Expression and significance of caveolin-1 in hepatitis B virus-associated hepatocellular carcinoma

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Abstract. Caveolin-1 (Cav-1) is a major component of caveolae and has been recently identified as a tumor suppressor. As little is known about Cav-1 in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC), the aim of the present study was to investigate the expression and significance of Cav-1 in HBV-associated HCC. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect the mRNA expression level of Cav-1 in 40 cases of HBV-associated HCC, the corresponding 11 non-tumor cases of HBV-associated chronic hepatitis, 29 non-tumor cases of HBV-associated cirrhosis and 6 cases of normal liver tissues. Immunohistochemical analysis indicated the expression of Cav-1, cluster of differentiation 34 and vascular endothelial growth factor (VEGF) in HBV-associated HCC tissue samples. In addition, the association of Cav-1 expression with angiogenesis and clinicopathological characteristics of HBV-associated HCC was also analyzed. RT-PCR results demonstrated that the expression rate of Cav-1 mRNA in HBV-associated HCC, non-tumor HBV-associated chronic hepatitis and cirrhosis liver tissues and control normal liver tissues from patients with metastatic carcinoma was 92.5, 85.0 and 16.7%, respectively. mRNA expression level of Cav-1 was significantly increased in chronic hepatitis, cirrhosis and HBV-associated HCC livers compared with normal control livers ($P<0.05$ and $P<0.01$, respectively). Cav-1 protein was detected by immunohistochemistry in 80% of the samples of HBV-associated HCC. Furthermore, Cav-1 and VEGF protein expression levels were correlated with microvessel density (MVD; $\gamma_s<0.46$, $P=0.01$ and $\gamma_s<0.31$, $P=0.05$, respectively). In addition, Cav-1 expression and MVD were significantly associated with metastasis ($P=0.031$ and $P=0.046$, respectively). In conclusion, Cav-1 may have an important role in the carcinogenesis and progression of HBV-associated HCC and angiogenesis may be affected by Cav-1 during this process.

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Introduction

Caveolae are 50-100-nm in size, non-clathrin-coated, flask-shaped invaginations of the plasma membrane that are involved in vesicular transport and signal transduction (1). Caveolins (CAVs) were identified as essential proteins involved in the formation of invaginations (2). Numerous studies have indicated that CAVs associate with several signaling factors, including heterotrimeric G-protein α-subunits, endothelial nitric oxide synthase (eNOS), receptor and non-receptor tyrosine kinases and protein kinase C (3-5). A previous study have suggested that signaling interactions of CAVs with these factors are mediated by the CAV scaffolding domain, which is a membrane-proximal region (residues 82-101 in Cav-1) of the CAVs (6). Currently, Cav-1, -2 and -3 have been identified as members of the CAV family (1). Cav-1 also exists in non-caveolar, cellular or extracellular forms (1). The Cav-1 isoform is particularly abundant in endothelial cells (ECs), where it regulates various functions, including transcytosis, permeability, vasculartone and angiogenesis (7). Previous results have demonstrated that Cav-1 is a growth-inhibitory protein that may act as a tumor suppressor (8,9). Cav-1 expression is downregulated in some forms of cancer, including mesenchymal tumors and sarcomas (8,10); however, in other cancer types; for example, oral squamous cell carcinoma, Cav-1 expression is high (11,12). These findings suggest that Cav-1 has multiple actions in human cancer cells.

Angiogenesis is the process of generating novel blood vessels derived as extensions from the existing vasculature (13). The principal cells involved are ECs, which line all blood vessels and constitute virtually the entirety of capillaries (13). Non-caveolar Cav-1 has an important role in the regulation of EC proliferation, differentiation and tube formation (14). In addition, eNOS is a CAV-interacting protein that has a central role in angiogenesis (15), and Cav-1 abundance and its cellular distribution in ECs may be altered in nitric oxide (NO)-mediated angiogenesis (16). Our previous experimental study demonstrated that Cav-1 was important for

NO-mediated angiogenesis (17). However, the exact molecular mechanisms of Cav-1 in the process of angiogenesis have not been thoroughly explored.

