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The relationship between integrin avß6 and HBV infection in patients with liver cirrhosis and hepatocellular carcinoma: a preliminary report

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ABSTRACT

Objective: the aim of this study was to investigate the expression of integrin αvβ6 in normal, hepatitis B, HBV-associated cirrhosis and HBV-associated HCC liver tissues.

Methods: immunohistochemistry and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) were used to study the expression of integrin αvβ6 in HBV-associated cirrhosis (*n* = 88), chronic hepatitis B (*n*= 11), HBV-associated HCC (*n* = 84) and normal (*n* = 10) human liver tissues.

Results: the expression of integrin αvβ6 was significantly upregulated in HBV-associated liver cirrhosis and the expression increased with an increase in severity of cirrhosis. Furthermore, it was moderately or weakly expressed in chronic hepatitis B and HBV-associated HCC liver tissues when compared to normal liver tissue.

Conclusion: integrin αvβ6 could be a predictive marker for the progression of liver cirrhosis associated with HBV infection. Further studies are needed to determine the association between the expression of integrin αvβ6 in hepatitis B and HBV-

associated HCC liver tissues.

Keywords: Hepatitis B. Hepatitis B virus. Liver cirrhosis. Integrin beta chain. Hepatocellular carcinoma.

INTRODUCTION

In 2015, approximately 240 million people were diagnosed with a chronic hepatitis B virus (HBV) infection worldwide (1). Approximately 75 % of people infected with HBV reside in the Asia-Pacific region, resulting in a huge challenge for public health (2). Furthermore, 15-40 % of patients with chronic HBV infection eventually develop hepatic cirrhosis (3,4). 10-25 % of chronic HBV carriers develop hepatocellular carcinoma (HCC) in their lifetime (5,6). The development of HBV-associated hepatic cirrhosis has a strong association with the level of HBV replication (6-9). The pathogenesis of HBV-related fibrosis and cirrhosis is complex. HBV induces the degeneration and necrosis of hepatocytes and activates hepatic stellate cells (HSCs) to produce increased amounts of collagen, other extracellular matrix components and inflammatory cytokines, leading to liver fibrosis and cirrhosis (10-12).

Integrins are heterodimeric cell adhesion molecules (13). Integrin αvβ6 is almost undetectable in normal adult epithelial cells (14). However, integrin αvβ6 is expressed during the development of some tumors and during fetal development, wound healing and fibrosis (14). Recent studies have shown that the combined integrin αvβ6 and transforming growth factor beta (TGF-β) pathway is involved in pulmonary and renal fibrogenesis (15,16). The expression of integrin αvβ6 is elevated in acute biliary fibrosis (17). A previous study in a mouse model showed that integrin αvβ6 was not detected in normal liver tissues and was upregulated in liver cirrhosis, which was reversed after the use of an integrin αvβ6 antagonist. These findings support the possible effects of integrin αvβ6 in liver fibrosis (18). However, the role of integrin αvβ6 in cirrhosis and HCC associated with chronic HBV infection remains unknown. Therefore, this study investigated the relationship between integrin avß6 and HBV infection in patients with liver cirrhosis and hepatocellular carcinoma.

METHODS

Patients and tissue samples

This clinical study was performed between March 2014 and October 2016 at Qilu Hospital of Shandong University. A total of 84 patients diagnosed with HBV-associated HCC were included in the study. HBV was diagnosed pathologically, which was based on the revised standard of the National Conference of Parasitic and Infectious Diseases in 2000 (Chinese Society of Infectious and Parasitic Diseases, Hepatology, 2000). The liver resection specimens of these 84 patients were comprised of tumor tissue and adjacent liver tissue that did not contain tumors (19). The liver samples were obtained from adjacent non-tumor liver tissue. The present study complied with the requirements of the Ethics Committee of our hospital. The liver tissue was obtained by liver biopsy in the clinic (Table 1). The participants were not on antiviral treatment and all participants provided a signed informed consent.

Antibodies and reagents

The monoclonal mouse anti-human IgG antibody 442.5C4 against β6-integrin was purchased from Calbiochem (Darmstadt, Germany) and an immunohistochemical kit was purchased from Beyotime (Shanghai, China).

