	M	28	1	1	1	1	5.2
15	F	35	1	2	1	1	5.1
16	M	30	1	2	1	1	5.3
17	M	45	1	3	1	1	5.2
18	M	38	1	4	1	1	5.6
19	M	54	1	4	1	1	5.2
20	M	48	1	4	1	1	5.4

Abbreviations: F, female; HBV, hepatitis B virus; M, male; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3.

*Hepatocytic Smad3 phosphorylation is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation.

†Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.

pSmad3L, paired with sections immunostained for HBx and c-Myc.

In specimens from patient 3 in Table 2 with chronic hepatitis B, pSmad3L, HBx, and c-Myc were distributed in hepatocytes throughout liver lobules (Fig. 3A and Supplementary Fig. 2). Double immunofluorescence studies in chronic hepatitis B specimens confirmed that pSmad3L was colocalized in HBx- and c-Myc-immunoreactive hepatocytes (Fig. 3B). HBx and c-Myc expression increased in hepatocytes of hepatitis B specimens as Smad3 showed more phosphorylation at the linker region (Fig. 3C).

Increased JNK/pSmad3L/c-Myc Oncogenic Signaling and Impaired pSmad3C/p21^{WAF1} Tumor-Suppressive Signaling as Chronic Hepatitis B Progresses From Cirrhosis to HCC. We further investigated tumor-suppressive and oncogenic Smad3 signaling in biopsy specimens during HBV-related hepatocarcinogenesis by staining sections using anti-pSmad3L Ab and anti-pSmad3C Ab, paired with sections stained for anti-c-Myc Ab and anti-p21^{WAF1} Ab. Although pSmad3L accelerates tumor growth by up-regulating c-Myc, pSmad3C participates in tumor suppression by up-regulating p21^{WAF1} transcription.^{16, 20}

In specimens from a patient with chronic hepatitis B, the distribution of pSmad3L fit well with the pattern shown by c-Myc immunolabeling (Fig. 4A, chronic hep-

atitis panel): both were strong in hepatocytes throughout liver lobules. Linker phosphorylation and c-Myc staining increased further as chronic liver disease progressed through cirrhosis to HCC (Fig. 4A, cirrhosis and HCC panels).

Distribution of pSmad3C resembled the pattern obtained by p21^{WAF1} staining in chronic hepatitis B specimens (Fig. 4B, chronic hepatitis panel). As with pSmad3C distribution, hepatocytes showed increased p21^{WAF1} staining in nuclei. In contrast to intense staining for pSmad3L and c-Myc, pSmad3C and p21^{WAF1} staining decreased in hepatocytic nuclei in cirrhotic liver (Fig. 4B, cirrhosis panel). Nuclear pSmad3C and p21^{WAF1} immunostaining showed only a scattered distribution throughout HCC specimens (Fig. 4B, HCC panel). Semiquantitative analyses of positivity for pSmad3L, pSmad3C, and c-Myc in HBV-related chronic liver disease showed increasing pSmad3L/c-Myc and decreasing pSmad3C as chronic hepatitis B progressed from cirrhosis (F4) to HCC (Table 1).

We next quantified the extent of phosphorylation at Smad3L and Smad3C by immunoblotting with domain-specific Abs against phosphorylated Smad3 in tissue samples representing various stages of HBV-related chronic liver disorders. The linker region of Smad3 showed very little phosphorylation in normal liver (Fig. 4C, α pSmad3L panel). Remarkable up-

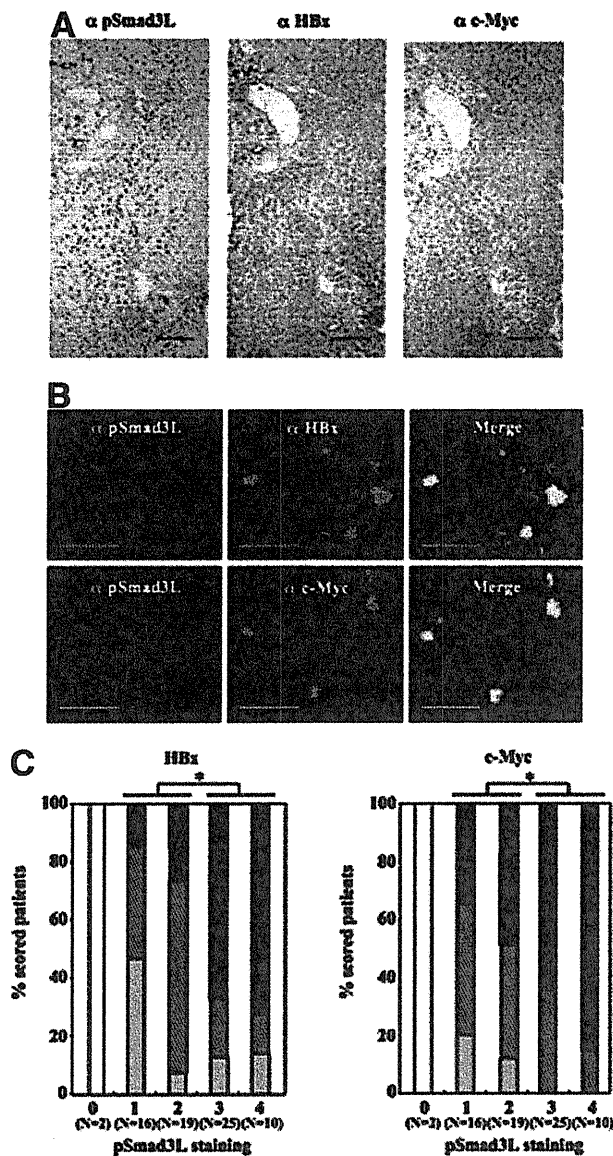


Fig. 3. HBx might be involved in c-Myc-mediated oncogenic activity in human chronic hepatitis B via the pSmad3L pathway. (A) Hepatocytes of chronic hepatitis B specimens from patient 3 in Table 2 showed diffuse immunostaining for pSmad3L, HBx, and c-Myc. All sections were counterstained with hematoxylin (blue). Brown color indicates specific Ab reactivity. Bar = 50 μ m. (B) pSmad3L in hepatocytic nuclei of chronic hepatitis B specimens was colocalized with HBx and c-Myc proteins. Sections of chronic hepatitis B tissues were stained for immunofluorescence to simultaneously detect pSmad3L (red) and HBx or c-Myc (green). Yellow color indicates overlap of proteins. Hepatocytes immunoreactive for pSmad3L showed colocalization of HBx (upper column) and c-Myc (lower column). Bar = 50 μ m. (C) HBx and c-Myc expression increased in hepatocytes of chronic hepatitis B specimens as Smad3 was increasingly phosphorylated at the linker region. HBx and c-Myc expression was greater in hepatocytes with high phosphorylation at Smad3L (staining scored as 3 or 4) than in hepatocytes with staining scored as 0 to 2. The extent of HBx and c-Myc expression is indicated as that of pSmad3L positivity: \square , 0; \square , 1; \square , 2; \square , 3; \blacksquare , 4. * $P < 0.05$.

regulation of pSmad3L was seen with progression of hepatic fibrosis and carcinogenesis. In cirrhotic liver and HCC, pSmad3L was far more abundant than in chronic hepatitis. In contrast, pSmad3C gradually decreased as disease stages progressed toward HCC (Fig. 4C, α pSmad3C panel).

We previously reported that Smad3L served as a substrate for JNK.¹² To address the functional relationship between activated JNK and Smad3L phosphorylation during hepatocarcinogenesis, we presently assayed kinase activity *in vitro*. Although JNK from normal liver showed little ability to phosphorylate Smad3 at the linker region, JNK from livers involved by chronic hepatitis B, cirrhosis, and HCC could directly phosphorylate Smad3L (Fig. 4D). These results suggested that JNK in preneoplastic liver tissues and HCC directly phosphorylated the linker region of Smad3.

