RESEARCH COMMUNICATION

Smad4 Expression in Hepatocellular Carcinoma Differs by Hepatitis Status

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Abstract

Aims: Primary hepatocellular carcinoma (HCC) is a common malignancy often related to hepatitis viral infection. Smad4 is known to mediate the TGF-β pathway to suppress tumorigenesis. However, the function of Smad4 in HCC is still controversial. In this study we compared levels of Smad4 in HCC tissues with or without hepatitis virus infection and adjacent normal-appearing liver. Methods: Samples from HCC patients were analyzed for Smad4 protein and mRNA expression by immunohistochemistry (IHC), RT-PCR and Western blotting. Results: We found that tumor tissues expressed less Smad4 mRNA and protein than the adjacent tissues. Most HCC tumor tissues were negative for Smad4 in IHC staining, while the majority of adjacent tissues were positively stained. Interestingly, protein levels were higher in HCC tissues with viral hepatitis than those without virus infection. Suppression of expression appeared closely related to HCC, so that Smad4 appears to function as a tumor suppressor gene (TSG). Conclusion: Patients with hepatitis viral infection, at higher risk for HCC, exhibited increased Smad4 protein expression suggesting hepatitis virus may modulate Smad4 expression, which is functionally distinct from its putative role as a TSG. Smad4 expression may thus be an applicable marker for diagnosis and/or a target to develop therapeutic agents for HCC.

Keywords: Liver neoplasms/genetics - SMAD4 - liver cirrhosis/virology - tumor suppressor gene (TSG) - viral/genetics

Asian Pacific J Cancer Prev, **13**, 1297-1303

Introduction

Primary hepatocellular carcinoma (HCC), one of the most common malignancies, is a viral infection related cancer. HCC prevalently happens in Asia and Africa and has an increasing mortality rate in China. Patients in Asia with HCC follow the progress from hepatitis either viral or non-viral to hepatic fibrosis, cirrhosis, and carcinogenesis (Lai et al., 2003; Vince, 2005). Although hepatitis B virus (HBV), hepatitis C virus (HCV), and aflatoxin have been confirmed as the main causes of HCC (Shin et al., 2006; Feitelson and Lee, 2007; Keasler et al., 2007), the mechanisms underlying the malignant transformation of hepatocytes are still largely unknown (Ozturk, 1995). Transforming growth factor beta (TGF-β) has a central role in the growth of hepatocytes (Rossmanith and Schulte-Hermann, 2001), where the Smad family of proteins, particularly Smad4, is the key mediator of the TGF-β pathway (Lee et al., 2001; Torbenson et al., 2002; Yakicier et al., 1999; Lönn et al., 2009; Yang and Yang, 2010). The Smad4 protein is encoded by the DPC4 gene, which is located in chromosome 18q21.1 and thought to be a putative TSG, while mutating or deleting the Smad4 gene interrupts the transmission of signals from the TGF-β pathway (Hahn et al., 1996a, 1996b; Maurice et al., 2001). Loss or inactivation of Smad4 is related to pancreatic cancer (Ang et al., 2010; Hahn et al., 1996a, 1996b), colorectal cancer (Ang et al., 2010), and also occurs in some cases of extrahepatic cholangiocarcinoma (Argani et al., 2001), gastrointestinal cancers (Lei et al., 1996; Maitra et al., 2000) as well as other malignancies (Schutte et al., 1996; Miyaki and Kuroki, 2003; Waite and Eng, 2003).

In HCC, Smad4 mutations that alter the TGF-β pathway have been observed (Yakicier et al., 1999). The over-expressed Smad4 protein observed in HCC (Torbenson et al., 2002; Lu et al., 2008; Yamazaki et al., 2011) has been correlated with poor prognosis (Hiwatashi et al., 2009), and suggested to contribute to HBV-associated liver fibrosis through enhanced TGF-β signaling that pathologically accelerates collagen gene transcription (Inagaki et al., 2001; Lee et al., 2001; Yamazaki et al., 2011). Torbenson and colleagues (2002) found that Smad4 was over-expressed in 10 of 20 HCC patients, while Ji and colleagues found that the expression of Smad4 was lower in HCC tissue than in its adjacent tissue (Ji et al., 2006). Thus, the exact function of Smad4 in HCC is still controversial.

