Original Research

Phospho-Smad3 signaling is predictive biomarker for hepatocellular carcinoma risk assessment in primary biliary cholangitis patients

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1. Abstract

Introduction: Patients with primary biliary cholangitis (PBC) are at increased risk for development of hepatocellular carcinoma (HCC), particularly in the presence of comorbidities such as excessive alcohol consumption. Although liver fibrosis is an important risk factor for HCC development, earlier predictors of future HCC development in livers with little fibrosis are needed but not well defined. The transforming growth factor (TGF)-β/Smad signaling pathway participates importantly in hepatic carcinogenesis. Phosphorylated forms (phospho-isoforms) in Smad-related pathways can transmit opposing signals: cytostatic C-terminally-phosphorylated Smad3 (pSmad3C) and carcinogenic linker-phosphorylated Smad3 (pSmad3L) signals. **Methods and results**: To assess the balance between Smad signals as a biomarker of risk, we immunohistochemically compared Smad domain-specific Smad3 phosphorylation patterns among 52 PBC patients with various stages of fibrosis and 25 non-PBC patients with chronic hepatitis C virus infection. HCC developed in 7 of 11 PBC patients showing high pSmad3L immunoreactivity, but in

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only 2 of 41 PBC patients with low pSmad3L. In contrast, 9 of 20 PBC patients with minimal Smad3C phosphorylation developed HCC, while HCC did not occur during follow-up in 32 patients who retained hepatic tumor-suppressive pSmad3C. Further, PBC patients whose liver specimens showed high pSmad3L positivity were relatively likely to develop HCC even when little fibrosis was evident. **Conclusion**: In this study, Smad phospho-isoform status showed promise as a biomarker predicting likelihood of HCC occurrence in PBC. Eventually, therapies to shift favorably Smad phospho-isoforms might decrease likelihood of PBC-related HCC.

2. Introduction

Worldwide, hepatocellular carcinoma (HCC) is third cause of deaths from malignant tumor, while hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the major promoting factor for HCC [1, 2]. More effective treatments for HBV and HCV have decreased HCC occurrence. Although less common, primary biliary cholangitis (PBC) is another important cause of HCC. In Japan, HCV accounts for 67% of all HCC, followed by HBV at 16%; and then nonviral causes at 15.8% [3]. These nonviral chronic liver diseases include PBC, autoimmune hepatitis, alcoholic liver disease, and non-alcoholic steatohepatitis. In PBC, HCC usually arises after development of cirrhosis following prolonged chronic inflammation [4–6]; cirrhosis complicates more than 80% of such chronic cases [7]. Importantly, PBC-related HCC can occur even in livers with only mild fibrosis [8]. Early identification of patients at high risk for HCC within a PBC cohort therefore is highly important.

Transforming growth factor (TGF)-β regulates cell-cycle in hepatocytes as well as extracellular matrix (ECM) synthesis. TGF-β also involved in fibrosis and carcinogenesis [9–12]. Smads protein is consisted with MH1, MH2, and linker domains. Smad transmit signals from cell-surface receptors for TGF-β superfamily members to the cell nucleus [13]. TGF-β binds to type II cell-surface receptors, and activates type I receptors (TβRI).

Once activated, TβRI can phosphorylate receptor-activated Smads at the C-terminal region. These proteins include Smad2 and Smad3. On the other hands, Ras-related kinases such as c-Jun N-terminal kinase (JNK) phosphorylate the linker domains of Smads 2 and 3 [14]. Thus, TβRI and JNK act upon Smad3 to produce 2 different phospho-isoforms: C-terminally phosphorylated Smad3 (pSmad3C) and Smad3 with linker-phosphorylation (pSmad3L) [15, 16]. TGF-β-dependent pSmad3C signals impede cell-cycle progression by activating transcription of $p15^{INK4B}$ and $p21^{CIP1}$, while de-activating c-Myc gene transcription [17–19]. Thus, pSmad3C-mediated TGF-β signaling is cytostatic and tumor-suppressive [20–22]. On the other hands, JNK-mediated pSmad3L up-regulates transcription of c-Myc, which promotes hepatocytic proliferation leading to liver cancer [23]. Finally, pSmad3L induced hepatocyte proliferation opposes cytostatic pSmad3C signaling [15].

A key therapeutic aim in countering hepatic carcinogenesis is to restore tumor-suppressive signaling as seen in normal hepatocytes. Several investigators have sought to achieve this goal. Treating rats with a JNK inhibitor SP600125, following chemically induced liver carcinogenesis, suppressed phosphorylation in the oncogenic pSmad3L region to favor tumor suppression by pSmad3C [24]. In clinical studies of HCV- or HBV-infected patients, chronic inflammation and certain hepatitis virus components had shifted Smad signaling to favor carcinogenic pSmad3L, promoting liver fibrosis and HCC development [25, 26], while effective antiviral treatment redirected Smad phospho-isoform signaling to favor tumor suppression by the pSmad3C pathway [27]. Thus, TGF-β-mediated Smad signaling can reduce likelihood of HCC development and possibly the aggressiveness of these tumors.

In this study, we retrospectively analyzed 43 PBC patients without HCC and 9 who developed HCC, and clarify the potential risk factors for PBC associated HCC. Among them, we especially focused on changes in domain-specific hepatocytic phospho-Smad3 signaling in patients with PBC. Here we report that in PBC, Smad phospho-isoforms favor cell-proliferative pSmad3L signaling during the fibrotic stages of the disease, resulting in HCC development.

3. Materials and methods

3.1 Patient cohorts

We retrospectively enrolled 43 PBC patients without HCC and 9 patients with PBC-associated HCC. All patients had undergone liver biopsy between 1992 and 2019 in the Department of Gastroenterology and Hepatology at Kansai Medical University Hospital, Osaka Japan. All patients were diagnosed with PBC according to liver histology using international criteria [28]. Among these 52 patients, 30 could be followed up continuously for at least 10 years or until onset of HCC. To detect HCC, ultrasonography (US) or computed tomography (CT) was performed at least yearly. Laboratory tests during follow-up were performed using standard clinical laboratory techniques, including blood cell counts, serum concentration measurements for alanine aminotransferase (ALT). In addition to the 52 patients with PBC, our immunohistochemical assessment of phospho-Smad3, included 20 random patients including each stage of HCV-related fibrotic liver disease (F1–F4) and 5 other HCV-related patients who underwent liver biopsy between 2007 and 2018. The definition of alcohol consumption was 20 g/day or more as positive.

All subjects gave their informed consent for study participation. The study protocol was reviewed and approved by the Human Research Committees of Kansai Medical University, and is consistent with the 1975 Declaration of Helsinki. Enrolled patients underwent ultrasonically guided percutaneous liver biopsy.