Collectively, JNK/pSmad3L/c-Myc oncogenic signaling in hepatocytes came to predominate while the tumor-suppressive pSmad3C/p21^{WAF1} pathway became quiescent as chronic hepatitis B progressed to cirrhosis and then HCC.

Selective Blockade of Linker Phosphorylation Abolishes pSmad3L-Mediated Cell Growth in HBx-Expressing Hepatocytes. pSmad3L, HBx, and c-Myc were colocalized in preneoplastic lesions including chronic hepatitis and cirrhosis (Fig. 3). These findings suggest that HBx oncoprotein might alter hepatocytic TGF- β signaling in chronic hepatitis B. We investigated this hypothesis using HBx-expressing hepatocytes. Selective blockade of linker phosphorylation by a mutant Smad3 lacking the JNK-dependent linker phosphorylation sites abolished pSmad3L-mediated cell growth in HBx-expressing hepatocytes (Supplementary Figs. 3-5). These results suggest that HBx activated the JNK/pSmad3L pathway, further promoting cell proliferation by up-regulating c-Myc transcription (Fig. 5).

Activation of the pSmad3L/c-Myc Pathway as HBx Transgenic Mouse Livers Progress Through Hyperplasia to HCC. We further investigated localization of pSmad3L, HBx, and c-Myc during HBx-induced hepatocarcinogenesis in HBx transgenic mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding the central veins where bromodeoxyuridine was uptaken into the hepatocytes.⁶

In this hyperplastic mouse liver, phosphorylation at Smad3L was observed in hepatocytic nuclei in the centrilobular region, and distribution of pSmad3L was similar to those of HBx and c-Myc (Fig. 6A). pSmad3L, HBx,

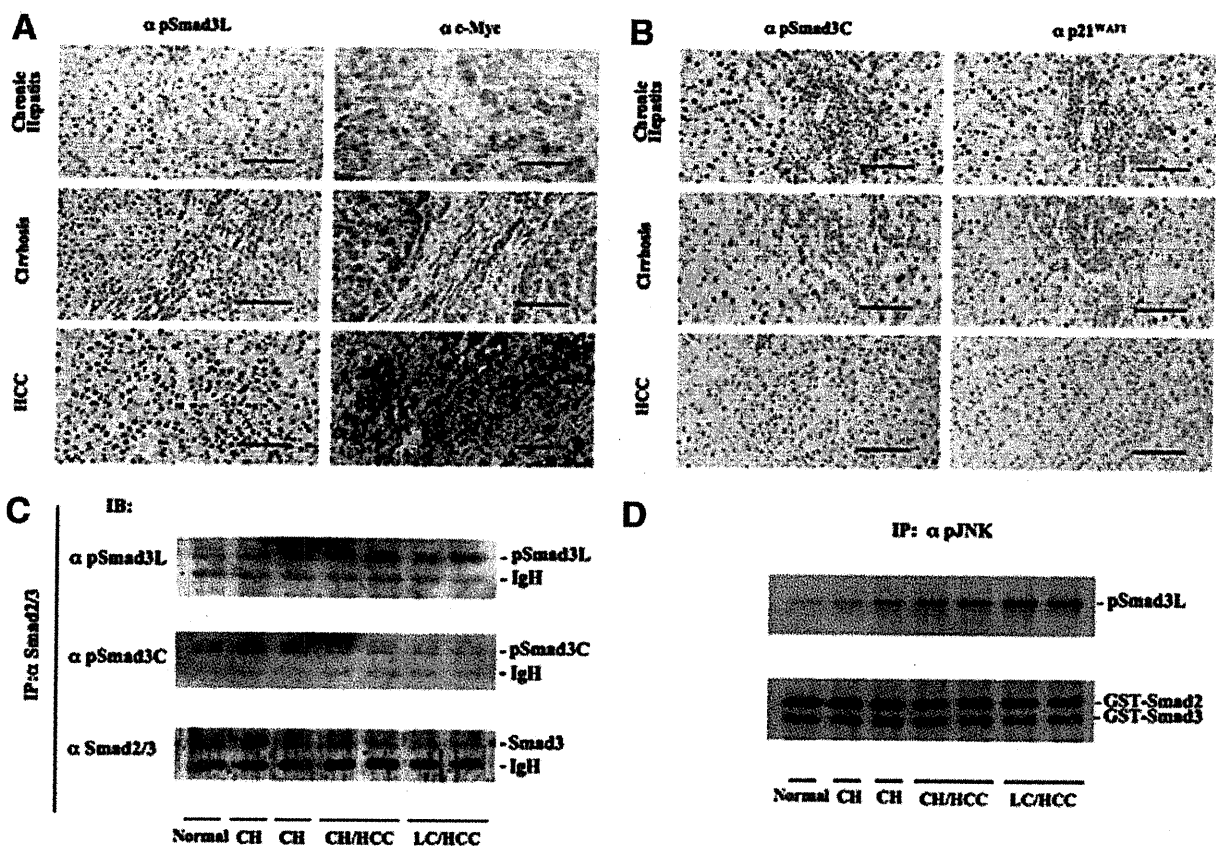


Fig. 4. As chronic hepatitis B progressed through cirrhosis to HCC, JNK/pSmad3L/c-Myc oncogenic signaling started to increase, whereas the tumor-suppressive pSmad3C/p21^{WAF1} pathway decreased. (A) pSmad3L and c-Myc increased as human chronic hepatitis B progressed through cirrhosis to HCC. (B) pSmad3C and p21^{WAF1} decreased as human chronic hepatitis B progressed through cirrhosis to HCC. All sections in A and B were counterstained with hematoxylin (blue). Brown staining indicates specific Ab reactivity. Bar = 50 μ m. (C) Immunoblotting of pSmad3L and pSmad3C in HBV-related chronic liver diseases. Cell lysates obtained from hepatocellular carcinoma (HCC) and surrounding nonneoplastic liver tissues including chronic hepatitis B (CH) or liver cirrhosis (LC) as well as uninvolved normal liver tissues from a patient with a metastatic liver tumor were subjected to anti-Smad3 immunoprecipitation (IP) and were then immunoblotted with each anti-pSmad3 Ab (upper panels). Relative amounts of endogenous Smad3 were determined via immunoblotting using anti-Smad3 Ab (bottom panel). (D) JNK in human HBV-related chronic liver tissue directly phosphorylated Smad3 at the linker region. Cell lysates obtained from HCC and surrounding nonneoplastic liver tissue including chronic hepatitis (CH) and liver cirrhosis (LC) from HBV-infected patients, as well as uninvolved normal liver tissue from a patient with a liver metastasis, were subjected to anti-phospho-JNK1/2 immunoprecipitation (IP), and were then mixed with bacterially expressed GST-Smad3 and GST-Smad2. After *in vitro* kinase assay, phosphorylation of Smad3 was analyzed via immunoblotting using anti-pSmad3L antibody (upper panel). Total Smad3 and Smad2 were determined via immunoblotting using anti-Smad2/3 Ab (lower panel).

and c-Myc were distributed diffusely in HCC specimens (Fig. 6B, HCC panel). Semiquantitative analyses of positivity for these molecules in HBx transgenic mouse livers also revealed that hepatocytic pSmad3L, HBx, and c-Myc increased as mouse liver progressed through hyperplasia to HCC (Fig. 6C). Double immunofluorescence studies in hyperplastic specimens confirmed that pSmad3L was colocalized in HBx- and c-Myc-immunoreactive hepatocytes (Fig. 6D).

Success in comparative study of HBx, pSmad3L, and c-Myc positivity during human and mouse hepatocarcinogenesis identified pSmad3L as a key regulatory element

that offers a general framework for understanding the origins of HBV-related HCC.