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, particularly in the Asia Pacific region (18). Due to late diagnosis and high rate metastasis of HCC, HCC is still associated with poor survival rate (18). At present, the 5-year survival rate of individuals with HCC is very low at 34% (19). The major risk factor for HCC in China is infection with hepatitis B virus (HBV) (20).

The aim of the present study was to investigate the expression and significance of Cav-1 in HBV-associated HCC. TRIzol reagent was used for RNA extraction; semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and the mRNA expression evels of Cav-1 mRNA were detected. Immunohistochemistry analysis and microvessel counting was used for exploring the expression of Cav-1, cluster of differentiation (CD)34 and vascular endothelial growth factor (VEGF).

Materials and methods

Patients and tissue collection. The present study was approved by the Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China), and informed written consent was obtained from all subjects. Tissue samples, including HBV-associated HCC, non-tumor HBV-associated chronic hepatitis and cirrhosis, were all obtained from 40 patients with HBV-associated HCC (33 males and 7 females) who had consecutively undergone surgical resections between June 2002 and June 2006 in our hospital. The patients were selected according to the following criteria: i) having primary HCC, ii) having a history of HBV infection and tested positive for serum hepatitis B surface antigen (HBsAg). All the 40 patients were diagnosed and histopathologically confirmed with HCC (40 patients), including chronic hepatitis (11 patients) and cirrhosis (29 patients). The control normal liver (non-tumor) specimens were obtained from patients (n=6; Group 1) with metastatic liver carcinoma without HBV infection. The corresponding non-tumor tissues were obtained from the same 40 patients with HBV-associated HCC, which were subsequently divided into the HBV-associated chronic hepatitis group (n=11; Group 2) and cirrhosis group (n=29; Group 3). The patients' clinical records and histopathologic diagnoses were fully reviewed. The mean age of the patients was 50±11 years. Tumor size varied from 2-15 cm in diameter and the tumor diameter was determined as the longest diameter of the specimen measured at the time of pathological examination. Serum α-fetoprotein concentrations were measured using an ELISA kit (EL0018; Huzhou Innoreagents Co., Ltd., Huzhou, China) according to the manufacturer's instructions, with normal AFP concentration defined as <20 ng/ml. Cancer tissues and adjacent non-tumor liver tissues of the 40 patients with HBV-associated HCC were excised from each surgical specimen immediately after liver resection. Half of the tissue was flash-frozen in liquid nitrogen for RT-PCR analysis. The other half of the tissue was fixed in 10% neutral formalin for 2 weeks at 4˚C and subjected to histopathological examination and immunohistochemical study. All the tissues were prepared from paraffin blocks as described below. The scoring system of pathological grade and differentiation was performed according to Edmonson Steiner grading system proposed in 1954. The following scoring was applied: Grade 1, minor differentiation between tumor cells and hyperplastic liver cells; Grade 2, tumor cells resemble normal hepatic cells while the nuclei are larger and more hyperchromatic, and cell characteristics indicate sharp, clear-cut borders; Grade 3, larger and more hyperchromatic nuclei are present with a higher proportion of nuclei to existing cytoplasm; and Grade 4, cells are filled with nuclei that are intensely hyperchromatic. The diagnosis of tumors with cancerous thrombi in the portal vein or intrahepatic metastasis by computed tomography in HCC met the diagnosis criteria of the American Association for the study of Liver Diseases (21).

RNA extraction. Total RNA from frozen tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration of RNA extracted was determined at wavelength of 260 nm using a spectrophotometer (Eppendorf, Hamburg, Germany).