3.2 Antibodies against phosphorylated Smad3 domains

To develop specific anti-phospho-Smad3 antibodies, we immunized rabbits with synthetic phosphorylated peptides as described previously [29]. Immunotitration was performed as part of that protocol [29] (also unpublished data). Affinity purification was performed for antibodies against pSmad3L (Ser 208/213) and pSmad3C (Ser 423/425) as described previously [29]. These antibodies have been reported to distinguish phosphorylation at linker or C-terminal region of Smad3 [29].

3.3 Histologic and immunohistochemical analyses

After formalin-fixed, paraffin-embedded liver tissues were processed, serial sections were cut at a thickness of 4 μM and stained with hematoxylin and eosin (HE), as well as Azan stain to delineate fibrosis. PBC was histologically staged as described by Scheuer: stage I, proliferative bile duct lesions; stage II, ductular proliferation; stage III, fibrotic scarring; and stage IV, cirrhosis [30]. In HCV-infected livers, necroinflammatory activity and fibrotic stage were graded histologically according to the classification of Desmet and colleagues. Fibrotic stages were defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis) [31]. Histologic grading was performed by two blinded pathologists kept unaware of clinical data (Koichi Tsuneyamaand Mayuko Ichimura).

Immunohistochemical processing and scoring have been described in detail [25]. Nonenzymatic antigen retrieval was performed by heating the sections to 121 °C in 0.01 mol/L sodium citrate buffer (pH 6.0) for 10 minutes. Briefly the primary antibodies (Abs) that we used included affinity-purified rabbit polyclonal anti-pSmad3L (2 μg/mL) and anti-pSmad3C (0.5 μg/mL). The anti-pSmad3C Ab cross-reacted weakly with C-terminally phosphorylated Smad2: to block such binding, anti-pSmad3C Ab was adsorbed with C-terminally phosphorylated Smad2 peptide (1 μg/mL). After exposure to primary Abs for overnight at 4 °C, sections were incubated with peroxidase-labeled, polymeres conjugated goat anti-mouse or anti-rabbit immunoglobulin G (IgG; DAKO, Glostrup, Denmark) for 1 hour at room temperature. Then the peroxidase reactions was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA). Finally, sections were stained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and coverslipped. We scored pSmad3 positivity according to percentage of hepatocytes showing immunostaining: 0, 0%; 1, <25%; 2, 25% to 50%; 3, 50% to 75%; and 4, >75%. Similarly to evaluation of fibrosis, phosphorylation positivities among hepatocytes were scored by two pathologists blinded to clinical data. Each immunochemical determination included positive and negative controls.

3.4 Statistical analyses

The Mann-Whitney U test was used to assess differences in hepatocytic pSmad3L and pSmad3C positivity between stages of fibrosis and patients developing or not developing HCC. During clinical follow-up after liver biopsy, occurrences of HCC were plotted using the Kaplan-Meier method. The optimum cut-off point was determined by plotting ROC curves. HCC development was compared between patients whose liver specimens showed abundant (score 3 to 4) and sparse (score 0 to 2) Smad3L/C phosphorylation by means of the log-rank test. p values below 0.05 were considered indicative of statistical significance. The Mann-Whitney U test and the Kaplan-Meier method were performed using GraphPad Prism ver.9 (GraphPad, San Diego, CA 92108, USA). The ROC curve was plotted by using JMP (SAS institute, Cary, NC 27513, USA). Optimal cut-off point was determined by Youden index [32].

4. Results

4.1 Distribution of Smad phospho-isoforms in liver specimens from patients according to underlying disease

Tables 1,2 present clinical information and positivity ratings for pSmad3L and pSmad3C in liver specimen for 52 patients with PBC and 25 patients with chronic liver disease related to HCV. Although, platelet count tended to decrease as PBC progression, there was no statistical difference. Most of stage 1 PBC showed low pSmad3L and high pSmad3C. As the stage progress to Stage 3 and 4, pSmad3L statistically increase and pSmad3C decrease. Notably, most of HCC developed from high pSmad3L and low pSmad3C patients in PBC. Distribution of pSmad3L and pSmad3C in PBC specimens showed 1 of 2 patterns of fibrosis severity. For example, in a mildly fibrotic liver specimen (stage 1) from patient 5 (**Supplementary Table 1**), who did not develop HCC during 7.5 follow-up years, hepatocytes retained phosphorylation of Smad3C, while showing little phosphorylation at Smad3L (Fig. 1A). Another patient with similarly mild fibrosis (**Supplementary Table 1**, patient 44) was diagnosed with HCC within 5 years of histopathologic diagnosis of PBC, pSmad3L immunostaining in hepatocytic nuclei was intense throughout liver lobules, while C-terminal Smad3 phosphorylation was reduced (Fig. 1B). Similarly, in a severely fibrotic specimen showing stage 4 PBC (**Supplementary Table 1**, patient 49), the patient already was diagnosed with HCC when liver biopsy was performed, Smad3L phosphorylation was high, while Smad3 C-terminal phosphorylation was low (Fig. 1C). An-

Table 1. Summary of clinical and pathological characteristics with scoring of phosphorylated Smad3L and Smad3C in PBC patients' livers.

Scheuer stage	Stage 1	Stage 2	Stage 3	Stage 4	HCC (stage1:4, stage2:1, stage4:4)
Total cases	15 (28.8%)	13 (25%)	12 (23.1%)	3 (5.8%)	9 (17.3%)
Male	3	1	2	0	4
Female	12	12	10	3	5
Age mean	56.33	58.92	59.25	47.33	68.78
(range) (year)	(34–77)	(35–76)	(46–77)	(42–54)	(60–77)
Follow-up period (year)	12.37	13.01	10.38	9.55	
Time to HCC (year)					4.36
HBs Ag					
(+)	0	0	0	0	0
(–)	15	13	12	3	9
HCV Ab					
(+)	0	1	0	0	2
(–)	15	12	12	3	7
HCV-RNA					
(+)	0	0	0	0	0
Alcohol					
(+)	3	3	2	1	3
(–)	9	6	9	1	6
N/A	3	4	1	1	0
ALT mean	94	56.8	80.8	88	50.8
(range) (IU/L)	(13–509)	(25–130)	(11–378)	(7–179)	(15–105)
Plt mean	208×10^9	205×10^9	188×10^9	163×10^9	155×10^9
(range) (/L)	($99–280 \times 10^9$)	($90–310 \times 10^9$)	($90–440 \times 10^9$)	($120–220 \times 10^9$)	($100–259 \times 10^9$)
pSmad3L stainig mean	0.93	1.62	1.83	1.67	3
(range)	(0–2)	(0–3)	(0–3)	(1–2)	(2–4)
pSmad3C stainig mean	3.13	3.31	2.33	2.33	1.78
(range)	(2–4)	(2–4)	(0–4)	(2–3)	(1–2)
pSmad3L/3C, low (0–2), high (3–4)					
low/high	14	9	5	1	0
low/low	1	2	5	2	2
high/low	0	0	1	0	7
high/high	0	2	1	0	0

PBC, primary biliary cholangitis; HCC, hepatocellular carcinoma; HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; ALT, alanine aminotransferase; Plt, platelets; M, male; F, female; N/A, data not available.

other severely fibrotic liver specimen at a similar stage from patient 41 in **Supplementary Table 1**, who did not develop HCC during 12.8 years following the liver biopsy, demonstrated low phosphorylation at Smad3C and Smad3L (Fig. 1D).