Chronic Hepatitis B Patients with Hepatocytes Positive for pSmad3L and Negative for pSmad3C Increased Risk of HCC Development. We finally investigated whether phosphorylation levels of Smad3 could affect the risk of neoplastic evolution in the patients with chronic hepatitis B (Table 2). To compare HCC incidence, patients were classified into those with abundant (scores 3 to 4) and limited (scores 0 to 2) Smad3 phosphorylation in hepatocytic nuclei. HCC developed in six of 28 patients with abundant Smad3L phosphory-

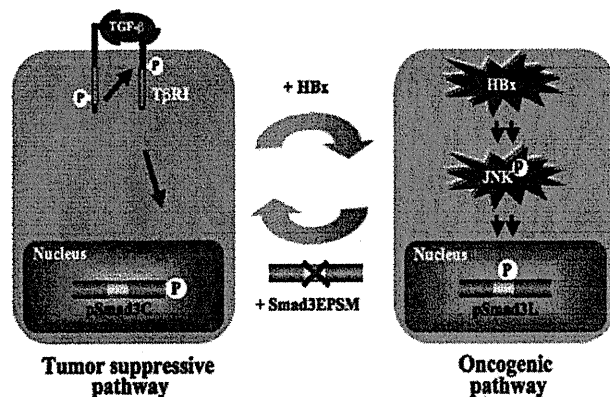


Fig. 5. Reversibility of Smad3-dependent signaling between tumor suppression and oncogenesis in HBx-expressing hepatocytes. Hepatocytes exhibit TGF- β -dependent Smad3 phosphorylation at the C-terminal region, which results in growth inhibition by repression of c-Myc. High expression of HBx protein in hepatocytes tends to shut down pSmad3C-mediated signaling and favor acquisition of constitutively active JNK-mediated pSmad3L signaling, which fosters cell growth by up-regulating c-Myc. Selective blockade of linker phosphorylation by a mutant Smad3 lacking the JNK-dependent linker phosphorylation sites (Smad3EPSM) restores the TGF- β -dependent tumor-suppressive response involving pSmad3C that is shown by parental hepatocytes.

lation, but in only one of 32 patients with limited Smad3L phosphorylation (log-rank = 0.03) (Fig. 7A). In contrast, HCC developed only in the patients with limited Smad3C phosphorylation, and no patients with abundant hepatocytic pSmad3C developed HCC (log-rank = 0.009) (Fig. 7B).

Several studies have analyzed risk factors for HCC occurrence in patients with HBV-related chronic liver disease, including elevated plasma HBV DNA²⁴ and seropositivity for hepatitis B e antigen.²⁶ In the univariate analysis, HCC occurrence in high pSmad3L positivity ($P = 0.01$), low pSmad3C positivity ($P = 0.03$), and plasma HBV-DNA levels of more than 5.0 log copies/mL ($P = 0.05$) showed P values less than 0.10, thus being significantly associated with HCC (Table 5). All variables with statistical significance in the univariate analysis were entered in the multivariate analysis, and high pSmad3L and low pSmad3C positivity were considered significantly predictive of HCC development within 12 years. Hepatocytic positivity for pSmad3L and pSmad3C should allow us to distinguish chronic hepatitis B patients at high and low risk for the development of HCC in near future.

Discussion

In patients with chronic hepatitis B, persistent HBV infection is clearly the primary inducer of HCC.¹⁻⁷ Com-

parative studies that seek to identify conserved oncogenic signaling common to HCC in both humans and experimental animals will help to eventually identify the molecular pathways that drive the development of HCC.²⁷ Much is known about the morphologic changes of cells and tissues that precede and accompany development of HCC in humans, allowing earlier diagnosis in some instances.²⁸ A variety of molecular alterations have been detected in fully developed HCC and to a lesser extent in morphologically defined preneoplastic precursor lesions.²⁹ Our current studies compared pSmad3L- and pSmad3C-mediated signaling in biopsy specimens of chronic hepatitis, cirrhosis, or HCC from 90 patients with chronic HBV infection versus signaling in preneoplastic and neoplastic liver lesions of HBx transgenic mice. Taken together with the results of *in vitro* experiments using HBx-expressing hepatocytes, our findings indicate that the HBx oncoprotein participates directly in hepatocarcinogenesis by shifting hepatocytic Smad3-mediated signaling from tumor suppression to oncogenesis in patients with early chronic hepatitis B (Fig. 7C). According to the two-step model of carcinogenesis (initiation and promotion), tumor formation can be explained by permanent HBx-dependent activation of the JNK/pSmad3L cascade that has a tumor promoter-like action.

HCC is a human neoplasm associated with viral infection.^{1,3} At present, hepatitis virus-associated carcinogenesis can be seen as a multifactorial process that includes both direct and indirect mechanisms.¹⁹ A major factor in the process of HCC development is the host immune system.³⁰ Chronic inflammation, degeneration, and regeneration induced by the host cellular immune response are common to a variety of human liver diseases, and subsequent cellular proliferation might increase the risk of cancer. We previously reported that increased phosphorylation of Smad3L and decreased phosphorylation of Smad3C were associated with an increased risk of HCV-related HCC.²⁰ Similarly to HCV-related chronic liver disease, strong pSmad3L positivity was observed in the late stages of HBV-related chronic liver disease (F3 to F4) (Fig. 1C). Considering the development of HCC in HCV core gene-transgenic mice,³¹ hepatitis viruses themselves together with the host immune response might promote human hepatocarcinogenesis via the JNK/pSmad3L pathway during the late stage of the carcinogenic process in both HBV- and HCV- related chronic liver disease.

However, HBV and HCV have different roles in human hepatocarcinogenesis when early chronic hepatitis (F1 to F2) is considered. The histological severity of HCV-related liver disease correlates closely with the risk