Semi-quantitative RT-PCR. The cycle number was set to 28, 30, 34 and 36 to determine the plateau (22). cDNA were synthesized using a Reverse Transcription System (Promega Corp., Madison, WI, USA), according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed to first-strand cDNA in 20 μl of mixture containing 25 mM $MgCl_2$ (4 μl), reverse transcription 10X buffer (2 μl), 10 mM dNTP mixture (2 μl), recombinant RNase inhibitor (0.5 μl), AMV reverse transcriptase (15 μl and Oligo (dT) primers (0.5 μg). The reaction conditions were as follows: 42˚C for 60 min, followed by 95˚C for 5 min. Semi-quantitative analysis for the expression of Cav-1 mRNA was performed using RT-PCR technique and β-actin used as an internal control (22). PCR was performed using the following program: 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, 58˚C for 60 sec and 72˚C for 45 sec. The primers for RT-PCR were as follows: Cav-1, forward 5'-GACTTTGAAGATGTGATTGC-3' and reverse 5'-AGATGGAATAGACACGGCTG-3'; and β-actin, forward 5'-CTACAATGAGCTGCGTGTGGC-3' and reverse 5'-CAG GTCCAGACGCAGGATGGC-3'. The PCR products were 254 and 275 bp, respectively. RT-PCR was performed using a DNA thermal cycler MJ Research PTC-200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR samples were analyzed using a 1% agarose gel and visualized by ethidium bromide staining. The intensity of the bands was measured by densitometry utilizing Tobias TBX1000 scanning densitometer (Tobias Associates, Inc., Miami Beach, FL, USA).

Immunohistochemistry and microvessel counting. A light microscope was utilized in the following experiment. Serial sections (4-μm thick) from tumor and corresponding non-tumor tissues tissue samples obtained from all patients previously fixed in formalin were prepared from paraffin blocks. Sections were deparaffinized and rehydrated in Tris-buffered saline. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature. Antigen retrieval was performed by microwave pretreatment with Trisodium citrate 2.94 g, 0.2 M hydrochloric acid solution (22.0 ml), UltraPure sterile

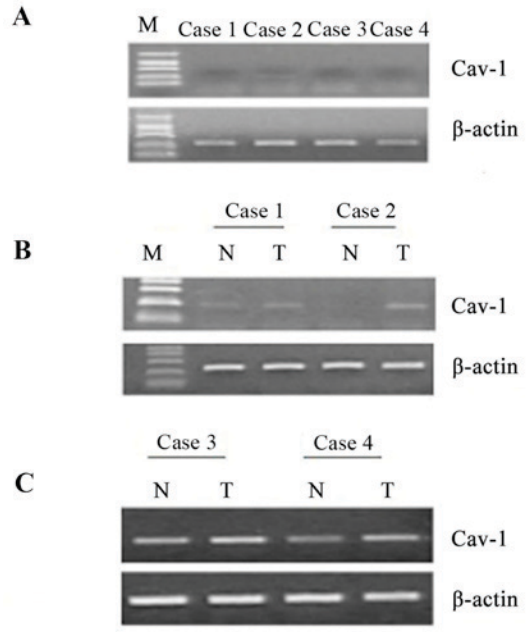

Figure 1. Cav-1 mRNA expression level in (A) non-tumor normal liver tissue from 4 cases of patients with metastasis carcinoma without HBV infection. Cav-1 mRNA expression levels in HBV-associated HCC tumor and adjacent non-tumor in 2 cases of patients with (B) HBV-associated chronic hepatitis and (C) HBV-associated cirrhosis tissues were compared. The mRNA expression levels of Cav-1 were normalized to β-actin expression and tests were performed in triplicate. Cav-1, caveolin-1; M, maker; N, non-tumor liver tissue; T, tumor tissue.

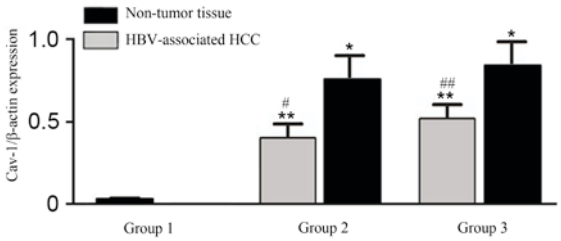

Figure 2. mRNA expression levels of Cav-1 relative to β-actin in groups 1-3 comparing their respective non-tumor groups with HBV-associated HCC tumor. Data are presented as mean + standard deviation (n=3). *P<0.05 and **P<0.01 vs. group 1 non-tumor tissue; $^{\#}$P=0.019 vs. group 2 non-tumor tissue; $^{\#\#}$P=0.045 vs. group 3 non-tumor tissue. Cav-1, caveolin-1; HCC, HBV-associated hepatocellular carcinoma. Group 1, non-tumor normal liver tissue from patients with metastasis carcinoma without HBV infection; group 2, non-tumor liver tissue from patients with HBV-associated chronic hepatitis; group 3, non-tumor tissue from patients with HBV-associated cirrhosis.