The aim of present study is to analyze the expression of Smad4 in HCC tissue as well as its adjacent tissue and compare HCC with or without viral infection to delineate the association between Smad4 and the occurrence/progression of primary HCC.

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Materials and Methods

Tumors and adjacent non-tumour tissues

This study was approved by the Ethics Committee of Second Affiliated Hospital of Harbin Medical University, and informed consent was obtained from all the patients who participated. The samples of primary liver cancer tissues and adjacent tissues were collected from 48 patients of our hospital. The tissues were immediately frozen in liquid nitrogen, and stored at -80 °C. All tissues were sectioned, stained with hematoxylin and eosin (H&E), and histopathologically confirmed as hepatic cellular carcinoma. The UICC liver cancer staging system (Santiago et al., 2011) was applied to divide the HCC tissues into stage I cancer, stage II cancer, stage III cancer, and stage IV cancer. Viral or non-viral hepatitis and AFP were routinely examined in hospitalized HCC patients. Tumor sizes were estimated by liver ultrasound or CT before surgery and directly measured after surgery. Thrombosis was primarily determined by liver ultrasound or CT before surgery combined with observations during surgery. Conditions including thrombus was removed from the portal vein or detective portal vein thrombus but unable to be removed were considered thrombosis.

Pathological determination of immunohistochemistry (IHC)

Two pathologists who were blind to the study performed the pathological analysis and determined the staining intensity. A total of 14 HCC tissue sections and their adjacent tissues were stained with the SP-9003 HistostainTM-Plus Kit (Zhongshan Goldbridge Co., Ltd, Beijing, China) and Smad4 antibody (ab40759) (Abcam, Cambridge Science Park, Cambridge, UK). Normal lung tissues served as positive controls, and normal liver tissues without primary antibody staining were negative controls. Positive cells had yellowish brown granules in the cytoplasm. The staining intensity of Smad4 in the cytoplasm was graded as: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of positive cells was also graded: 1, 0-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. To minimize the bias in the scoring, the Smad4 expression in the cytoplasm was evaluated with the immunoreactive score, calculated as staining intensity × proportion of positive cells, and scored as: 0, negative; 1-4, weak; 6-8, moderate; and 9-12, strong (Remmele and Stegner, 1987; de Caestecker et al., 2000).

Extraction of total RNA and RT-PCR

The tissue samples were ground in liquid nitrogen and then total RNA was extracted with the Tri Reagent RNA/DNA/Protein Isolation reagent (Invitrogen, Carlsbad, CA, USA). The RNA quality was monitored by the RNA/DNA ratio with the RNA/DNA calculator (Pharmacia,Uppsala, Sweden), and its integrity was detected by electrophoresis on a 1% agarose gel, the qualified preparations were subject to RT-PCR.

The RT-PCR solution from the One-step SYBR RT-PCR kit (TaKaRa, Dalian, China) was prepared on ice according with the 20 μL reaction mixture containing 10 μL of 2×One Step SYBR RT-PCR Buffer III, 0.4 μL of TaKaRa Ex Taq HS (5 U/μl), 0.4 μL of PrimeScript RT Enzyme Mix II, 0.4 μL of PCR Forward Primer (10 μM), 0.4 μL of PCR Reverse Primer (10 μM), 0.4 μL of ROX Reference Dye or Dye II (50×), 2 μL of total RNA, and 6 μL of water. The primers were synthesized by Sangon (Shanghai, China), and their sequences were: Smad4-F: 5'-CGGAATTCATGGACAATATGTCTATTACG-3'; Smad4-R: 5'-GCGGATCCTCAGTCTAAAGGTTGTGG-3'; β-actin-F: 5'-CGGTTTGGTCGTATTGGG-3'; and β-actin-R: 5'-TCTCGCTCCTGGAAGATGG-3.

The reaction mixture was vortexed and centrifuged, and then reverse transcription was performed at 42°C for three minutes and 95 °C for 10 seconds. The PCR conditions were: 40 cycles of 95 °C for five seconds and 58 °C for 30 seconds, with isolation at 95 °C for 15 seconds, at 55 °C for one minutes, and 95 °C for 15 seconds. The relative expression of Smad4 in cancer tissue in comparison to its adjacent tissue was represented using the equation $2^{-\Delta\Delta Ct}$.