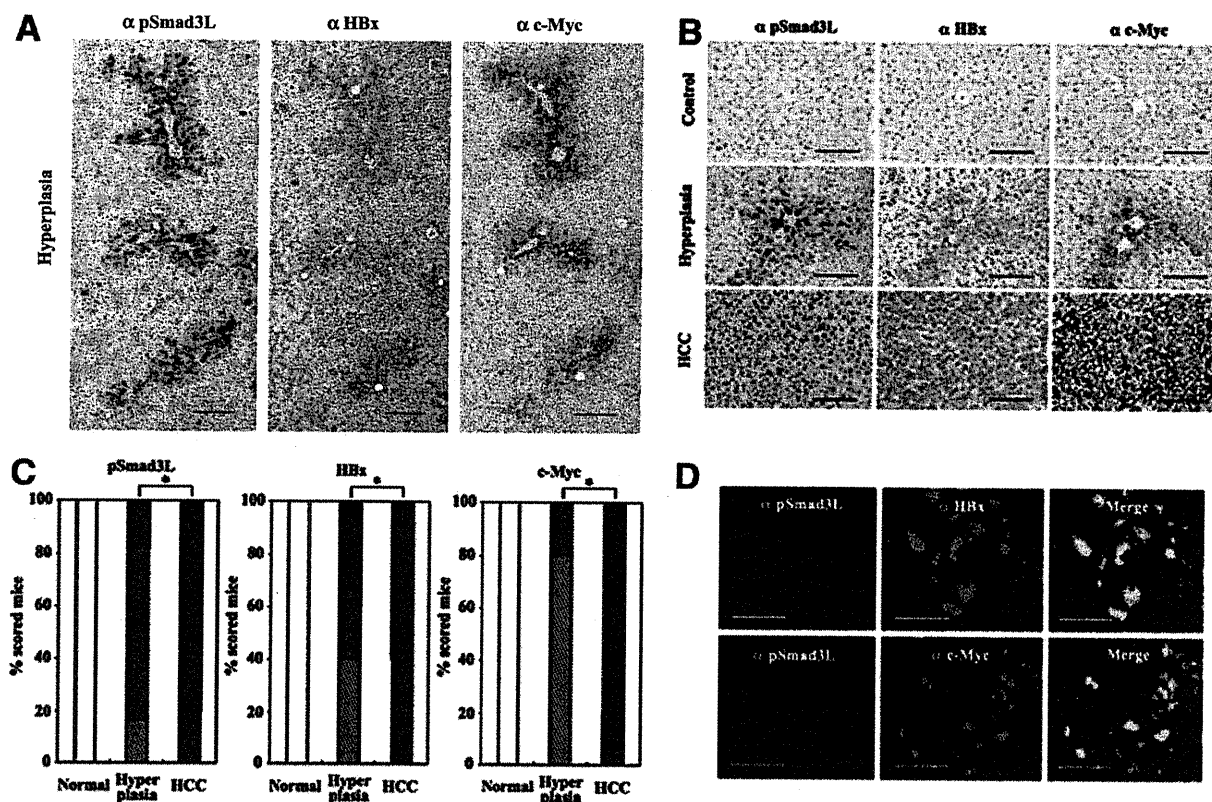


Fig. 6. The pSmad3L/c-Myc pathway was activated as HBx transgenic mouse liver progressed through hyperplasia to HCC. (A) Distribution of pSmad3L, HBx, and c-Myc in hyperplastic specimens from HBx transgenic mouse liver. (B) Distributions of pSmad3L, HBx, and c-Myc in normal liver, hyperplasia, and HCC specimens from HBx transgenic mice. Immunostaining for pSmad3L, HBx, and c-Myc was present in hyperplastic hepatocytes surrounding central veins in HBx transgenic mouse liver [(A) and (B), hyperplasia panels], and was distributed diffusely in HCC specimens [(B), HCC panel]. All sections were counterstained with hematoxylin (blue). Brown color indicates specific Ab reactivity. Bar = 50 μ m. (C) Hepatocytic pSmad3L, HBx, and c-Myc increased as HBx transgenic mouse liver progressed from hyperplasia to HCC. Staining for pSmad3L, HBx, and c-Myc was detected minimally in normal mouse livers, but was strongly up-regulated in neoplastic livers. In HCC, pSmad3L, HBx, and c-Myc were significantly greater than in livers with hyperplasia. * $P < 0.05$. Extent of pSmad3L, HBx, and c-Myc: □, 0; ▤, 1; ▥, 2; ▦, 3; ▧, 4. (D) Hepatocytic pSmad3L in hyperplastic specimens from HBx transgenic mouse liver was colocalized with HBx and c-Myc. Hyperplasia sections of HBx transgenic mouse livers were stained for immunofluorescence to simultaneously detect pSmad3L (red) and HBx or c-Myc (green). Yellow color indicates overlap of proteins. Hepatocytes immunoreactive for pSmad3L showed colocalization of HBx (upper column) and c-Myc (lower column). Bar = 50 μ m.

of HCC.³² In contrast, HCC occasionally develops in healthy HBV surface antigen carriers, who are persistently infected with HBV but have normal liver function parameters and no necroinflammation.³³ This indicates that HBV itself has a direct influence on hepatocarcinogenesis in early chronic hepatitis B. Although integration of the viral genome into chromosomal DNA has not been reported in patients with HCV infection, integration of HBV has been detected in almost all cases of chronic hepatitis B,³ leading to activation of the HBx-mediated oncogenic pathway.⁴ It is noteworthy that HCC developed in patient 10 (Table 2), who showed strong pSmad3L positivity of hepatocytic nuclei but had minimal necroinflammatory activity (A1) or fibrosis (F1). In summary, HCV contributes indirectly to the development of HCC through chronic inflammation in early

chronic hepatitis C. In contrast, HBV directly triggers the JNK/pSmad3L oncogenic pathway in early chronic hepatitis B, thus playing a role beyond mere stimulation of the host immune response.

Our findings also open up a new avenue to understanding the development and progression of hepatic fibrogenesis.³⁴ Whereas HSCs have traditionally been considered as the principal source of liver fibrosis, mature hepatocytes can acquire a mesenchymal phenotype and perform the functions of activated HSC—that is, they can contribute to fibrogenesis.^{35,36} In support of this notion, pSmad3L-mediated signaling promotes liver fibrosis by hepatocytes as well as activated HSCs during long-standing carcinogenesis.^{13,18,20} In this manner, either HBV- or HCV-related chronic hepatitis progresses through fibrogenesis to HCC.

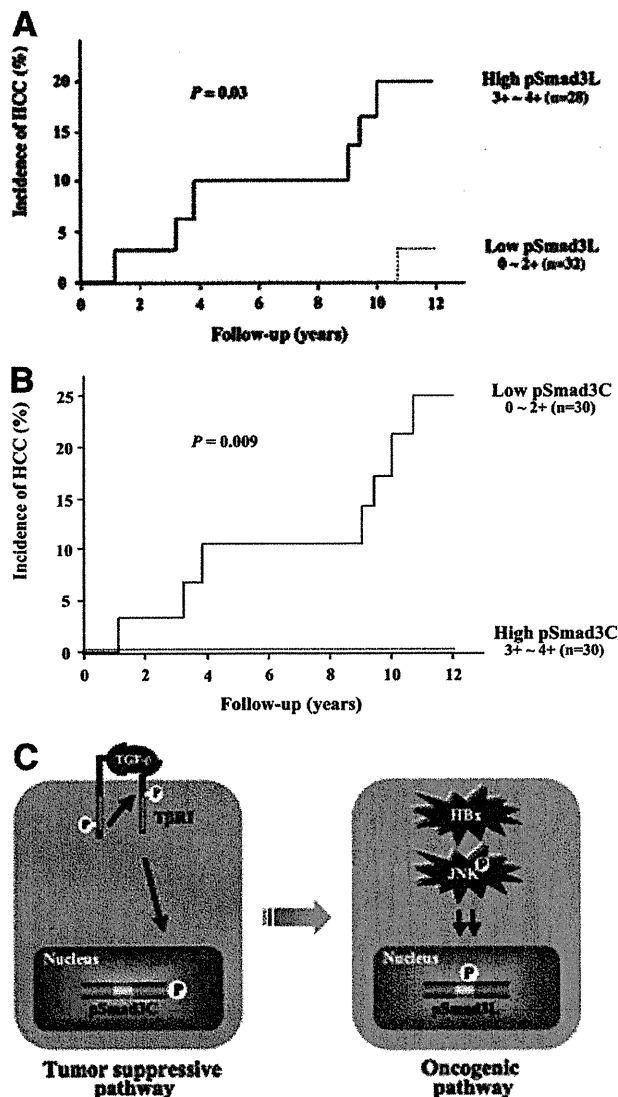


Fig. 7. Chronic hepatitis B patients with hepatocytes positive for pSmad3L and negative for pSmad3C increased risk of HCC development. (A) HCC occurred subsequently among patients whose hepatocytes in chronic hepatitis B specimens were strongly positive for pSmad3L. Incidence of HCC was significantly higher in patients with abundant Smad3L phosphorylation (scores 3 to 4, solid line) in hepatocytic nuclei versus those with sparse Smad3L phosphorylation (scores 0 to 2, dotted line). (B) HCC did not occur subsequently among patients whose hepatocytes in chronic hepatitis B specimens were strongly positive for pSmad3C. HCC occurred only in patients with sparse Smad3C phosphorylation (scores 0 to 2, solid line) in hepatocytic nuclei, while no patients with abundant Smad3C phosphorylation (scores 3 to 4, dotted line) have developed HCC. Cumulative rates of HCC occurrence from chronic hepatitis B were compared between cases with high and low phosphorylation of Smad3L and Smad3C (Kaplan-Meier analysis and log-rank test). (C) HBx protein shifted hepatic TGF- β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic JNK-dependent pSmad3L pathway in early stages of chronic hepatitis B. Normal hepatocytes exhibited TGF- β -dependent Smad3 phosphorylation at the C-terminal region, which is related to growth inhibition by up-regulation of p21^{WAF1}. HBx protein activates JNK, promoting the oncogenic pSmad3L signaling, which fosters cell growth by up-regulating c-Myc, in a mean time reducing tumor-suppressive pSmad3C-mediated signaling.