4.2 Tumor-suppressive pSmad3C decreased as liver fibrosis progressed, while oncogenic pSmad3L increased

As liver specimens with HCV infection progressed from chronic hepatitis to cirrhosis, occurrence of HCC increased. Among HCV-infected livers, pSmad3L in those with early fibrosis (F1 and F2) was significantly less abundant than in livers with advanced fibrosis (F3 and F4) and those with HCC (Fig. 2A; **Supplementary Table 2**). In contrast to scant phosphorylation at Smad3L, mildly fibrotic livers showed high Smad3C phosphorylation (Fig. 2B; **Supplementary Table 2**). In severely fibrotic livers (F3 and F4) and those with HCC, positivity for pSmad3C was lower than in livers with mild fibrosis (F1 and F2, Fig. 2A; **Supplementary Table 2**).

Incidence of HCC increased with PBC progression, as occurred in HCV. As was true in HCV infection, phosphorylation of Smad3L in PBC livers with marked fibrosis (stages 3 and 4) also was greater than in less fibrotic livers with PBC (stages 1 and 2, Fig. 2A). In contrast, phosphorylation at Smad3C in highly fibrotic PBC livers (stages 3 and 4) was less abundant than in PBC showing stage 1 or 2 fibrosis (Fig. 2B). Incidence of HCC increased with PBC progression, as occurred in HCV. HCC occasionally developed in PBC livers with only early fibrosis. Overall, the oncogenic pSmad3L pathway was increasingly activated as liver fibrosis progressed, but it sometimes was still up-regulated in PBC irrespective of fibrosis.

Table 2. Summary of clinical and pathological characteristics with scoring of phosphorylated Smad3L and Smad3C in HCV patients' livers.

Fibrosis stage		F1	F2	F3	F4	HCC, (F3:2, F4:3)
Total cases		5 (20%)	5 (20%)	5 (20%)	5 (20%)	5 (20%)
	Male	1	2	2	2	5
	Female	4	3	3	3	0
Age mean		57	53.4	49.6	62.6	73.6
(range) (year)		(39–68)	(36–68)	(20–66)	(47–70)	(61–91)
HBs Ag						
	(+)	0	0	0	0	0
	(–)	5	5	5	5	5
HCV genotype						
	1	3	1	4	4	4
	2	2	4	1	1	1
Alcohol						
	(+)	1	1	1	0	1
	(–)	1	4	2	3	0
	N/A	3	0	2	2	4
ALT mean		66.6	148.8	83.4	81.2	54.6
(range) (IU/L)		(34–158)	(37–338)	(54–111)	(33–166)	(30–86)
Plt mean		171×10^9	152×10^9	148×10^9	75×10^9	98×10^9
(range) (/L)		($105–248 \times 10^9$)	($83–228 \times 10^9$)	($120–178 \times 10^9$)	($50–109 \times 10^9$)	($57–141 \times 10^9$)
pSmad3L stainig mean		2	3.2	3.2	3.4	3.6
(range)		(1–3)	(3–4)	(2–4)	(2–4)	(3–4)
pSmad3C stainig mean		3.4	3.2	2.8	2.4	1.8
(range)		(3–4)	(2–4)	(2–4)	(1–4)	(1–3)

4.3 Oncogenic pSmad3L was increased while tumor-suppressive pSmad3C was decreased during PBC-related hepatocarcinogenenesis, irrespective of degree of liver fibrosis

Three of five patients with stage 1 and 2 PBC but high level of pSmad3L developed HCC during follow-up (Fig. 3A), while HCC did not develop in 13 PBC patients whose livers were severely fibrotic but showed only limited Smad3L phosphorylation (Fig. 3A). In contrast, 5 of 8 PBC patients whose livers were mildly fibrotic but showed only limited Smad3C phosphorylation developed HCC (Fig. 3B).

To further investigate how hepatocytic pSmad3L vs. C phosphorylation affects HCC occurrence in PBC, we divided liver specimens into groups showing mild or severe fibrosis (Fig. 4). The 5 of 33 PBC patients with mild fibrosis (stages 1 and 2) developing HCC by the time of biopsy or during follow-up showed significantly more Smad3L phosphorylation (scores 3 to 4) and significantly less Smad3C phosphorylation (scores 1 to 2; Fig. 4A,B) than the other 28 patients. On the other hand, those with more marked fibrosis (stages 3 and 4) who did not develop HCC showed significantly less Smad3L phosphorylation (Fig. 4C). Thus, hepatocytes strongly positive for pSmad3L can give rise to HCC even when fibrosis is mild, while hepatocytes showing less positivity for pSmad3L did not develop HCC despite advanced fibrosis.

We then compared phosphorylation profiles in livers with cirrhosis according to etiology (HCV vs. PBC). No difference was evident between etiologies of cirrhosis for pSmad3C, but pSmad3L was significantly more abundant in HCV-related than in PBC-related cirrhosis (Fig. 5A,B). This relative excess of pSmad3L suggested that hepatitis C virus infection promoted HCC through the pSmad3L pathway.

4.4 Abundant hepatocellular pSmad3L and sparse pSmad3C predicted increased risk of HCC development in PBC patients

We next investigated whether Smad3 phosphorylation profiles influenced neoplastic evolution in patients with PBC. The ROC curves were blotted to determine the optimal cut off value of phosphorylation level of Smad3L and Smad3C for HCC prediction. We have defined score 0–2 were low, and 3–4 were high levels in both pSmad3L and pSmad3C by using Youden index (**Supplementary Fig. 1**).

We compared HCC incidence between patients with high and low for both patterns of hepatocytic Smad3 phosphorylation. HCC was diagnosed in 7 of 11 patients with abundant Smad3L phosphorylation, but in only 2 of 41 patients with low Smad3L phosphorylation (log-rank $p < 0.0001$, Fig. 6A). On the other hands, HCC developed in 9 of 20 patients with low Smad3C phosphorylation, but in none of 32 patients with abundant Smad3C phosphorylation (log-rank $p < 0.0001$, Fig. 6B).