Protein extraction and analysis by Western blot

Total protein was extracted by grinding 50 mg of tumor or adjacent tissues in liquid nitrogen, and then dissolving the mixture in 1 ml lysis buffer containing 150 mmol/L NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 1% (w/v) SDS, and protease inhibitors in 50 mmol/L Tris base solution (all from Sigma, St. Louis, MO, USA). The tissues were homogenized and then sonicated on ice a total of three times. The homogenate was kept on the ice for one hour, and then centrifuged at 14,000 g for 40 minutes at 4 °C. The supernatant was collected for use.

Western blots used primary antibodies against Smad4 and β-actin (ab3280) and Smad4 antibody (ab40759) (both were from Abcam), and corresponding rabbit secondary antibody (1:2000; Sigma, St. Louis, MO, USA). Representative photos were captured by the Odyssey infrared fluorescence imaging system (LI-COR, Lincoln, Nebraska, USA) and analyzed with Scion image software (Scion, Maryland, USA).

Statistical analysis

Patients' clinical characteristics and demographics were summarized as n (%) by hepatitis status; Difference among hepatitis status were compared using Fisher's exact test due to at least one of cell numbers was less than 5. The mRNA expression of Smad4 was presented as median with inter-quartiles (IQR: Q1, Q3) due to not normally distributed. Smad4 mRNA expression with considering patients'characteristics was compared using Mann-Whitney U test and Kruskal-Wallis test due to not follow normal distribution. The protein expression of Smad4 was represented as mean± standard deviations (SD) as for tumor and adjacent tissues, respectively; Smad4 protein expression was compared between tumor and adjcent tissues with paired t-test. Furthermore, Smad4 protein expression with considering patients' characteristics were compared using two-sample t-test or one-way ANOVA test. Immunohistochemistry analyses of Smad4 expression were compared with Wilcoxon signed-rank test. All statistical assessments were two-tailed and considered

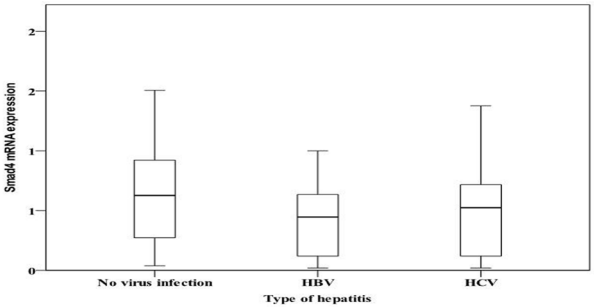


Figure 1. Distribution of Fold Change of Smad4 mRNA Expression in HCC Tissues in Comparison to Its Adjacent Tissue with Considering Types of Hepatitis by Real Time PCR. Due to the abnormality of Smad4 expression, the data were represented as Box-Plot as for the median with inter-quartiles for a given types of hepatitis. Smad4 mRNA expression in HCC was calculated as the equation of $2^{-\triangle\triangle Ct}$ of HCC and adjacent tissues. The Kruskal-Wallis test shows the Smad4 mRNA relative expression was not significantly different among types of Heptatis (P=0.567)

Table 1. Clinical Characteristics and Demographics of Patients with Hepatitis Status (N=48)