The general biomedical approach to HCC is shifting away from population risk assessment and empirical treatment of patients to predictive personalized medicine based on molecular classification and targeted therapy.²⁹ Better knowledge of the risk factors associated with the occurrence of HCC can improve the effectiveness of surveillance programs. Our approach has identified pSmad3L and pSmad3C as prognostic markers that may prove to be clinically useful. Such predictive markers could allow us to select patients with chronic hepatitis B who have a high or low risk of developing HCC. Although the latter group could be followed up on an annual basis, the patients with a high risk require targeted surveillance measures to allow early diagnosis of HCC.

Phosphorylation of many transcription factors is controlled by the dynamic interplay between kinases and phosphatases. In this regard, we studied the kinetics of both linker and C-terminal phosphorylation of Smad3 in parental and HBx-expressing hepatocytes in response to TGF- β (unpublished observation). In parental hepatocytes, the levels of linker and C-terminal phosphorylation peaked at 30 minutes after the start of exposure to TGF- β and then gradually declined. However, HBx-expressing hepatocytes showed constitutive phosphorylation at Smad3L during continuous exposure to TGF- β . Several lines of evidence have identified small C-terminal domain phosphatase (SCP1-3) and protein phosphatase magnesium 1A (PPM1A) as the linker and C-terminal phosphatases, respectively.^{37,38} Accordingly, SCP1-3 and PPM1A may reverse domain-specific phosphorylation in normal hepatocytes. In contrast, HBx-expressing hepatocytes may not show induction or activation of SCP1-3. Alternatively, linker phosphorylation in HBx-expressing hepatocytes might be resistant to SCP1-3.

Many researchers have been seeking key transcription factors regulating tumor-suppressive pathways that are altered in cancer. Our current model of JNK/pSmad3L signaling during HBV-related chronic liver disease suggests that specific inhibitors of the JNK/pSmad3L pathway might inhibit the progression of HCC. With respect to molecular targeting therapy for human HCC, pSmad3L and pSmad3C should be assessed as biomarkers to evaluate the benefit from specific inhibition of the JNK/pSmad3L pathway.

Acknowledgment: We thank Dr. Rik Derynck (University of California at San Francisco) and Dr. Seishi Murakami (Kanazawa University) for providing us with complementary DNAs encoding human Smad3 and HBx. We also thank Chiaki Kitano for assistance to construct ecotropic retrovirus and Natsuko Ohira for assistance with immunoblotting.

Table 5. Variables with Independent Predictive Value for HCC in Univariate and Multivariate Analyses

Characteristics	n	No. of Patients with HCC (%)	Univariate Analysis		Multivariate Analysis	
			Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
pSmad3L positivity*						
Low (1 and 2)	32	1 (3)	1.00		1.00	
High (3 and 4)	28	6 (21)	3.8 (1.4-10.6)	0.01	14.8 (1.8-118.5)	0.01
pSmad3C positivity*						
High (3 and 4)	30	0 (0)	1.00		1.00	
Low (1 and 2)	30	7 (23)	2.8 (0.001-7.0)	0.03	16.4 (1.0-125.0)	0.04
Fibrotic stage†						
Low (F1 and F2)	39	4 (10)	1.00		1.00	
High (F3)	21	3 (14)	1.9 (0.7-5.4)	0.24	3.9 (0.4-38.6)	0.24
Inflammatory activity†						
Low (A0 and A1)	23	1 (4)	1.00		1.00	
High (A2 and A3)	37	6 (16)	1.8 (0.7-4.8)	0.27	0.2 (0.02-1.1)	0.06
HBV DNA (copies /mL)						
<10 ⁵	42	3 (7)	1.00		1.00	
>10 ⁵	18	4 (22)	1.9 (1.0-3.5)	0.05	2.5 (0.9-6.9)	0.08
HBeAg						
Negative	42	4 (10)	1.00		1.00	
Positive	18	3 (17)	2.1 (0.5-9.5)	0.32	9.9 (1.1-89.3)	0.03

Abbreviations: CI, confidence interval; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3.

*Hepatocytic Smad3 phosphorylation in chronic hepatitis B specimens is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation.

†Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.

References

- Llover JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; 362:1907-1917.
- El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004;127(Suppl):27S-34S.
- Brechot 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;286:533-535.
- Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 1991;351:317-320.
- Yu DY, Moon HB, Son JK, Jeong S, Yu SL, Yoon H, et al. Incidence of hepatocellular carcinoma in transgenic mice expressing the hepatitis B virus X-protein. *J Hepatol* 1999;31:123-132.
- Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *HEPATOLOGY* 1994;19:810-819.
- Benn J, Su F, Doria M, Schneider RJ. Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-related and c-Jun N-terminal mitogen-activated protein kinases. *J Virol* 1996;70:4978-4985.
- Roberts AB, Sporn MB. The transforming growth factor- β s. In: Sporn MB, Roberts AB, eds. *Peptide Growth Factors and Their Receptors*. Berlin: Springer-Verlag, 1990:419-472.
- Heldin CH, Miyazono K, ten Dijke P. TGF- β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390:465-471.
- Massagué J. TGF- β signal transduction. *Annu Rev Biochem* 1998;67:753-791.
- Kretschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGF- β /Smad signaling by oncogenic Ras. *Genes Dev* 1999; 13:804-816.
- Mori S, Matsuzaki K, Yoshida K, Furukawa F, Tahashi Y, Yamagata H, et al. TGF- β and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. *Oncogene* 2004;23:7416-7429.
- Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, et al. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *HEPATOLOGY* 2003;38:879-889.
- Matsuura I, Denisova NG, Wang G, He D, Long J, Liu F. Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 2004;430:226-231.
- Yamagata H, Matsuzaki K, Mori S, Yoshida K, Tahashi Y, Furukawa F, et al. Acceleration of Smad2 and Smad3 phosphorylation via c-Jun NH(2)-terminal kinase during human colorectal carcinogenesis. *Cancer Res* 2005; 65:157-165.
- Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, Seki T, et al. Reversible Smad-dependent signaling between tumor suppression and oncogenesis. *Cancer Res* 2007;67:5090-5096.
- Arany PR, Rane SG, Roberts AB. Smad3 deficiency inhibits v-ras-induced transformation by suppression of JNK MAPK signaling and increased farnesyl transferase inhibition. *Oncogene* 2008;27:2507-2512.
- Yoshida K, Matsuzaki K, Mori S, Tahashi Y, Yamagata H, Furukawa F, et al. 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;166:1029-1039.
- Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene* 2003;22:5093-5107.
- Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, et al. Chronic inflammation associated with hepatitis C viral infection perturbs hepatic TGF- β signaling, promoting cirrhosis and hepatocellular carcinoma. *HEPATOLOGY* 2007;46:48-57.
- Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *HEPATOLOGY* 1994;19:1513-1520.
- Cox DR. Regression models and life-tables. *J R Stat Soc (B)* 1972;34:187-220.
- Pardali K, Moustakas A. Actions of TGF- β as tumor suppressor and prometastatic factor in human cancer. *Biochim Biophys Acta* 2007;1775:21-62.

24. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. REVEAL-HBV study group. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006;295:65-73.
25. Feitelson MA. c-Myc overexpression in hepatocarcinogenesis. *Human Pathology* 2004;35:1299-1302.
26. Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, et al. Taiwan community-based cancer screening project group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* 2002;347:168-174.
27. Thorgeirsson SS, Lee JS, Grisham JW. Functional genomics of hepatocellular carcinoma. *HEPATOLOGY* 2006;43:145-150.
28. Theise ND, Park YN, Kojiro M. Dysplastic nodules and hepatocarcinogenesis. *Clin Liver Dis* 2002;6:497-512.
29. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002;31:339-346.
30. Chisari FV, Klopchin K, Moriyama T, Pasquinelli C, Dunsford HA, Sell S, et al. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell* 1989;59:1145-1156.
31. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065-1067.
32. Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. *HEPATOLOGY* 1997;26 (Suppl):34S-38S.
33. Popper H, Shafritz DA, Hoofnagle JH. Relation of the hepatitis B virus carrier state to hepatocellular carcinoma. *HEPATOLOGY* 1987;7:764-772.
34. Inagaki Y, Okazaki I. Emerging insights into transforming growth factor β Smad signal in hepatic fibrogenesis. *Gut* 2007;56:284-292.
35. Kaimori A, Potter J, Kaimori JY, Wang C, Mezey E, Koteish A. Transforming growth factor- β 1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro. *J Biol Chem* 2007;282:22089-22101.
36. Weng HL, Ciuculan L, Liu Y, Hamzavi J, Godoy P, Gaitantzi H, et al. Profibrogenic transforming growth factor- β /activin receptor-like kinase 5 signaling via connective tissue growth factor expression in hepatocytes. *HEPATOLOGY* 2007;46:1257-1270.
37. Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, et al. PPM1A functions as a Smad phosphatase to terminate TGF β signaling. *Cell* 2006;125:915-928.
38. Wrighton KH, Willis D, Long J, Liu F, Lin X, Feng XH. Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor- β signaling. *J Biol Chem* 2006;281:38365-38375.