water, 0.1 M sodium hydroxide 1N solution (pH=13, 0.1 M hydrochloric acid solution (pH=1, xylene and methanol under 98˚C prior to staining. Non-specific binding was blocked with 5% normal bovine serum (Jackson ImmunoResearch Laboratories, Inc., Shanghai, China) for 10 min at room temperature. The β-actin was used as internal reference obtained from GenScript Co. Ltd. (Nanjing, China; cat. no. A00702-100; 1:20). Subsequently, sections were incubated with anti-CD 34 (1:30; cat. no. MS-363-P0; Leica Microsystems Ltd., Milton Keynes, UK) at room temperature for 45 min, and anti-Cav-1 (1:100; cat. no. 610406; BD Biosciences; Franklin Lakes, NJ, USA) and anti-vascular endothelial growth factor (VEGF; 1:200; cat. no. SC-7269; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. Subsequently, corresponding secondary biotinylated immunoglobulin was applied and then reacted with a streptavidin biotinylated horseradish peroxidase complex (1:5,000; cat. no. ab96895; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at 37˚C for 30 min. The sections were stained with a freshly prepared diaminobenzidine solution and then counterstained with Mayer's hematoxylin. Negative control was obtained by omitting the primary antibodies. A semi-quantitative system was employed to evaluate the level of Cav-1 and VEGF expression: Intensity was scored as absent (grade 0), weakly positive (grade 1), moderately positive (grade 2) or strongly positive (grade 3) based on the proportion (percentage of positive cells) and intensity, as described previously (23). Microvessel density (MVD) was determined with CD34-stained slides using the procedure outlined by Weidner *et al* (24). Individual microvessels were counted in the area of highest vascularity at magnification x200 in three selected microscopic fields. The microvessel count was expressed as the mean number of vessels in the selected area.

Statistical analysis. All data were expressed as the mean ± standard deviation as indicated. Data were analyzed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were made between two groups using the Student's test and between multiple groups with one-way analysis of variance followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cav-1 mRNA expression levels. Cav-1 gene expression was evaluated in different types of liver diseases Cav-1 mRNA expression was detected in 1/6 (16.7%) control non-tumor normal liver tissues from patients with metastatic carcinoma without HBV infection, 34/40 (85.0%) non-tumor liver tissues and 37/40 (92.5%) HBV-associated HCC. The Cav-1 mRNA expression levels in the control normal liver tissues were negative or very low (Figs. 1 and 2). However, the expression level of Cav-1 mRNA was increased in HBV-associated chronic hepatitis (Group 2), HBV-associated cirrhosis (Group 3) and HBV-associated HCC compared with non-tumor normal liver tissue from patients with metastatic liver carcinoma without HBV infection (Group 1; Figs. 1 and 2). The expression levels of Cav-1 mRNA in HBV-associated chronic hepatitis and cirrhosis were significantly elevated compared with that in non-tumor normal liver tissue from patients with metastatic carcinoma without HBV infection (P<0.001); however, there was no significant difference between HBV-associated chronic hepatitis and cirrhosis (P=0.076). The expression levels of Cav-1 mRNA in HBV-associated HCC were significantly decreased compared with that of corresponding non-tumor tissues in HBV-associated chronic hepatitis and cirrhosis (P=0.019 and P=0.045, respectively). But there was no significant difference in the expression levels of Cav-1 mRNA in HBV-associated HCC between Group 2 and Group 3 (P=0.41; Fig. 2).

Immunohistochemical analysis for Cav-1 and VEGF in HBV-associated HCC and their correlation with angiogenesis. Cav-1 was expressed primarily in the cytoplasm of tumor cells

Table I. Association between Cav-1 mRNA expression and MVD and clinicopathological features of the patients.