Variables	No virus infection (n=8)	HBV (n=32)	P values
Age			
<50 years (n=25)	4 (50%)	16 (50%)	0.91
≥50years (n=23)	4 (50%)	16 (50%)	
Gender			
Males (n=35)	7 (87.5%)	22 (69.8%)	0.711
Females (n=13)	1 (12.5%)	10 (31.2%)	
Tumor size			
>5cm (n=30)	3 (37.5%)	22 (68.8%)	0.347
<5cm (n=18)	5 (62.5%)	10 (31.3%)	
AFP			
>20ug/ml (n=38)	6 (75%)	26 (81.3%)	0.772
<20ug/ml (n=10)	2 (25%)	6 (18.7%)	
UICC stage			
I (n=9)	2 (25%)	6 (18.7%)	0.305
II (n=14)	0 (0%)	10 (31.3%)	
III (n=21)	5 (62.5%)	14 (43.8%)	
IV (n=4)	1 (12.5%)	2 (6.2%)	
Thrombosis			
No (n=38)	6 (75%)	25 (78.1%)	1
Yes (n=10)	2 (25%)	7 (21.9%)	
Localization			
Limited (n=37)	6 (75%)	26 (81.3%)	0.466
Infiltration (n=11)	2 (25%)	6 (18.8%)	
Differentiation			
High (n=6)	0 (0%)	6 (18.8%)	0.293
Moderate (n=39)	7 (87.5%)	25 (78.1%)	
Low (n=3)	1 (12.5%)	1 (3.1%)	

Data were summarized as n (%) by hepatitis status; Difference among hepatitis status were compared using Fisher's exact test due to at least one of cell numbers was less than 5

significant when $P < 0.05$. Statistics were analyzed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

Results

Patients' demographic and disease characteristics were summarized by hepatitis status in Table 1. There were 8 HCC patients (16.7%) were not infected by virus while 32 patients (66.7%) were infected by HBV and

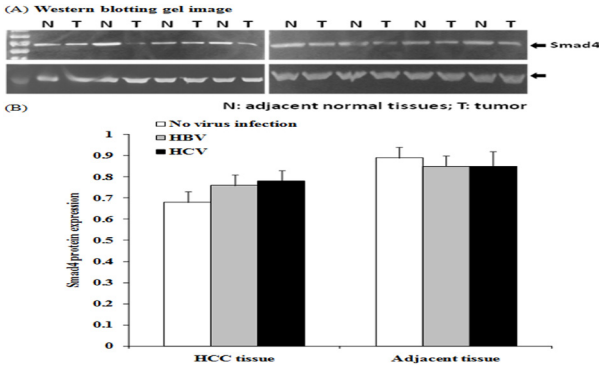


Figure 2. Distribution of Smad4 Protein Expression with Considering Types of Hepatitis. The data were represented as bar chart as for the mean±SD for a given types of hepatitis. Smad4 protein expression was calculated as ratio relative β-actin for both HCC tissue and adjacent tissue. Difference among hepatitis was compared using one-way ANOVA. The Smad4 protein expression was significantly different among hepatitis types in HCC tissue (HCC tissue: P<0.001; Adjacent tissue: P=0.164). HCC tissue and adjacent tissue from each given hepatitis types were compared with paired t-test. The Smad4 protein expression between HCC and adjacent tissues were significantly different in no virus infection and HBV patients, but not in HCV patients (No virus infection patients: P<0.001; HBV patients: P<0.001; HCV patients: P=0.128)

left 8 patients (16.7%) infected by HCV. Majority of patients with viral hepatitis were diagnosed for the first time and did not receive any specific anti-virus treatments before the samples were collected. In overall, twenty-five (52.1%) patients were younger than 50 years old while the left 23 (47.9%) patients were≥ 50 years; thirty-five (64.6%) were males and 13 (35.4%) were females. Thirty (62.5%) patients had HCC more than 5 cm in size; the cancer cells in majority patients (81.3%) were in moderate differentiation status and 25 (52%) patients were at UICC stage III or IV. Ten (20.8%) patients had thrombosis; and the cancer cells in majority patients (93.7%, 45/48) were in moderate or high differentiation status. There was no significant difference in patients' demographics and characteristics among hepatitis types (Table 1). Further analysis of the 48 HCC cases by the statues of viral hepatitis versus the UICC stage, as shown in Table 1, viral hepatitis dominantly contributed to the occurrence of HCC. However, viral hepatitis showed no obvious effect on the UICC stage in our limited number of cases (P=0.305).

We first detected Smad4 mRNA expression in tumor tissue and its adjacent tissue by RT-PCR. For all 48 HCC patients, their tumor tissues expressed average 0.47 (0.12, 0.72) times Smad4 mRNA in comparison to the adjacent tissues (Table 2). The distribution of fold change of Smad4 mRNA expression in HCC tissues in comparison to its adjacent tissue with considering types of hepatitis by real time PCR was no significant difference (Figure 1). Furthermore, the Smad4 mRNA in tumor tissues relative to its adjacent tissues were not significantly different when compared within groups by patients' demographic and disease characteristics (Table 2, all P>0.05).