Expression of α -taxilin in hepatocellular carcinoma correlates with growth activity and malignant potential of the tumor

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DOI: 10.3892/ijo_XXXXXXX

Abstract. The membrane traffic system has been recognized to be involved in carcinogenesis and tumor progression in several types of tumors. α -taxilin is a newly identified membrane traffic-related molecule, and its up-regulation has been reported in embryonic and malignant tissues of neural origin. In the present study, we analyzed the expression of α -taxilin in relation to clinicopathological features of hepatocellular carcinomas (HCC) and proliferative activity of the tumor determined by proliferating cell nuclear antigen labeling index (PCNA-LI). Twenty-nine surgically resected nodules of HCC (8 well-, 11 moderately-, and 10 poorly-differentiated) were studied. Fifteen cases showed 'strong staining', while 14 cases showed 'weak staining' for α -taxilin. A significantly higher expression of α -taxilin was observed in less-differentiated ($p=0.005$), and more invasive ($p=0.016$) HCCs. The 'strong staining' group showed significantly higher PCNA-LI than the 'weak staining' group (the medians of PCNA-LI were 59.4% vs. 14.4%, $p<0.0001$). We also evaluated the expression of α -taxilin in hepatoma cell lines (PLC/PRF/5, Hep G2 and HuH-6) in association with cell proliferation. The expression levels of α -taxilin protein were correlated with their growth rates. In conclusion, the expression of the α -taxilin protein was related with an increased proliferative activity and a less-differentiated histological grade of HCC. α -taxilin may be involved in cell proliferation of HCC, and its expression can be a marker of malignant potential of HCC.

Introduction

The molecular mechanisms of carcinogenesis and tumor progression have been extensively investigated to find novel targets for anti-tumor therapy as well as useful predictors of tumor growth and biological aggressiveness. Involvement of intracellular signaling pathways, cell cycle regulators, growth factors and angiogenic factors has been shown in a variety of carcinomas (1,2). Furthermore, recent studies have revealed that membrane traffic-related molecules play a role in the processes of carcinogenesis and tumor progression in several types of tumors (3-5).

Membrane traffic is a fundamental intracellular transport system in eukaryotic cells (6). Small transport vesicles bud from membranes of a donor compartment, and subsequently fuse with membranes of a target compartment. Cargo molecules in the vesicles as well as biomembranes are dynamically exchanged between organelles with temporal and spatial selectivity. These processes are essential for multiple cellular functions such as endo- and exocytosis, maintenance of organelle homeostasis and cell growth, division, and motility. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), located on vesicles and the target membranes, are the central coordinators of membrane traffic (7), and syntaxin family proteins are their main components.

Recently, taxilin was identified as a novel binding partner of syntaxins (8). α -taxilin, one of the isoforms, was proposed to be involved in Ca^{++} -dependent exocytosis in neuroendocrine cells, although its actual function *in vivo* is not yet known. Over-expression of α -taxilin mRNA has been reported in human glioblastoma compared to normal tissues of the central nervous system (CNS) (9). Prominent up-regulation of α -taxilin protein has also been reported in proliferating neural stem cells during embryonic development in rats followed by a rapid decrease of the expression level as development proceeds (10). These findings imply that α -taxilin is related to cell proliferation of mesenchymal cells, especially in the CNS. However, significance of the expression of α -taxilin protein has not yet been studied in tissues or malignancies of epithelial origin.

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Key words: hepatocellular carcinoma, taxilin, vesicular transport proteins

In the present study, we analyzed the expression of α -taxilin in hepatocellular carcinomas (HCC), which is one of the most common malignancies of epithelial origin worldwide and is known to have wide varieties of differentiation and growth activity. We found a correlation of α -taxilin expression with proliferative activity as well as with malignant potential of HCC.

Patients and methods

Patients. Twenty-nine patients of HCC were studied who underwent curative or non-curative hepatectomy at the Hepato-Biliary-Pancreatic and Transplantation Division between May 2003 and August 2008. No patients received preoperative treatments for HCC. Diagnosis of HCC was confirmed by histology based on sections stained with hematoxylin and eosin. The degree of tumor differentiation and other pathological features were assessed according to the Liver Cancer Study Group of Japan (11), and reviewed by a pathologist who was not informed of the results of α -taxilin expression. When there were multiple nodules of HCC in the resected tissues, the largest one was considered to be representative. The clinical and pathological features of the study population are shown in Table I. Informed consent was obtained from each participant. This study protocol was approved by the institutional review board of the University of Tokyo.

Immunohistochemistry of α -taxilin. The polyclonal anti-human α -taxilin antibody raised in a rabbit was used for immunohistochemistry, as previously described (12).

The formalin-fixed, paraffin-embedded liver specimens containing HCC nodules were sliced into 3 μ m-thick sections, immersed in xylene and graded alcohols, and washed in 10 mM PBS. The sections were heated using a microwave processor MI-77 (Azumaya, Tokyo, Japan) in 10 mM citrate buffer (pH 6.0) at 95°C for 30 min. The sections were treated with 0.3% v/v H₂O₂ in methanol at room temperature for 30 min, blocking solution (10% v/v normal goat serum and 1% w/v BSA in 10 mM PBS) at room temperature for 1 h, and then incubated with a primary antibody diluted in the blocking solution overnight at 4°C.

A standard avidin-biotin-peroxidase complex (ABC) technique with Vectastain® ABC elite kit (Vector Laboratories, Burlingame, CA, USA) was applied. Briefly, a biotinylated antibody against rabbit IgG diluted in the blocking solution and ABC solution were applied at room temperature for 25 and 30 min, respectively. Staining was visualized with 3,3'-diaminobenzidine working solution (Vector Laboratories) with nuclear counterstaining in Mayer's hematoxylin (Wako Pure Chemical Industries Ltd., Osaka, Japan).

The sections were studied with a light microscope Eclipse 80i (Nikon, Tokyo, Japan) at magnifications x40, x100 and x200 with a digital camera DXM1200F (Nikon). Intensity of α -taxilin staining was evaluated independently by three of the authors. The intensity of staining in HCC tissues was classified into two categories: 'strong staining' where almost all the cancerous cells were stained (Fig. 1A), and 'weak staining' where no stained cells or few weakly stained cells were observed in cancerous tissues (Fig. 1B).

Immunohistochemistry of proliferating cell nuclear antigen (PCNA) in HCC. Immunohistochemistry of PCNA was performed on the serial section of the specimens as mentioned above except for blocking solution (10% v/v normal horse serum and 1% w/v BSA in 10 mM PBS), the primary antibody (mouse monoclonal anti-PCNA antibody (clone PC10; dilution 1:200; Dako, Glostrup, Denmark)), the secondary antibody (a biotinylated anti-mouse IgG) and the duration of microwave heating (10 min).

PCNA labeling index (PCNA-LI) was determined by random evaluation of at least 1,000 HCC cell nuclei at magnification x100. All of the stained nuclei were regarded as positive. PCNA-LI was expressed as the percentage of positive nuclei (13,14).