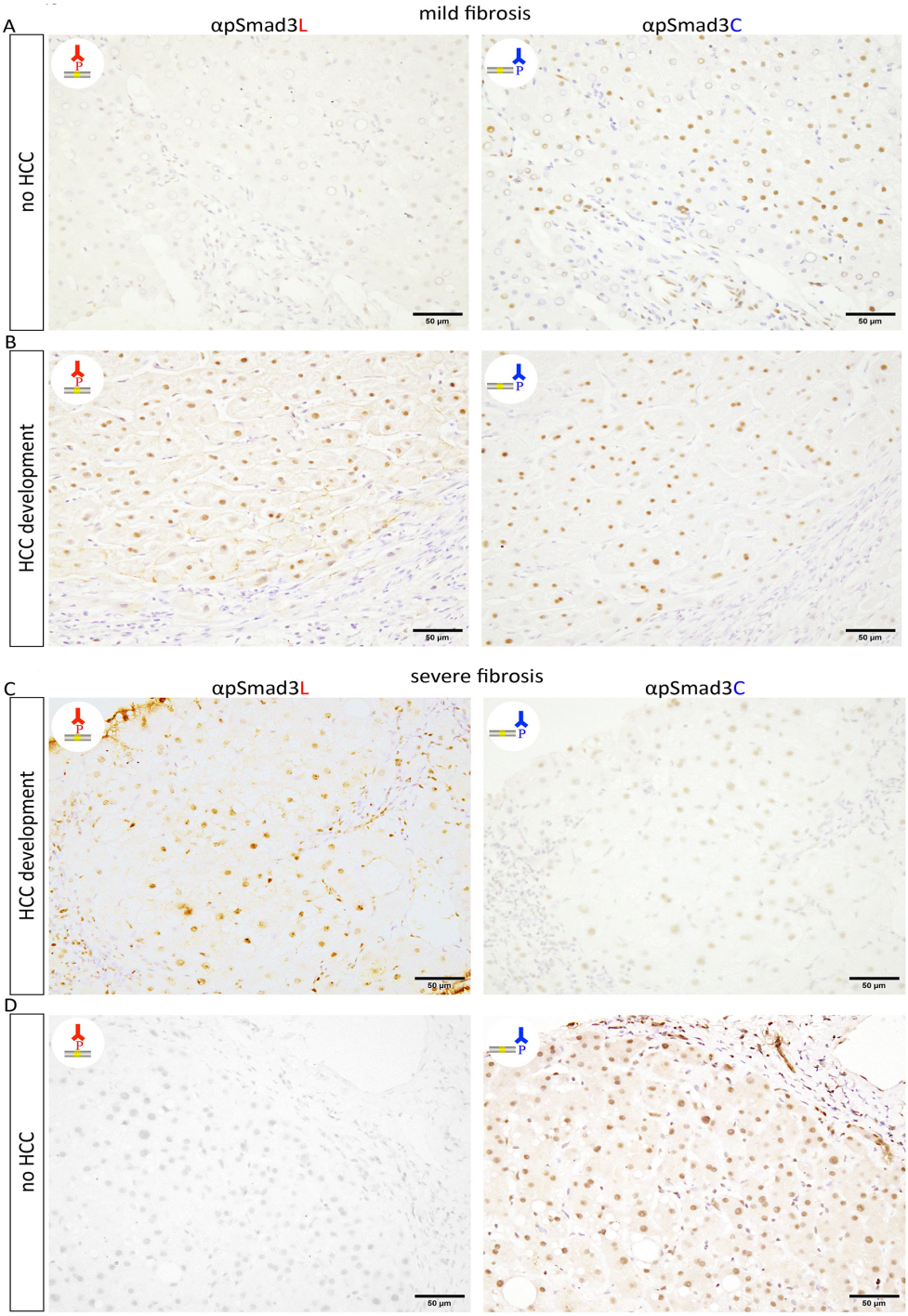

Fig. 1. Two contrasting hepatocytic phospho-Smad signaling patterns were present among liver specimens from patients wtih PBC: pSmad3L-dominant and pSmad3C dominant. (A) Patient 5 in **Supplementary Table 1**, free of HCC development during 7.5 years following histopathologic diagnosis of PBC. Hepatocytes showed considerable level of pSmad3C (α pSmad3C column), but scant level of pSmad3L (α pSmad3L column). (B) Patient 44 in **Supplementary Table 1**, had onset of HCC within 5 years after liver biopsy. Smad3 in hepatocytic nuclei was sparsely phosphorylated in the C-terminal region (α pSmad3C column) but highly phosphorylated in the linker region (α pSmad3L column). (C) Cirrhotic liver specimen from patient 49 in **Supplementary Table 1**, who was diagnosed with HCC when liver biopsy was performed. Phosphorylation of Smad3L was high, while C-terminal phosphorylation of Smad3 was low. (D) Cirrhotic liver specimen from patient 41 in **Supplementary Table 1**, No HCC arose during 12.8 years after liver biopsy. Hepatocytic nuclei showed low phosphorylation at Smad3C and low phosphorylation at Smad3L. Scale bars: 50 μM. Formalin-fixed, paraffin-embedded sections of liver specimens were stained with anti-pSmad3L antibody (α pSmad3L column) and anti-pSmad3C antibody (α pSmad3C column), as described in the Methods section.