Variables	n	Cav-1 expression[a]	P-value	MVD	P-value
Age, years			0.72		0.45
<60	26	0.72±0.18		148.4±14.1	
>60	14	0.65±0.17		139.3±10.3	
Gender			0.42		0.67
Male	33	0.68±0.20		143.3±17.4	
Female	7	0.75±0.13		154.4±20.1	
α-fetoprotein, μg/l			0.12		0.98
<363	16	0.63±0.19		141.1±16.4	
≥363	24	0.73±0.12		148.3±19.2	
Tumor diameter, cm			0.25		0.37
<5	23	0.65±0.21		138.1±18.8	
≥5	17	0.75±0.11		152.6±17.1	
Edmondson grading system			0.09		0.05
I+II	24	0.63±0.18		134.9±24.1	
IIIa+IIIb	16	0.78±0.12		163.2±14.7	
Differentiation			0.06		0.41
Well-differentiated	6	0.60±0.10		141.7±20.4	
Moderately + poorly differentiated	34	0.71±0.12		149.3±17.7	
Metastasis			0.03		0.05
Yes	19	0.80±0.18		164.8±33.9	
No	21	0.59±0.11		127.2±16.5	

[a]Relative to β-actin. Data are presented as the mean ± standard deviation. Cav-1, caveolin-1; MVD, microvessel density; Yes, tumors with cancerous thrombi in portal vein or intrahepatic metastasis; No, tumors without cancerous thrombi in portal vein or intrahepatic metastasis.

obtained from HBV-associated HCC tissues, as evidenced by the presence of stained granular immunoreaction products. The majority of tumors exhibited extensive staining for VEGF: seven tumors (17.5%) were grade 0, nine (22.5%) were grade 1, 13 (32.5%) were grade 2 and 11 (27.5%) were grade 3 (Fig. 3A and B). Cav-1 immunoreactivity was indicated in 32/40 (80%) HBV-associated HCC tissues: 8 tumors (20%) were grade 0, 11 (27.5%) were grade 1, 9 (22.5%) were grade 2 and 12 (30%) were grade 3 (Fig. 3C and D). MVD was 145.2±16.2 (Fig. 4). The expression levels of Cav-1 and VEGF significantly correlated with MVD (r_s=0.46, P=0.01; and r_s=0.31, P=0.05, respectively), which was not shown in figure.

Correlation of the expression of Cav-1 mRNA and MVD with clinicopathological characteristics of HBV-associated HCC. The patients' clinical records and histopathologic diagnoses were fully reviewed. As demonstrated in Table I, the expression levels of Cav-1 mRNA and MVD exhibited a significant association with metastasis (P=0.031 and P=0.046, respectively). However, no significant association was noted between Cav-1 mRNA expression levels and MVD and the other clinicopathological variables.

Discussion

A study by Koleske *et al* (25) demonstrated that Cav-1 mRNA and protein expression levels were reduced in NIH 3T3 cells transformed by various oncogenes, and caveolae were also absent from these transformed cells. A previous study has indicated that Cav-1 gene mutations were identified in breast carcinomas and that the Cav-1 gene localizes to a suspected tumor suppressor locus on chromosome 7q31.1, which is commonly deleted in a variety of types of human cancer (26). Prior reports have suggested that Cav-1 may function as a tumor suppressor gene (27,28). However, this finding is inconsistent with the fact that Cav-1 is highly expressed in multiple cancer types and cancer cell lines (29,30). In the present study, positive Cav-1 mRNA expression was detected in 37/40 tumors, and the expression of Cav-1 was elevated in HBV-associated HCC compared with non-tumor liver tissues from patients with metastatic liver carcinoma without HBV infection. In addition, 32/40 (80%) HBV-associated HCC specimens indicated Cav-1 immunoreactivity in tumor cells. These results suggested that increased Cav-1 expression was detected in HBV-associated HCC compared with the non-tumor tissue in group 1. Additionally, the results of the present study also demonstrated that Cav-1 expression was downregulated in HBV-associated HCC tissues compared with the corresponding non-tumor tissues. The exact reason of the decreased expression levels in HBV-associated HCC compared with corresponding non-tumor tissue was unclear; however, a previous study demonstrated that Cav-1 gene disruption was involved in promoting mammary tumor growth and enhancing cell metastasis (31), which may explain