Preparation of frozen tissue samples of HCC for Western blotting. Western blotting was performed on fresh frozen tissue samples from one representative case showing 'strong staining' of α -taxilin. The samples obtained at hepatectomy from the HCC nodule and adjacent non-cancerous liver tissue were snap-frozen in liquid nitrogen, and stored at -80°C. They were homogenized in ice-cold buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, Protease inhibitor cocktail Complete® (Roche Diagnostics, Basel, Switzerland), 1 mM DTT, and 2% w/v Triton X-100, incubated for 1 h with intermittent vortexing.

Preparation of lysates of hepatoma cell lines for Western blotting. The human hepatoma cell lines, Hep G2 (cell no. JCRB1054), PLC/PRF/5 (JCRB0406) and HuH-6 Clone 5 (JCRB0401) were purchased from Health Science Research Resources Bank (Osaka, Japan) in November 2008. The cell lines were authorized by Multiplex PCR method using short tandem repeat by the cell bank.

The cells were plated on plastic culture dishes (BD, Franklin Lakes, NJ, USA) at a density of 3,000 cells/cm² in DMEM with 10% v/v FCS and grown to 50-70% confluency. The cells were lysed in ice-cold buffer containing 50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 0.5 mM DTT, protease inhibitor cocktail, and 1% v/v NP-40, incubated for 10 min with intermittent vortexing.

Western blotting for α -taxilin. Protein concentrations of the tissue homogenates and the cell lysates were determined by the Lowry's method with DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The samples were adjusted to the same protein concentration, boiled at 95°C for 5 min with a half volume of SDS buffer (186 mM Tris/HCl (pH 6.7), 9% SDS, 15% glycerol, 6% 2-mercaptoethanol, bromophenol blue), separated by SDS-PAGE on a 10% polyacrylamide gel, and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were treated with blocking buffer (10 mM PBS containing 5% w/v skim milk and 0.1% w/v Tween-20) for 1 h at room temperature, and incubated with 0.1 μ g/ml of anti-human α -taxilin antibody in the blocking buffer overnight at 4°C, and subsequently with HRP-conjugated anti-rabbit IgG (GE Healthcare) in the blocking buffer (dilution 1:2,000) for 45 min at room temperature. The chemiluminescent signals were visualized

Table I. α -taxilin expression in HCC and clinical and pathological features.

	All	Intensity of α -taxilin expression		P-value
		Weak (%)	Strong (%)	
Age ^a	68.0 (45-76)	68.0 (51-76)	68.0 (45-76)	
<68	14	7 (50)	7 (50)	0.860
≥68	15	7 (47)	8 (53)	
Gender				
Male	24	10 (42)	14 (58)	0.125
Female	5	4 (80)	1 (20)	
Background liver ^b				
Liver cirrhosis	17	10 (59)	7 (41)	0.395
Chronic hepatitis	10	4 (40)	6 (60)	
Normal liver	1	0 (0)	1(100)	
Number of tumors				
Solitary	20	10 (50)	10 (50)	0.786
Multifocal	9	4 (44)	5 (56)	
Tumor diameter cm) ^a	3.0 (0.9-7.5)	2.6 (1-5.5)	3.7 (0.9-7.5)	
<3.0	15	9 (60)	6 (40)	0.199
≥3.0	14	5 (36)	9 (64)	
Degree of tumor differentiation				
Well	8	7 (88)	1 (12)	0.005
Moderately	11	6 (55)	5 (45)	
Poorly	10	1 (10)	9 (90)	
Tumor invasiveness (vascular invasion and/or intrahepatic metastasis)				
Negative	16	11 (69)	5 (31)	0.016
Positive	13	3 (23)	10 (77)	
Fibrous capsular formation/infiltration				
Negative	11	3 (27)	8 (73)	0.216
Positive/negative	3	2 (67)	1 (33)	
Positive/positive	15	9 (60)	6 (40)	

Data shown are number of cases, and the numbers in parenthesis are percentage of cases unless otherwise indicated. ^aData are expressed as median (range). ^bData are not available in 1 case.

with ECL plus™ (GE Healthcare) and detected by LAS-1000 (Fujifilm, Tokyo, Japan).

Cell proliferation assay. The human hepatoma cell lines mentioned above were seeded into 96-well tissue culture plates (BD) at 1,000 cells/well in DMEM supplemented with 10% v/v FCS. After 24, 48 and 72 h, the numbers of viable cells were determined by a soluble tetrazolium/formazan assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) (15).

Statistical analysis. Statistical analysis was performed using StatView 5.0J software (SAS Institute Inc., Cary, NC, USA).

The Mann-Whitney U test was performed to compare variables of two categories. The Kruskal-Wallis test followed by the Scheffé's *post hoc* test was performed to compare variables of three or more categories. All statistical analyses were considered to be significant at $p < 0.05$.

Results

Immunohistochemical staining of α -taxilin in HCCs and non-cancerous liver tissues. Diffuse granular staining of α -taxilin was observed in the cytoplasm of HCC cells. Fifteen cases were classified as 'strong staining', where all the tumor cells

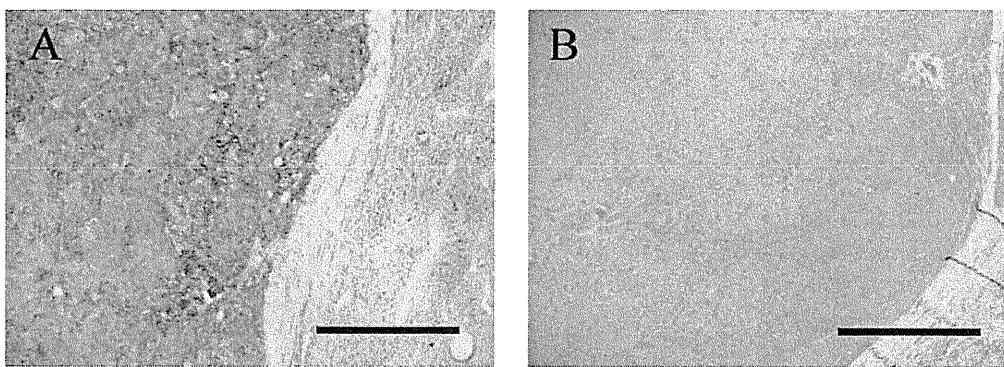


Figure 1. Expression of α -taxilin in HCCs. Intensities of staining were classified into 2 categories: (A) strong staining, almost all the cancerous cells were stained; (B) weak staining, no stained cells or few weakly stained cells were observed in cancerous tissues. Bar, 200 μ m.

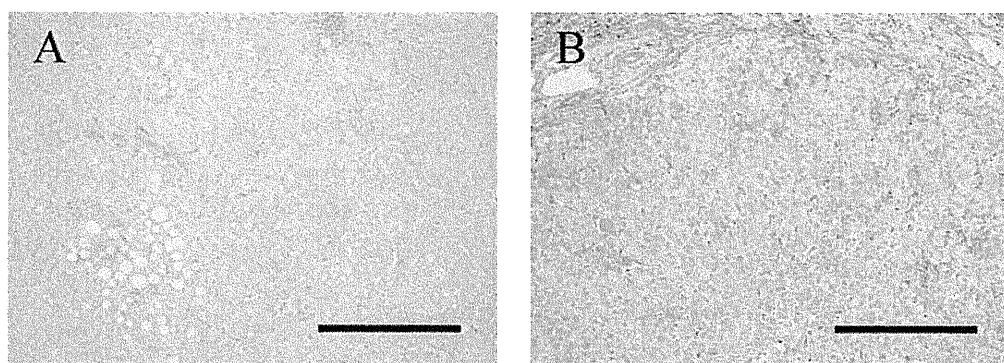


Figure 2. Expression of α -taxilin in non-cancerous tissues. Intensities of staining were evaluated as undetectable (A), or weakly detectable (B). Bar, 200 μ m.