Immunohistochemistry of liver tissues

All liver tissue specimens were fixed in formalin, processed and embedded in paraffin wax before tissue sectioning onto the glass slides. Subsequently, the tissues on the slides were de-paraffinated and hydrated and the endogenous peroxidase activity was blocked. The antigen was retrieved from the slides before incubation with the integrin αvβ6 primary antibody 442.5C4 (1:200) overnight at 4 °C. The negative controls did not include the primary antibody. After an overnight incubation, biotinylated anti-mouse IgG (1:500) was applied to the tissue sections, followed by incubation with horseradish peroxidase (HRP)-labeled streptoantibiotin (Dako) for 15 minutes. Subsequently, the slides were incubated for two minutes with 3,3-diaminobenzidine (DAB) solution, counterstained with hematoxylin and the glass

coverslips were mounted onto the sections before light microscopy.

Assessment of integrin αvβ6 immunohistochemical staining

Integrin αvβ6 immunostaining was independently evaluated by two histopathologists who were unaware of the clinical data and any disagreement was resolved by discussion. The percentage of positive cells was graded as follows, 0: 0 %; 1: < 25 %; 2: 25-50 %; 3: 51-75 % and 4: > 75 %. For statistical purposes, if the percentage of positive cells was < 50, this was considered as negative and if the percentage of positive cells was > 50, this was considered as positive. The immunohistochemical staining intensity was graded as follows, 0: no staining; 1: weak staining; 2: moderate staining; and 3: strong staining. The immunohistochemistry staining score was calculated by adding the percentage staining score and staining intensity score together.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

The fresh human liver samples (range: 5-10 mg) were homogenized and the total RNA was extracted from cells using Trizol (Invitrogen). Subsequently, 1 µg of total RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen), according to manufacturer's instructions. The cDNA obtained from the reverse transcription reaction was analyzed using an IQ5 real-time PCR thermocycler (Bio-Rad). A calibration scale was used in this study. The primers were designed using the Primer Express software (Perkin-Elmer, Wellesley, USA), according to previously published sequences (18) and these were synthesized at MWG-BiotechAG (Ebersberg, Germany). The sequences of the primers for integrin αvβ6 were as follows, forward: 5'-GCAGAACGCTCTAAGGCCAA-3' and reverse: 5'- AAAGTGCTGG TGGAA CCTCG -3'.

Statistical analysis

The statistical analysis was performed using SPSS version 16.0. Discontinuous variables, such as gender and staging, were expressed as percentages. The Chi-squared test was used for discontinuous variables. Continuous variables were expressed as the mean ± standard deviation (SD). For multiple comparisons, each

value was compared by one-way ANOVA. This was followed by Dunnett's test, when each datum conformed with a normal distribution. Whereas non-normally distributed continuous data were compared using non-parametric tests. $p < 0.05$ was considered as statistically significant.

RESULTS

Integrin αvβ6 was upregulated in liver cirrhosis

A total of 84 patients were included in the study. 63 tissue samples of HBV-associated cirrhosis, 10 tissue samples of normal hepatic tissues and 11 tissue samples that revealed histological changes of chronic hepatitis B were obtained from these patients. The severity and prognosis of the liver cirrhosis were assessed according to the Child-Pugh scoring system and the histological grading of hepatitis was performed, as previously described. These results showed that integrin αvβ6 expression was not detected in normal liver tissues but was upregulated in liver tissues that presented cirrhosis (89.8 %). The expression of integrin αvβ6 was moderate or weak in chronic HBV and HCC tissues (Table 1; Figs. 1 and 2). The samples were classified as follows, normal: 10, CHB: 11, HCC: 84, and cirrhosis: 88. Integrin αvβ6 expression levels could be used to distinguish liver cirrhosis from other conditions in the examined liver tissues.