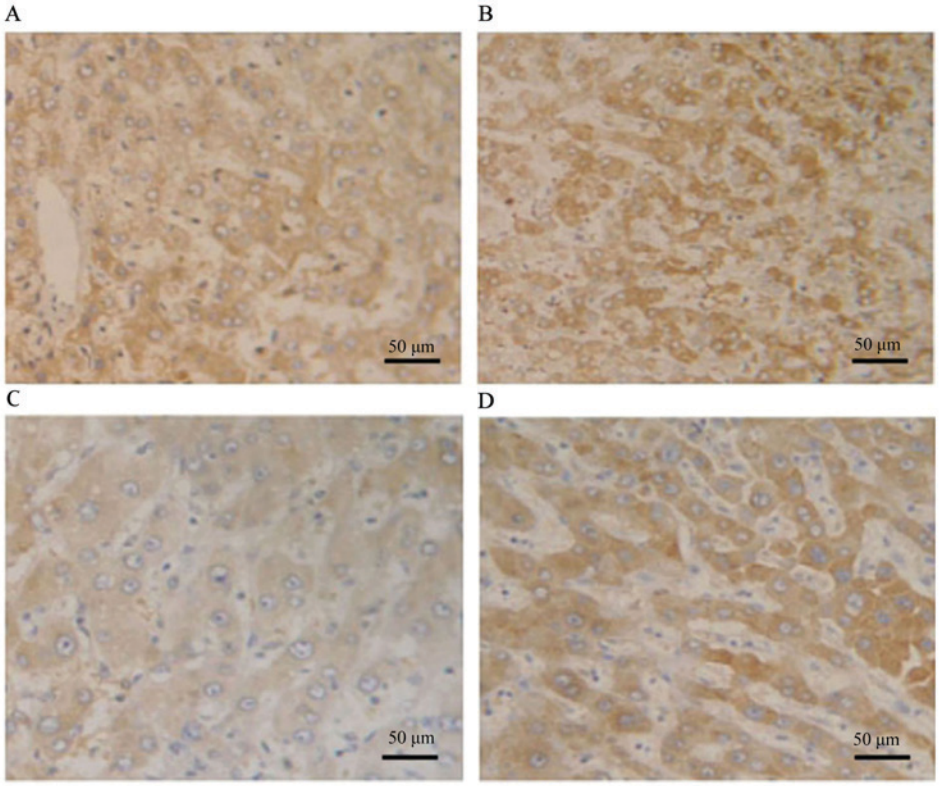

Figure 3. VEGF and Cav-1 immunostaining (magnification, x200). (A and B) VEGF and (C and D) Cav-1 were measured using a hematoxylin counterstain assay. Cav-1 immunoreactivity was detected predominantly in the cytoplasm of tumor cells. Consecutive sections of hepatitis B virus-associated hepatocellular carcinoma specimens were classified as grade 1 (C), grade 2 (A and D) and grade 3 (B). VEGF, vascular endothelial growth factor; Cav-1, caveolin-1.

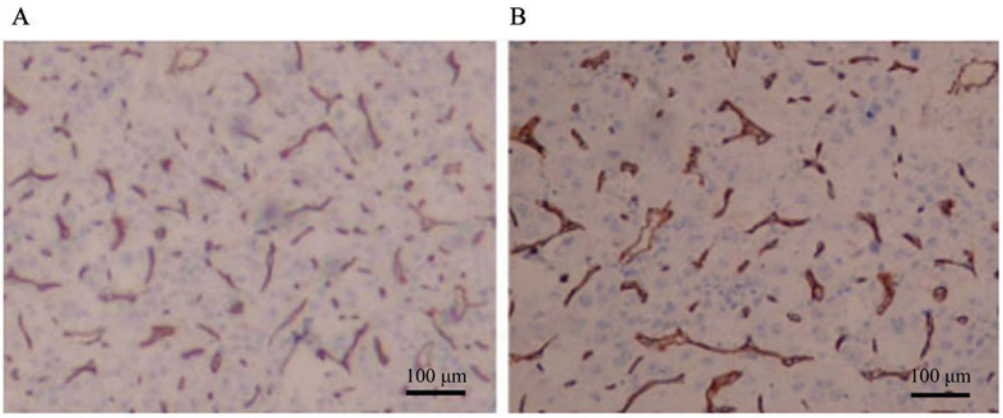

Figure 4. Microvessel immunoperoxidase staining. Microvessels were highlighted by staining endothelial cells with cluster of differentiation 34, using a standard immunoperoxidase technique. (A) Low-power view and (B) High-power view of intensively stained microvessels in a hepatitis B virus-associated hepatocellular carcinoma specimen.

the reduced Cav-1 expression observed in the HBV-associated HCC specimens. These effects of Cav-1 in different cancer specimens may be mediated by different regions of the Cav-1 molecule (32). Although various studies have suggested the correlation of Cav-1 with cancer, the exact role of Cav-1 in cancer remains to be elucidated (32).