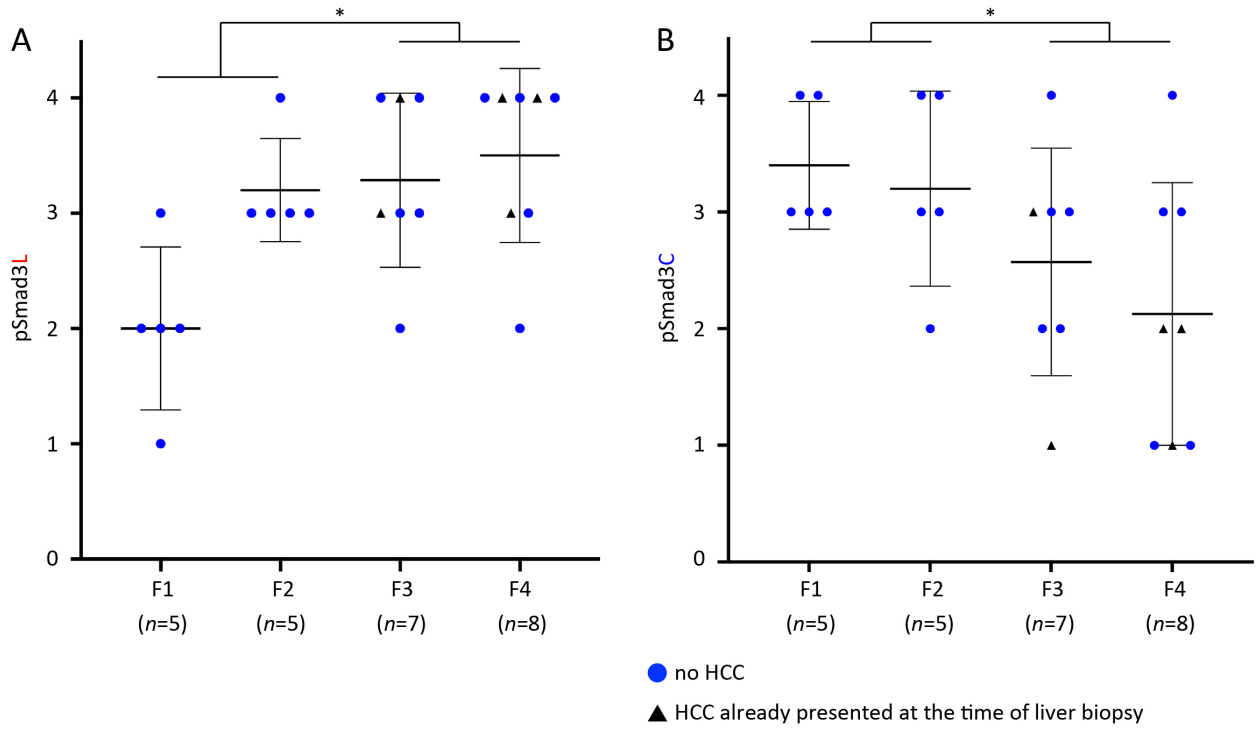

Fig. 2. Hepatocytic Smad signaling for pSmad3C and pSmad3L during progressive of HCV-related fibrosis. (A) Phosphorylation level of Smad3L in hepatocytes was up-regulated in tandem with fibrosis. Extent of pSmad3L in hepatocyte with severe fibrosis (F3 to 4) was statistically increased compared with in livers with less fibrosis (F1 to 2). (*$p < 0.05$). (B) Hepatocytic pSmad3C decreased while fibrosis increased in HCV-related chronic liver disease. Phosphorylation of Smad3C of hepatocytic nuclei in highly fibrotic livers (F3 to 4) was much less than in livers with F1 to 2 fibrosis (*$p <$ 0.05). Vertical positions of dots and triangles represent degree of pSmad3L (A) and pSmad3C (B) positivity for each liver specimen. Blue dots indicate cases where HCC had not developed. Black triangles indicate cases where HCC already was present at the time of liver biopsy. Horizontal bars indicate mean ± SD for phospho-Smad3 positivity in each fibrosis-defined group.

Because no one developed from high pSmad3C group, it was impossible to calculate odds ratios by multivariate logistic regression analysis using Prism. Instead, we calculated odds ratios by Haldane-Anscombe 1/2 correction [33]. The odds ratio was 26.333 (95% CI 4.659–148.829) in pSmad3L high group compared to the low group, and was 53.696 (95% CI 2.890–997.825) in pSmad3C low group compared to the high group. These data suggest strong correlation between HCC incidence and higher pSmad3L and lower pSmad3C in PBC patients. Furthermore, the odds ratio was 295 (95% CI 10.887–7993.750) in pSmad3L high/pSmad3C low group compared to the low/high group (Table 3). These findings suggest that Smad phospho-isoforms can help to predict which PBC patients are likely to develop HCC. Alternation of isoform balance then might contribute to prevention of PBC-related HCC.

Table 3. Phospho-Smad3L/3C are predictor of HCC occurrence.

Variables	Category	Odds ratio (95% CI)
pSmad3L positivity	high (3 and 4) low (0 to 2)	26.333 (4.659–148.829)
pSmad3C positivity	high (3 and 4) low (0 to 2)	53.696 (2.890–997.825)
pSmad3L/pSmad3C positivity	high/low low/high	295 (10.887–7993.750)

HCC did not occur in PBC when specimens were highly positive for pSmad3C, so odds ratios were calculated using Haldane-Anscombe 1/2 correction.

5. Discussion

This study deals with the comparative biology of Smad phospho-isoform signaling in PBC- and HCV-related chronic liver diseases. As these diseases progress from chronic hepatitis to cirrhosis, the pSmad3L pathway in hepatocytes becomes more active, while the pSmad3C pathway becomes more dormant. Interestingly, PBC patients whose livers demonstrate pSmad3L dominance in hepatocytic nuclei develop HCC even in mildly fibrotic livers. Alternatively, livers with scant pSmad3L livers are less likely to develop HCC even when cirrhosis occurs. These data suggest that Smad phospho-isoforms may serve as biomarkers for prediction of HCC in PBC; they also may represent a potential therapeutic target for prevention of PBC-related HCC.

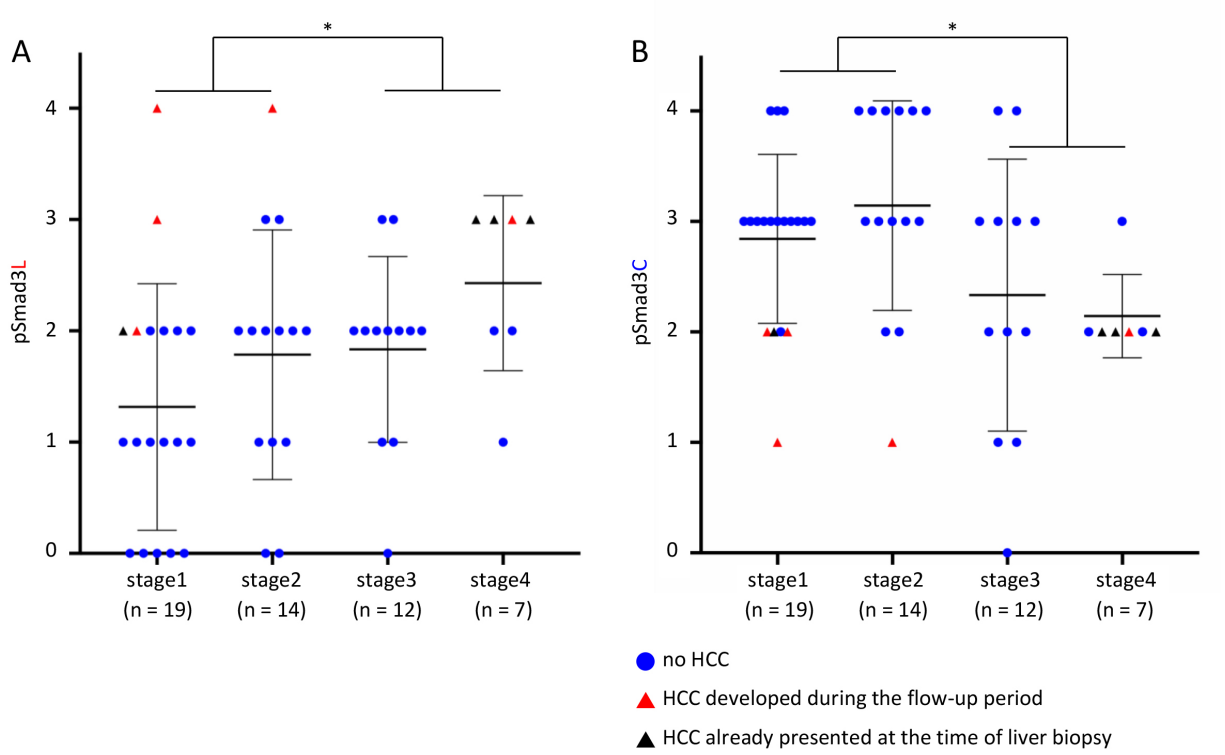

Fig. 3. Smad phospho-isoform signaling in hepatocyte increasingly favored the pSmad3L pathway over the pSmad3C pathway as disease progression in PBC. (A) Phosphorylation level of Smad3L in hepatocytes is up-regulated with intensity of fibrosis in PBC liver specimens. Smad3L phosphorylation in stage3 and 4 fibrotic livers was greater than with stages 1 and 2 hepatic fibrosis (*$p < 0.05$). (B) Smad3C phosphorylation decreased as fibrosis increased; phosphorylation of Smad3C in stage3 and 4 fibrotic livers was lower than in livers with stage 1 and 2 fibrosis (*$p < 0.05$). Vertical positions of dots and triangles represent degree of pSmad3L (A) and pSmad3C (B) positivity for each liver specimen. Blue dots, red triangles, and black triangles respectively indicate cases where HCC did not develop; HCC developed during the follow-up period; and HCC already existed at the time of liver biopsy. Horizontal bars indicate mean $\pm$ SD for phospho-Smad3 positivity in each fibrosis-defined group.