The expression of integrin αvβ6 is correlated with the stage of HBV-associated cirrhosis

The Child-Pugh grading system is a grading standard commonly used in the clinical practice for the quantitative evaluation of liver reserve function in patients with liver cirrhosis. Among the patient cohort, 25 patients (100 %) were Child-Pugh grade C, 22 of 26 patients (84.6 %) were Child-Pugh grade B and 28 of 37 patients (75.7 %) were Child-Pugh grade A. Subsequently, the relationship between the expression of integrin αvβ6 and the stage of HBV-associated cirrhosis was investigated. The expression of integrin αvβ6 was stage-dependent in HBV-associated cirrhosis. Integrin αvβ6 expression increased with the progression of liver cirrhosis in the tissue samples. Furthermore, the expression of integrin αvβ6 differentiated Child-

Pugh grade C liver cirrhosis from Child-Pugh grades B and A (Fig. 3).

DISCUSSION

The activation of TGF-β *via* integrin αvβ6 is a fundamental link in the pathogenesis of pulmonary fibrosis and high levels of integrin αvβ6 in patients with pulmonary fibrosis have been shown to be associated with a worse prognosis (20). Studies of biliary tract injury with animal models have shown that the expression of integrin αvβ6 was closely correlated to biliary fibrosis and the inhibition of integrin αvβ6 expression could reduce fibrosis (18,21). Furthermore, the upregulation of integrin αvβ6 expression has also been demonstrated in renal fibrosis and oral submucosal fibrosis (16,22).

Liver biopsy is an important diagnostic procedure in the evaluation of the severity of liver inflammation and liver fibrosis. However, this procedure is often limited due to its invasiveness, which can be associated with complications, sampling error and prolonged operation time. Furthermore, it has an intra-observer variability in the histological interpretation of such small amounts of tissues and may miss the cirrhotic nodules or tumor nodules (23,24). Therefore, non-invasive markers for liver inflammation, fibrosis, cirrhosis and tumors are needed. Recently, several liver function tests in peripheral blood samples have been investigated for the diagnosis of liver cirrhosis, including the FibroTest (FibroSure), FibroMeter blood test and Hepascore serum test. These may be helpful to supplement the liver biopsy (25).

The findings in the present study revealed that the expression of integrin αvβ6 was significantly upregulated in human HBV-related liver cirrhosis. Furthermore, the e xpression of integrin αvβ6 was detected in all patients. Among these patients, 25 (100 %) were at Child-Pugh grade C, 22 of 26 (84.6 %) were at Child-Pugh grade B and 28 of 37 patients (75.7 %) were Child-Pugh grade A. The detection of the transcription of integrin αvβ6 also showed that the expression of integrin αvβ6 increased with the progression of cirrhosis, according to the Child-Pugh scoring system. Furthermore, there were undetectable levels in normal liver tissues and moderate or weak levels in chronic hepatitis B and HBV-associated HCC. The findings of this study demonstrate the association between the expression of integrin αvβ6

progression of liver cirrhosis, which supports integrin αvβ6 as a potential biomarker for liver fibrosis and cirrhosis. However, further controlled clinical studies are required to evaluate this possibility.

The understanding of the mechanism that drives fibrosis in the liver continues to improve and has resulted in a growing list of agents that are being tested for anti-fibrotic activity. However, none of these new agents have been approved for the clinical practice at present (11,12,26,27). Furthermore, integrin αvβ6 has emerged as a promising pharmacological target in fibrotic biliary disease, due to its selective upregulation in biliary fibrosis and cancer (18). However, further studies are needed to explore the mechanism of integrin αvβ6 in HBV-related cirrhosis and its potential effects in the prevention of liver fibrosis and cirrhosis.

Limitations: First, the sample size was small. Second, the relationship between the expression of integrin αvβ6 and the stage (Staging 1/2/3/4) of HBV-associated cirrhosis should be further investigated. Third, the present study only included the Child-Turcotte-Pugh classification. There was insufficient data on the model for end-stage liver disease (MELD) or the MELD-Na, which should be further investigated. Fourth, there is insufficient data on viremia or antiviral therapies, which should be further investigated. Fifth, there is no information about the genotype, variant and viral load of these patients, which should be further investigated in the future. Sixth, there was no data to determine whether the expression of αvβ6 might increase in other causes of cirrhosis, which should be further investigated.