Numerous studies have demonstrated that Cav-1 may have an important role in the carcinogenesis of HCC (33,34). A study previously demonstrated that the expression of Cav-1 in cirrhotic livers was markedly enhanced at the protein and mRNA levels, whereas Cav-1 was almost undetectable in control liver tissue (35). Macroregenerative and dysplastic nodules (MDNs) are HCC precursor lesions and exhibit distinct vascular profiles relative to adjacent cirrhotic liver (36). It was determined that Cav-1 expression levels increased during the progression from normal to cirrhotic liver, and further increased in MDNs, whereas hepatitis C virus (HCV)-associated HCC liver exhibited similar or decreased Cav-1 expression relative to adjacent non-neoplastic liver (37). These findings suggested that Cav-1 may have a direct role in malignant transformation of hepatocytes. However, these studies were focused on HCV-associated tissues. The present results demonstrated that

the expression of Cav-1 was upregulated in HBV-associated HCC and the percentage and level of detectable Cav-1 mRNA was increased in non-tumor and HBV-associated HCC liver tissues compared with normal liver tissues. From the present results, it may be concluded that Cav-1 has a direct role in the malignant transformation of HBV-associated HCC.

Cav-1 has been identified to be a metastatic-associated gene with an independent prognostic value for various types of cancer (29,33,38). A study by Williams *et al* (39) revealed that loss of Cav-1 attenuated prostate development by significantly reducing primary tumor burden and metastatic disease in a transgenic prostate cancer model. However, another study demonstrated that Cav-1 gene disruption promoted mammary tumor growth and enhanced cell metastasis (31). These studies indicate that Cav-1 has tissue-or cell type-specific roles with regard to tumorigenesis. In the present study, the expression of Cav-1 was significantly correlated with metastasis, which suggests that Cav-1 may act in the progression of HBV-associated HCC.

The role of Cav-1 in angiogenesis has only been partially defined. A previous study indicated that angiogenic inhibition in pancreatic cancer was associated with the upregulation of Cav-1 (40). Furthermore, endothelial-specific expression of Cav-1 has been suggested to impair eNOS activation, endothelial barrier function and angiogenic responses to exogenous VEGF (41). Additionally, other research has shown that Cav-1 was essential for capillary formation but had different roles depending on the stage of angiogenesis (42).

HCCs are hypervascular tumors that exhibit distinctive vascular profiles relative to the surrounding liver in which they arise (36). HCV-associated HCC, in which Cav-1 may be involved, was indicated to be associated with angiogenesis in a previous study (36). A study by Mazzanti *et al* (43) demonstrated that angiogenesis was significantly varied in HCV-positive patients compared with HBV-infected subjects or controls. In light of these findings, the present study investigated the association between the expression of Cav-1 and angiogenesis in HBV-associated HCC. It has been demonstrated that VEGF-stimulated phosphorylation of extracellular signal regulated kinase 1/2 and eNOS was abrogated in $Cav^{-/-}$ ECs, but enhanced in the $Cav^{+/+}$ mice and ECs (41,44). Cav-1 expression is critical for VEGF-induced angiogenesis (35). MVD, an indicator of angiogenesis, has been correlated with Cav-1 expression in clear cell renal cell carcinoma (45). Similarly, the present study demonstrated a strong association between Cav-1 expression and MVD in HBV-associated HCC patients. As angiogenesis is fundamental to the growth and metastasis of solid tumors, we speculate that the increasing level of Cav-1 may act in the progression of HBV-associated HCC by affecting angiogenesis.

In conclusion, the present results indicated that Cav-1 may have an important role in the carcinogenesis and progression of HBV-associated HCC. The results suggested that Cav-1 may be associated with angiogenesis of HCC, and therefore Cav-1 may be an important target of anti-angiogenic therapy of HCC.

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