Next, we examined Smad4 protein expression in HCC tissue and its adjacent tissue by Western blotting (Figure 2A). Similar to the Smad4 mRNA expression levels,

Table 2. The Expression of Smad4 Protein and mRNA were Compared Based on Patients' Characteristics (N=48)

	n	mRNA expression $2^{-\triangle\triangle Ct}$	P1	Protein expression HCC tissue	Protein expression Adjacent tissue	P2	P3	P4
All patients	48	0.47 (0.12 , 0.72)	NA	0.75 ± 0.06	0.85 ± 0.06	<0.001	NA	NA
Sex								
Males	35	0.47 (0.12 , 0.79)	0.835	0.74 ± 0.06	0.86 ± 0.06	<0.001*	0.201	0.318
Females	13	0.48 (0.09 , 0.68)		0.77 ± 0.06	0.84 ± 0.05	0.005*		
Age								
<50 years	25	0.43 (0.11 , 0.78)	0.804	0.74 ± 0.06	0.85 ± 0.06	<0.001*	0.169	0.667
≥50years	23	0.49 (0.12 , 0.67)		0.76 ± 0.06	0.86 ± 0.06	<0.001*		
Type of hepatitis								
No virus infection	8	0.63 (0.16 , 0.95)	0.567	0.68 ± 0.05	0.89 ± 0.05	<0.001*	<0.001*	0.164
HBV	32	0.45 (0.11 , 0.65)		0.76 ± 0.05	0.85 ± 0.05	<0.001*		
HCV	8	0.52 (0.12 , 0.77)		0.78 ± 0.05	0.85 ± 0.07	0.128		
Tumor size								
>5cm	30	0.42 (0.12 , 0.6)	0.123	0.75 ± 0.06	0.85 ± 0.06	<0.001*	0.697	0.689
<5cm	18	0.66 (0.28 , 0.89)		0.75 ± 0.07	0.86 ± 0.05	<0.001*		
Differentiation								
High	6	0.21 (0.09 , 0.92)	0.789	0.79 ± 0.06	0.84 ± 0.07	0.246	0.318	0.606
Moderate	39	0.48 (0.12 , 0.74)		0.75 ± 0.06	0.86 ± 0.06	<0.001*		
Low	3	0.62 (0.04 , 0.82)		0.75 ± 0.1	0.83 ± 0.07	0.443		
Localization								
Limited	37	0.45 (0.11 , 0.8)	0.922	0.76 ± 0.06	0.85 ± 0.05	<0.001*	0.436	0.4
Infiltraction	11	0.49 (0.12 , 0.62)		0.74 ± 0.05	0.87 ± 0.07	<0.001*		
Thrombosis								
Non-thrombosis	38	0.47 (0.12 , 0.76)	0.644	0.76 ± 0.06	0.85 ± 0.06	<0.001*	0.072	0.138
Thrombosis	10	0.35 (0.06 , 0.68)		0.72 ± 0.06	0.88 ± 0.04	<0.001*		
AFP level								
>20ug/ml	38	0.46 (0.12 , 0.76)	0.851	0.76 ± 0.06	0.85 ± 0.06	<0.001*	0.206	0.749
<20ug/ml	10	0.51 (0.11 , 0.68)		0.73 ± 0.06	0.86 ± 0.04	<0.001*		
UICC stage								
I	9	0.64 (0.14 , 1.18)	0.329	0.74 ± 0.06	0.88 ± 0.04	<0.001*	0.728	0.363
II	14	0.56 (0.28 , 0.87)		0.77 ± 0.06	0.84 ± 0.05	0.010*		
III	21	0.45 (0.11 , 0.56)		0.75 ± 0.06	0.86 ± 0.07	<0.001*		
IV	4	0.25 (0.04 , 1.24)		0.74 ± 0.06	0.83 ± 0.05	0.064		