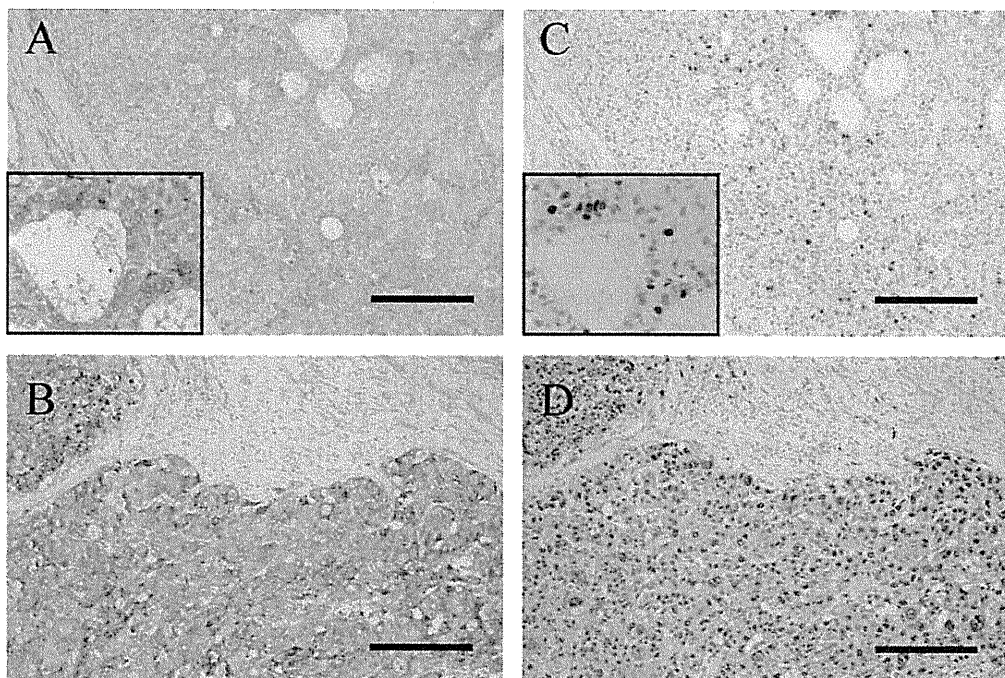


Figure 3. Expression of α -taxilin (A and B) and PCNA (C and D) in HCCs. Intensities of α -taxilin staining and PCNA-LI were determined in serial sections. Two representative cases are shown. Case 1, weak staining of α -taxilin (A) and 7.5% of PCNA-LI (C). Case 2, strong staining of α -taxilin (B) and 94.2% of PCNA-LI (D). Magnified pictures are shown in boxes (A and C). Bar, 500 μ m.

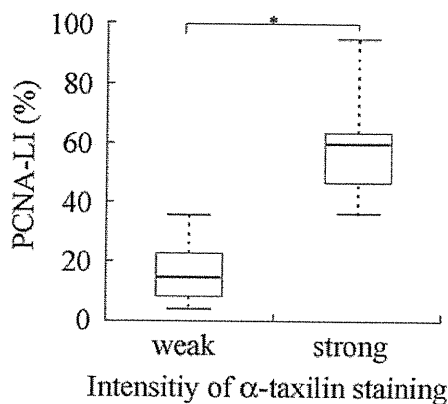


Figure 4. Relationship between α -taxilin expression and PCNA-LI. Tukey's box-and whisker plot are shown. Asterisk indicates a significant difference ($p < 0.0001$ by the Mann-Whitney U test).

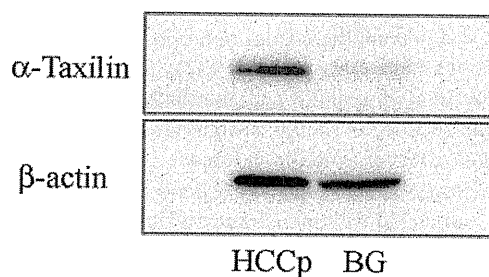


Figure 5. Expression of α -taxilin in HCC and surrounding non-cancerous liver tissue, determined by Western blotting. HCCp, cancerous tissue obtained from poorly differentiated HCC; BG, non-cancerous background liver tissue.

were clearly stained with or without strongly stained foci (Fig. 1A). Fourteen cases were classified as 'weak staining', where only a part of tumor cells showed weak staining mainly

in periphery of the tumor nodules (Fig. 1B). Relationships between the α -taxilin expression and various clinicopathological features were shown in Table I. Significantly higher expression of α -taxilin was observed in less differentiated HCCs ($p = 0.005$), and more invasive HCCs ($p = 0.016$).

In non-cancerous liver tissues surrounding HCCs, the staining was much weaker compared to HCCs. Eleven cases showed no detectable staining (Fig. 2A), 17 cases showed weak and scattered staining in the cytoplasm of hepatocytes in periportal and periseptal areas (Fig. 2B), and 1 case was not appropriate for evaluation because of the small specimen size of non-cancerous tissue.

Relationship between expressions of α -taxilin and PCNA in HCCs. PCNA-LI in HCCs ranged from 3.4 to 94.2%, and the median was 36.0%. α -taxilin-positive cells and PCNA-positive cells showed very similar distribution in the tumor nodules when comparing serial sections (Fig. 3A and C). The sections of 'strong staining' group of α -taxilin showed significantly higher PCNA-LI than the 'weak staining' group (the medians of PCNA-LI were 59.4% vs. 14.4%, $p < 0.0001$) (Fig. 4).

Detection of α -taxilin expression in HCC and non-cancerous tissues by Western blot analysis. Western blot analysis showed a single band consistent with α -taxilin (75 kDa) (8) in the tissue of poorly differentiated HCC (Fig. 5), which was strongly stained for α -taxilin by immunohistochemistry as shown in Fig. 3B. Much weaker signal was detected by Western blotting in the surrounding non-cancerous tissue.

Relationship between α -taxilin expression and cellular proliferation in hepatoma cell lines. All the cell lines in logarithmic-growth phase expressed a single band consistent with α -taxilin at various intensities by Western blotting (Fig. 6A and B). The rate of cell growth correlated with the intensity of α -taxilin expression (Fig. 6C).

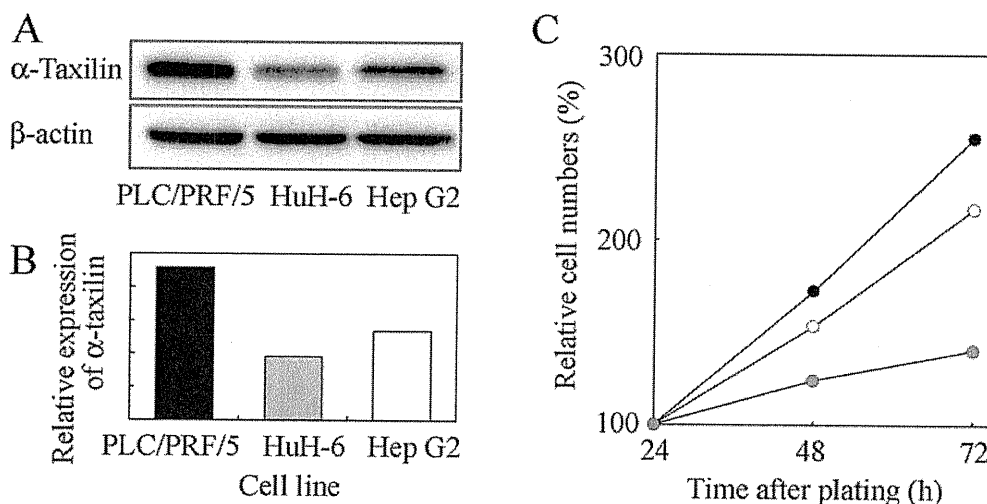


Figure 6. Expression of α -taxilin in hepatoma cell lines (A and B) and cell proliferation rate (C). Closed circles, open circles and shaded circles indicate PLC/PRF/5, Hep G2 and HuH-6, respectively. The expression levels of α -taxilin in the cell lines were correlated with their growth rates.