In conclusion, integrin αvβ6 could be a predictive marker for the progression of liver cirrhosis associated with HBV infection. Further studies are needed to identify the association between the expression of integrin αvβ6 in hepatitis B and HBV-associated HCC liver tissues.

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Table 1. Characteristics of patients and the expression of integrin αvβ6 in different hepatic tissues

Characteristics	Liver cirrhosis	Normal hepatic tissue	Chronic hepatitis	Hepatocellular carcinoma
Age (yr)	49 ± 10.3	50 ± 7.6	47 ± 12.3	48 ± 11.1
Gender (M/F)	47/31	7/3	8/3	49/35
n (positive; %; m)	88 (79; 89.8 %; 42)	10(0; 0 %; 0)	11 (4; 36.4 %; 3)	84 (17; 20.2 %; 10)
Child-Pugh score				
A	37 (28; 75.7 %)	10(0; 0 %)	8 (2; 25.0 %)	55 (9; 16.4 %)
B	26 (22; 84.6 %)	0	3 (2; 66.7 %)	29 (8; 27.6 %)
C	25 (25; 100 %)	-	-	-
Staging				
0	-	10(0; 0 %)	-	9 (0; 0 %)
1	-	-	4 (1; 25.0 %)	5 (1; 20.0 %)
2	-	-	4 (1; 25.0 %)	4 (2; 50.0 %)
3	-	-	3 (2; 66.7 %)	3 (3; 100 %)
4	88 (79; 89.8 %)	-	-	63 (13; 20.6 %)
Percentage of positive cells				
0 (0 %)	2 (0 %)	7(0 %)	1 (0 %)	19 (0 %)
1 (< 25 %)	1 (17.3 %)	3 (17.1 % ± 4.5 %)	2 (16.2 % ± 3.9 %)	37 (16.9 % ± 11.9 %)
2 (25-50 %)	5 (37.2 % ± 9.6 %)	0	4 (31.8 % ± 7.9)	11 (47.9 % ± 8.4 %)
3 (51-75 %)	34 (64.5 % ± 10.5 %)	0	3 (59.0 % ± 4.7 %)	13 (67.1 %7.4 %)
4 (> 75 %)	45 (84.6 % ± 12.3 %)	0	1 (81.6 %)	4 (77.3 ± 5.3 %)

Numbers are given as means ± SD. Age is given as means ± SD. Percentage of positive cells < 50 % (0,1,2) considered as negative, > 50 % (3,4) considered as positive.

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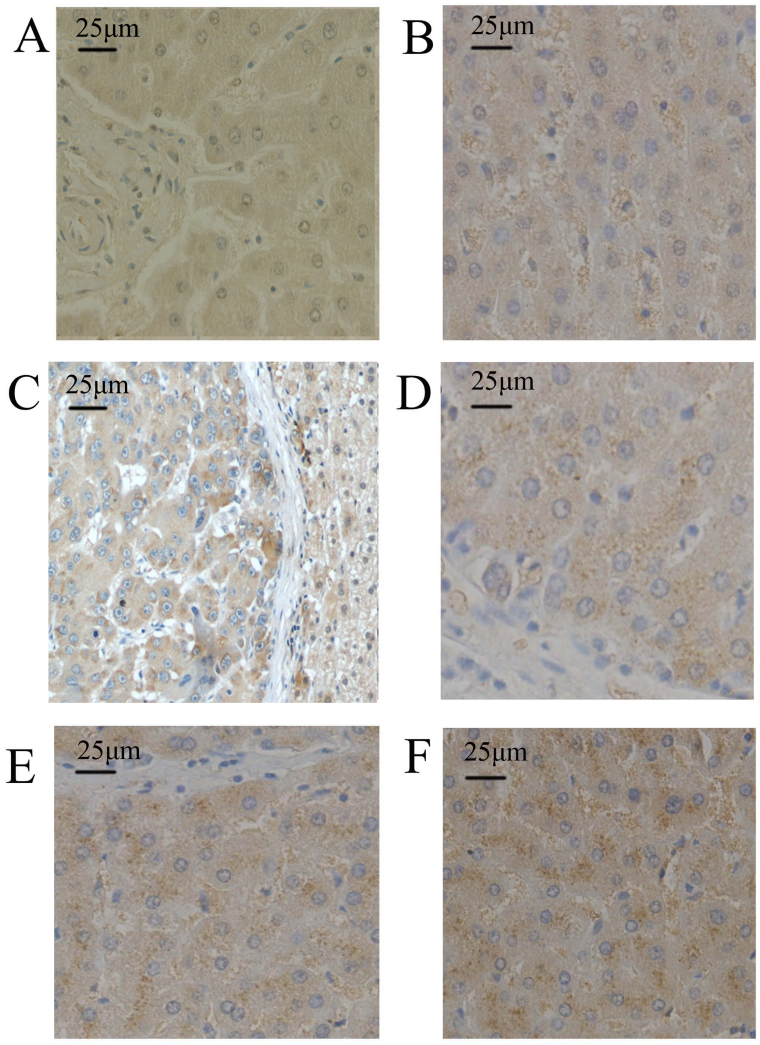