Data were summarized as median (IQR: Q1 to Q3) for mRNA expression due to not follow normal distribution; mean±SD for protein expression; P1, p-value of comparison of Smad4 mRNA expression with considering patients'characteristics using Mann-Whitney U test and Kruskall-Wallis test due to not follow normal distribution; P2, p-value of comparison of Smad4 protein expression between HCC tissue and adjacent tissue for each of given patients'characteristics using paired t-test; P3, p-value of comparison of Smad4 protein expression with considering patients'characteristics in HCC tissue using two-sample t-test or one-way ANOVA test; P4, p-value of comparison of Smad4 protein expression with considering patients'characteristics in adjacent tissue using two-sample t-test or one-way ANOVA test; NA, not assessed; * p-value <0.05, indicated significant difference

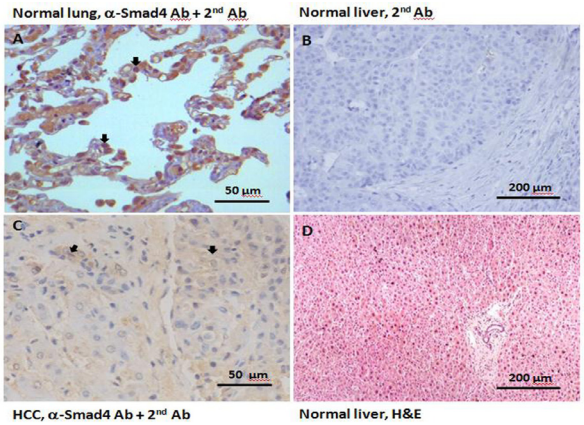
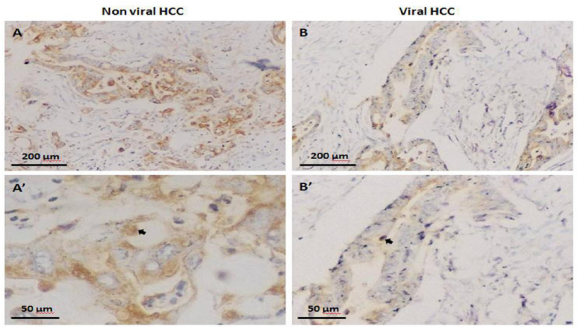


Figure 3. Immunohistochemistry Located Smad4 Protein in (A) normal lung (positive control), (B) Normal hepatocytes without primary antibody staining were negative controls, (C) HCC liver tissu, with scale bar indicating 50 μm and arrows indicating Smad4 staining, and (D) normal hepatocytes were visualized by H&E staining, with scale bar indicating 200 μm for both images

significant decreased relative protein expression was observed in the tumor tissues as compared to the adjacent normal ones (0.75 ± 0.06 vs 0.85 ± 0.06, P < 0.001) (Table 2). Moreover, the Smad4 protein in tumor tissues was found significantly decreased while compared to its adjacent tissues. Interesting, the Smad4 protein in HBV- or HCV-infected tumor tissues was found significantly increased in comparison to no virus infection tumor tissues (0.76 ± 0.05 or 0.78 ± 0.05 vs 0.68 ± 0.05, P<0.001) (Table 2, Figure 2B)

Localization of Smad4 in hepatocytes was visualized by immunohistochemistry analysis. It was found that tumor tissues had an altered Smad4 distribution, which is more abundant in the cytoplasm and less in the nucleus (Figure 3). Smad4 distribution in hepatocytes of viral or non-viral HCC was further examined. For tumor tissues that were not infected by hepatitis virus had weak and more diffused Smad4 expression pattern (Figure 4 A and A'); tumor tissues infected by hepatitis virus exhibited a stronger and more nucleus-restricted pattern of Smad4



C.