During progression of HCV infection or PBC, Smad phospho-isoform signaling typically changes from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway. The positivity of pSmad3L gradually increased as disease progress from stage1 to 4, in PBC patients. However, in PBC, which like HCV is characterized by liver fibrosis, yearly cumulative incidence of HCC is only about 2%, substantially less than in HCV [34]. Further, PBC-related HCC sometimes occurs in mildly fibrotic livers [6]. Thus, HCC occurrence in PBC is much less predictable than in HCV, where fibrosis is tightly linked to severity of hepatitis, sufficient viral elimination wtih treatment can halt fibrosis. While fibrosis in PBC can regress spontaneously in early stages, PBC with only early hepatic fibrosis still can lead to development of HCC [6]. On the other hand, even cirrhotic livers in PBC less often develop HCC than livers affected by HCV-related cirrhosis [34]. Differences in hepatocarcinogenesis between PBC and HCV include notably different Smad signaling patterns, which are less predictable in PBC.

In most of PBC patients, liver fibrosis progress for many years without HCC occurrence. Irrespective of etiology, hepatic fibrosis reflects ECM proteins accumulation. Matrix accrual during the course of diseases is dynamic, and may include phases of progression and regression [35]. When ECM synthesis exceeds degradation, the ECM excess results in liver fibrosis. Plasminogen activator inhibitor-1 (PAI-1) 1 is a potent inhibitor of plasminogen activator. Highly induced PAI-1 impedes degradation of ECM by down-regulating the plasminogen activation system [36, 37]. During chronic liver injury, hepatic stellate cells (HSC) are activated to become myofibroblast (MFB)-like cells which up-regulate PAI-1 transcription to promote ECM deposition [38]. Typically, MFB display fibrogenic dominant TGF-β signaling while no longer losing cell-cycle arresting signaling [9]. This signaling alteration in MFB, may be explained differential intracellular localization of pSmad2L and pSmad3L within MFB. JNK, activated by pro-inflammatory cytokines, increases nuclear localization of pSmad3L in MFB, inhibits pSmad3C signaling which suppress cell growth [25, 39, 40]. Although Smad2 and Smad3 are structurally very similar, Smad2 has several different functions. In contrast to nuclear localization of pSmad3L, pSmad2L remains in the cytoplasm. C-terminal phosphorylation of Smad2 is essential for nuclear localization [41]. Under sustained activation of JNK, both