Fig. 1. The immunohistochemical staining of integrin αvβ6 in different liver tissue samples: normal liver tissue (A); chronic hepatitis liver tissue (B); a section of HCC (C); a section of Child-Pugh grade A liver cirrhosis (relatively weak) (D); a section of Child-Pugh grade B liver cirrhosis (relatively moderate) (E); a section of Child-Pugh grade C liver cirrhosis (strong) (F).

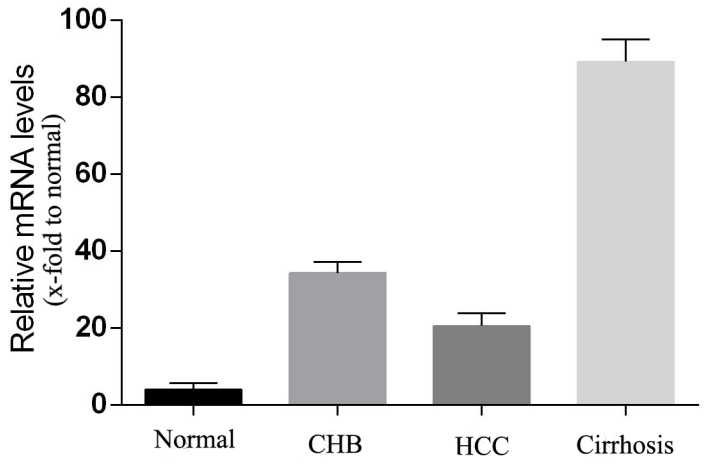

Fig. 2. mRNA expression of integrin αvβ6 in different hepatic tissues. Integrin αvβ6 mRNA was overexpressed in hepatic cirrhosis. The error bars represent the standard deviation (SD). The presented data was obtained from the PCR-based SYBR assay.

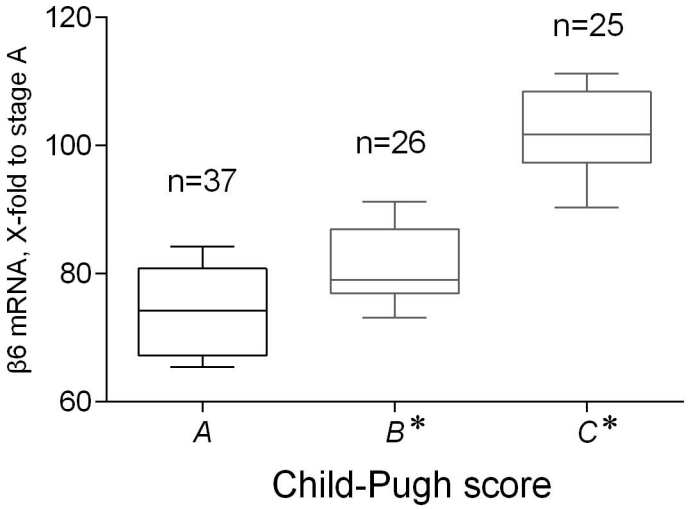

Fig. 3. The expression of integrin αvβ6 increases with the progression of HBV-associated cirrhosis. Data were expressed as the x-fold increase compared to Child-Pugh grade A. *$p < 0.05$ compared to Child-Pugh grade A.