	Smad 4				
Tissues	Negative	Weak	Moderate	Strong	P-value
Tumor (n=14)	11 (78.7)	1 (7.1)	1 (7.1)	1 (7.1)	<0.001*
Normal (n=14)	1(7.1)	2 (14.3)	5 (35.7)	6 (42.9)	

Figure 4. Immunohistochemistry Images of Non-viral (A, A') and Viral HCC (B, B') Stained with Smad4 Expression. (C) The summarization of immunohistochemical staining of Smad 4 in 14 patients whose tumor and adjacent tissues were available. The bars in (A) and (B) indicate 200 μm, and the bars in (A') and (B') indicate 50 μm. The arrows in (A') and (B') indicate z

staining (Figure 4 B and B'). The distribution of Smad 4 expression in HCV- and HBV-infected tumor tissues was not obviously different, majorly detected in the nucleus while majority staining of Smad 4 in tissues from non-viral tumor tissues was detected in the cytoplasm. A statistical analysis of the IHC results based on the intensity of Smad 4 proteins was performed in 14 patients whose tumor and adjacent tissues were available for IHC. The intensity of Smad4 expressions in adjacent tissues was significantly stronger compared to the tumors. In our data, tumor tissues dominantly revealed a negative to weak intensity of Smad4 expression (Figure 4C).

Finally, we analyzed overall survival and recurrence with considering Smad4 mRNA expression, and Smad4 protein expression. Among the 48 subjects, 7 subjects were lost follow-up. For the 41 subjects, 15 subjects (36.6%) had recurrence after operation and 12 subjects (29.3%) died after operation. Furthermore, we only had 35 subjects who recorded time to recurrence and 37 subjects who recorded time to overall survival. The mean time for recurrence and overall survival was observed as 63.2 months and 75.1 months, respectively. The estimated rate for one-year, two-year, three-year and five-year to recurrence were observed as 97.1%, 87.6%, 68.3%, and 56.9%. The estimated rate for 6 months, one-year, two-year, three-year, and five-year to overall survival were observed as 94.6%, 91.9%, 82.9%, 78.0%, and 73.2%. Table 3 presents the results of univariate cox-regression analysis of time-related data to overall survival and recurrence with considering Smad4 mRNA and protein expressions and clinical characteristics. It only shows the AFP level might be associated with overall survival [HR=0.23 (0.06 – 0.92), P<0.05]. However, there was no significant association found between Smad4 expression and overall survival or between Smad4 expression and time to recurrence (Table 3).

Table 3. Univariate Cox-regression Analysis of Time-related Data to Overall Survival and Recurrence with Considering Smade 4 Expression and Clinical Characteristics (N=48)

Variables		Overall survival HR (95%CI)
Smad4 mRNA expression		2.22 (0.63 – 7.80)
Smad4 protein expression of HCC tissue		1.30 (0 – 1.5×10^5)
Age	<50 years	Reference
	≥50years	1.49 (0.36 – 6.26)
Gender	Females	Reference
	Males	0.94 (0.19 – 4.67)
Type of hepatitis	No virus infection	Reference
	HBV	0.29 (0.06 – 1.46)
	HCV	0.63 (0.11 – 3.79)
Tumor size	<5cm	Reference
	>5cm	1.00 (0.24 – 4.17)
AFP	<20ug/ml	Reference
	>20ug/ml	0.23 (0.06 – 0.92)*
UICC stage	I	Reference
	II	1.66 (0.19 – 14.9)
	III	0.89 (0.09 – 8.55)
	IV[a]	Not derived
Thrombosis	No	Reference
	Yes	2.62 (0.61 – 11.4)
Localization	Limited	Reference
	Infiltration	2.49 (0.59 – 10.5)
Differentiation	Low	Reference
	Moderate[b]	Not assessed
	High[b]	Not assessed

Results were shown as estimated hazard ratio (HR) with 95% confidence interval for HR (95%CI) for outcomes, overall survival and recurrence, respectively; [ab]Not derived for a UICC stage IV or not assessed for b differentiation due to too low number for analysis; *P<0.05, indicated significantly association

Discussion

The current study investigated the expression of Smad4 in HCC patients with or without viral hepatitis. The results showed that the expressions of Smad4 mRNA and protein levels were both significantly down-regulated in tumor tissues compared with adjacent tissues, which suggests that suppressed expression of Smad4 is closely related to HCC and that Smad4 functions as a tumor suppressor gene. Interestingly, we found HBV or HCV virus infection contribute to the induction of Smad4 protein expression in HCC tissues (Figure 2B).