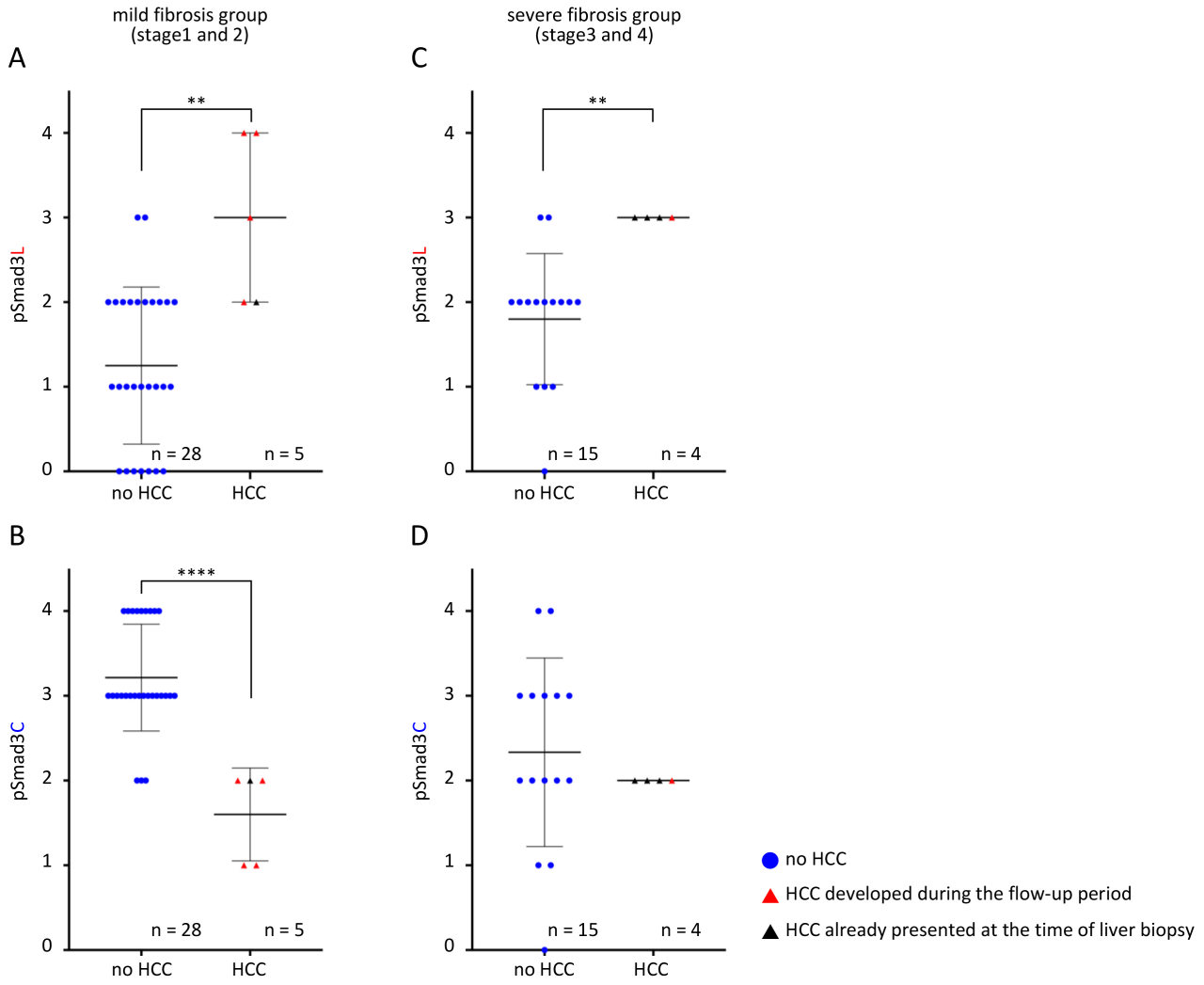

Fig. 4. Irrespective of fibrotic stage, HCC developed from hepatocytes with high level of pSmad3L in PBC. (A,B) HCC developed in the presence of high pSmad3L phosphorylation. (A) and low pSmad3C (B) in stage1 and 2 livers with PBC. Degree of pSmad3L (A) or pSmad3C (B) in cases where HCC developed was respectively greater or less when HCC did not arise (**$p < 0.01$, ****$p < 0.0001$). (C,D) HCC did not arise when Smad3L phosphorylation was limited (C), even when fibrosis was marked. In PBC with severe fibrosis, the level of pSmad3L (C) was greater when HCC developed than when HCC did not arise (**$p < 0.01$). Liver fibrosis in PBC was considered either mild (stages 1 to 2) or severe (stages 3 to 4). Vertical positions of dots and triangles represent degree of pSmad3L (A,C) and pSmad3C (B,D) positivity for each liver specimen. Blue dots, red triangles, and black triangles respectively indicate cases where HCC did not develop; HCC developed during the follow-up period; and HCC already existed at the time of liver biopsy. Horizontal bars indicate mean $\pm$ SD for phospho-Smad3 positivity in each fibrosis-defined group.

linker and C-terminal phosphorylated Smad (pSmad2L/C) can form complex with pSmad3L and Smad4 to up-regulate PAI-1 transcription and liver fibrogenesis [29]. Consequently, TGF-b dependent cell cytostatic response did not observe even when TGF-b continuously promoted ECM production in MFB.

Strong phospho-Smad3L in nuclei of α-smooth muscle actin-positive MFB was observed in HCV-infected and PBC livers samples. Both HCV and PBC result in cirrhosis. In the hepatocytic nuclei of HCV induced cirrhosis, high level of pSmad3L was observed. On the other hand, the level of pSmad3L was much lower in most of PBC related advanced fibrosis patients who was not observed HCC. When we compared phospho-Smad3 signaling between HCV- and PBC-induced liver cirrhosis, HCV and PBC showed similar staining for pSmad3C, while we found significantly less pSmad3L immunostaining in PBC than in HCV-related cirrhosis, contributing to a lower incidence of HCC in PBC-related cirrhosis.

Our group has reported phospho-Smad signaling in chronic liver disease caused by hepatitis B virus (HBV) and nonalcoholic steatohepatitis (NASH). As human HBV related chronic liver diseases progress, HBX gene and chronic inflammation additively shift hepatocytic Smad phospho-isoform signaling from tumor suppressive pSmad3C to carcinogenic pSmad3L pathways, accelerat-

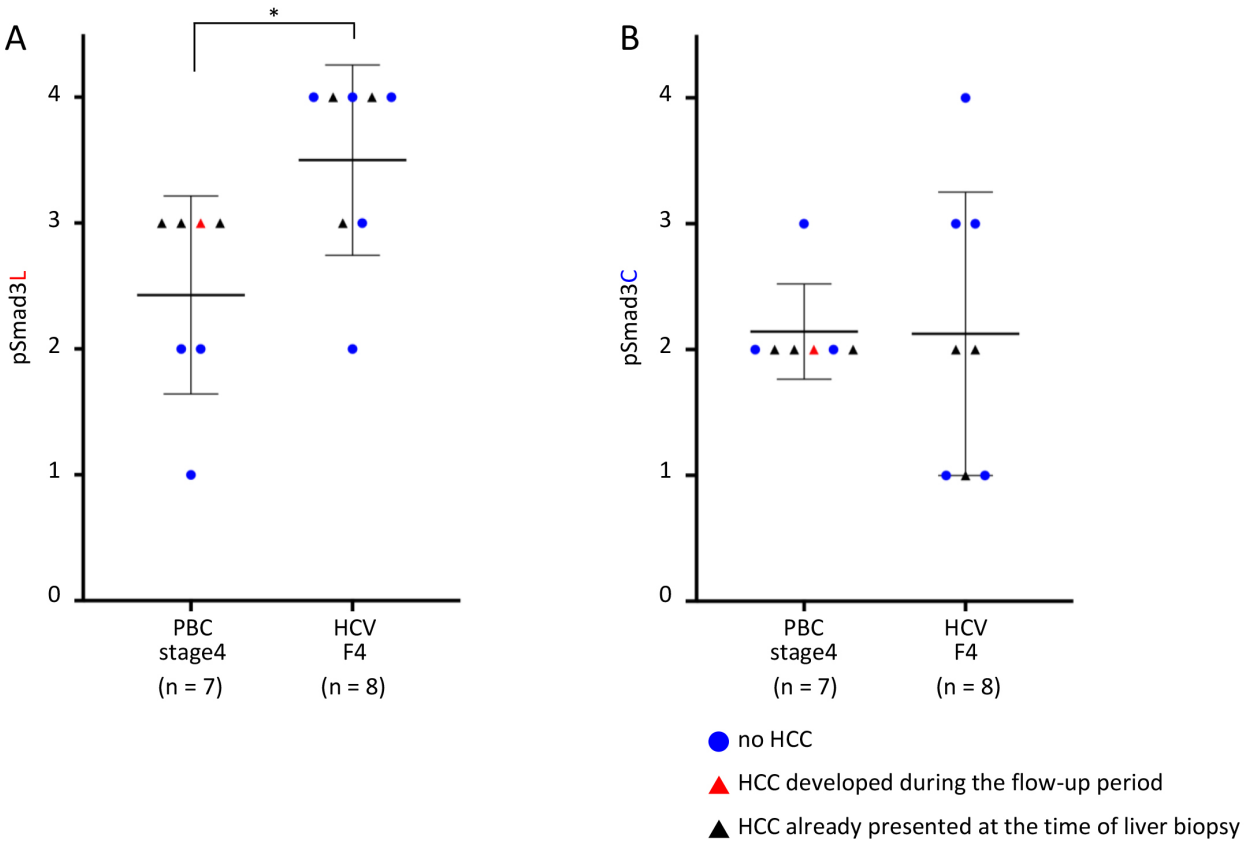

Fig. 5. Phosphorylation of Smad3L was higher in cirrhosis associated with HCV than in cirrhosis associated with PBC. Vertical positions of dots and triangles represent degree of pSmad3L (A) and pSmad3C (B) positivity for each liver specimen. $*p < 0.05$. Blue dots, red triangles, and black triangles indicate respectively cases where HCC did not develop; HCC developed during the follow-up period; and HCC already existed at the time of liver biopsy. Horizontal bars indicate mean ± SD for phospho-Smad3 positivity in each defined group.

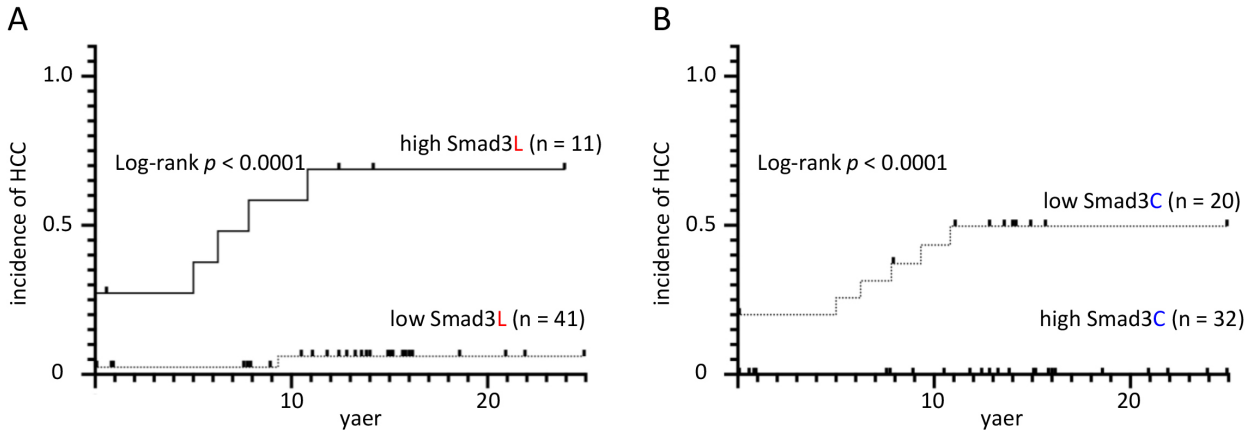

Fig. 6. PBC patients with high positivity for pSmad3L and negligible positivity for pSmad3C were likelier to develop HCC. (A) HCC was more likely in PBC when specimens were strongly positive for pSmad3L. HCC also was likelier in patients with plentiful Smad3L phosphorylation (scores 3 to 4, solid line) than in those with little such phosphorylation (scores 0 to 2, dotted line). (B) HCC did not occur in PBC when specimens were highly positive for pSmad3C, while HCC was more likely with sparse Smad3C phosphorylation. Occurrence was compared using Kaplan-Meier curves and log-rank tests.

ing liver fibrosis and increasing the risk of HCC [26]. These data similar to HCV-related chronic liver inflammation. We also found antiviral therapy can achieve reduction of fibro-carcinogenic pSmad3L and increase in tumor-suppressive pSmad3C signaling in HBV or HCV infected patients [27, 42]. In NASH patients, as fibrosis progressed hepatocytic pSmad3C had significantly decreased, but pSmad3L had not significantly changed. NASH livers with high risk of HCC occurrence showed high positivity in pSmad3L and low positivity in pSmad3C [43]. Interestingly, the NASH patients showing high pSmad3L in the liver, developed HCC in the near future even from low fibrotic livers. These data suggest chronic inflammation caused by NASH and PBC, without viral component, can shift slowly but steadily hepatocytic Smad3-mediated signaling from tumor suppression to oncogenesis [43].

Several kinds of clinical trials are testing novel biomarker for HCC occurrence in PBC patients [6, 44]. Advanced stage of liver fibrosis, alcohol intake, blood transfusion, male gender, and elder age have been reported to be risk factors of HCC [45]. However, they are not good predictive biomarker for HCC in near future. Because AMA and elevation of ALP are enough for diagnosis of PBC, liver biopsy is less frequency performed for PBC patients. In patients with high pSmad3L and low pSmad3C signaling progressed HCC despite early stage of PBC. In contrast, PBC patients with advanced fibrosis did not progress HCC, because hepatocytes maintained high pSmad3C and low pSmad3L. Even PBC patients with little hepatic fibrosis but high liver pSmad3L proved likely to develop HCC over time.

6. Conclusions

In HCV patients, the risk of HCC increases as the fibrosis progression. On the other hand, HCC eventually observed in early stage of PBC [6]. The difference reflects differential mechanism of PBC- and HCV related HCC, especially in early stages of chronic hepatitis. Because extent of Smad3L phosphorylation increases in step with fibrosis in chronic hepatitis C, Smad3L shows little phosphorylation in early disease. In contrast, marked linker phosphorylation of Smad3 was demonstrated in hepatocytic nuclei (scores of 3 or 4) in all PBC patients with mild liver fibrosis (stage 1 to 2) who developed HCC. The present study clearly identified high hepatocytic pSmad3L and low pSmad3C as risk factors for HCC in PBC (Fig. 7).

In PBC patients with HCC, hepatic pSmad3L positivity was much greater than in PBC without HCC. High pSmad3L phosphorylation was observed in PBC before HCC development. Our data suggest that Smad phospho-isoforms may be useful biomarkers for predicting HCC in PBC, and manipulation of phospho-isoform balance might offer a way to prevent HCC in PBC.

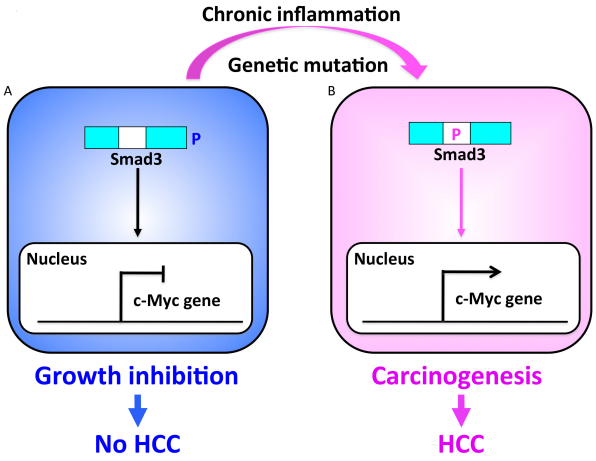

Fig. 7. Smad phospho-isoforms are useful biomarkers for HCC prediction in PBC patients. (A) HCC is unlikely to develop in PBC patients showing high hepatocytic nuclear positivity for tumor-suppressive pSmad3C signaling in their liver specimens. (B) HCC is more likely to develop in PBC patients showing high hepatocytic nuclear positivity for oncogenic pSmad3L signaling in their liver specimens.

7. Author contributions

NN, RT and KY designed the study and wrote the initial draft of the manuscript. MM and TY contributed to analysis and interpretation of data and assisted in the preparation of the manuscript. KS, MI, KT, KM, TN, JH, TS, KO, MG and MN have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

8. Ethics approval and consent to participate

Patients were enrolled in the study after informed consent and following the approval and recommendations of the Ethics Review Board of Kansai Medical University (code: 2006-0409).

9. Acknowledgement

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11. Conflict of interest

The authors declare no conflict of interest.

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Supplementary material: Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/26/12/10.52586/5042.

Keywords: TGF-β; PBC; Smad; HCC

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