The decreased Smad4 expression in tumor tissues agrees with previous work on HCC, which also found that Smad4 had low expression in HCC tumors (Lu et al., 2008). Smad4 acts on TGF-β, which has a complex role in cancer progression, acting as a tumor suppressor in early stages and a pro-tumorigenic factor at late stages (Massagué, 2008; Pardali and Moustakas, 2007). Smad4 mediates TGF-β signaling to suppress tumorigenesis, though its actions vary with the extracellular matrix, ligand concentration, and specific cofactors at different developmental stages (Yang and Yang, 2010).

The higher Smad4 protein levels in patients with viral hepatitis suggest that Smad4 expression might be also modulated by hepatitis viruses, and that increased Smad4 expression in patients with viral hepatitis predicts a higher risk for HCC than for those without hepatitis. This increased risk may be due to the close relationship

between hepatic fibrosis and inflammatory damage to the liver. It is supported by in vitro data that suggests hepatitis to facilitate liver fibrosis by upregulating TGF-β (Guo et al., 2009). The ability of HBV to promote the nuclear translocation of Smad proteins may enhance TGF-β signaling, which may contribute to HBV-associated liver fibrosis and the chronicity of hepatitis B (Lee et al., 2001). Moreover, Hepatitis B virus p X was found to facilitate the nuclear translocation of Smad4 protein even independent of TGF-β, and it enhanced fibrogenic Smad2/3 signaling on TGF-β stimulation (Lee et al., 2001). Further studies showed that chronic inflammation associated with HCV infection could shift hepatocytic TGF-β signaling from tumor-suppression to fibrogenesis, accelerating liver fibrosis and increasing risk for HCC. Since TGF-β plays "double-edged sword" effects in carcinogenesis. During the stage of tumor initiation and early progression, TGF-β serves more likely as a tumor suppressor by inhibiting proliferation and accelerating apoptosis. When tumors come to the later stages of progression, activation of TGF-β may promote tumor formation by facilitating migration, invasion, and angiogenesis. The hepatocytes affected by HCV related chronic inflammation may undergo a transition from the tumor-suppressive Smad pathway to fibro-carcinogenic Smad pathway. Thus the activation of TGF-β-Smad4 signal pathway in patients with hepatitis may be associated with inflammation and poor prognosis. In one word, Smad4 may have harmful instead of protective functions in HCC with hepatitis viral infection present.

The patients with thrombosis had lower Smad4 levels than those without thrombosis. Previous studies showed that when Smad4 is over-expressed, the platelet-derived growth factor B is induced by TGF-β in vascular endothelial cells and suggested that the Smad proteins may play a role in the vascular response to injury (Taylor and Khachigian, 2000). Our data suggested that Smad4 levels are lower in patients with thrombosis which were in line with previous findings. Previous reports found the expression of Smad4 in tumor tissue was contradicted (Torbenson et al., 2002; Ji et al., 2006). Our studies found Smad4 was decreased in tumor tissues in comparison to the adjacent tissue. This might be due to heterogeneous characteristics of HCC tissue. In this study, we found hepatitis virus-infected HCC tissue would express more Smad4 protein in comparison with those tissues without hepatitis infection.

The aberrant expression of Smad4 which we detected in different liver tissues may indicate the cell phenotype transforming from hepatitis to hepatic cirrhosis to HCC. However, the potential molecular mechanisms are still largely unknown. More studies are required to elucidate the underlying mechanisms. This study is limited by its descriptive nature and by a lack of detailed data on the prognosis of the patients. Furthermore, it will be interesting to elucidate the roles of Smad4 in HCC with hepatitis virus infection.

Strong expression of Smad4 in HCC patients has been correlated with poor prognosis after surgery (Hiwatashi et al., 2009). Together with our findings, Smad4 levels are affected by HCC, by infection with hepatitis virus, and by the presence of thrombosis. Collectively, Smad4 may have potentials to serve as biomarkers for prognosis, diagnosis as well target to develop pharmaceutical agents for HCC.

Acknowledgements

This study was supported by Heilongjiang Department of Health Funded Project (2010-081) and Harbin Science and Technology Commission Outstanding Subject Leaders Project (2012RFXYS015). We declare that there is no competing interest in the submission and no funding support was received for the study.

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