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Original Article

Expression of Integrin β1, Focal Adhesion Kinase, and PDZ-Binding Motif in Human Liver Cirrhosis and Simple Steatosis

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Abstract

Background: Integrins are transmembrane mechanosensitive proteins that negatively contribute to the pathogenesis of different types of chronic liver disease and can activate focal adhesion kinase (FAK).

Objectives: This study aimed to determine the hepatic integrin β1 and FAK mRNA as well as the transcriptional coactivator with PDZ-binding motif (TAZ) protein expressions in cirrhotic patients and simple steatosis.

Methods: In this case–control study, liver tissues were collected from 30 cirrhotic patients with various etiologies (i.e., nonalcoholic steatohepatitis-, primary sclerosing cholangitis-, alcoholic-, autoimmune hepatitis [AIH]- and hepatitis B virus [HBV]/hepatitis C virus [HCV]-related cirrhosis [six per group]), liver samples with simple steatosis (n = 6), and control liver tissues (n = 9).

Results: Integrin β1 gene expression was significantly up-regulated in all cirrhotic groups compared to control group ($P < 0.05$), with the exception of AIH cirrhosis. However, hepatic FAK gene expression and TAZ protein level in the cirrhotic groups were not significantly different than those in the control group. Furthermore, hepatic integrin β1 and FAK gene expressions as well as TAZ protein level in simple steatosis were significantly lower than those in nonalcoholic steatohepatitis (NASH) cirrhosis and control ($P < 0.05$).

Conclusion: Integrin β1 was up-regulated in cirrhotic liver tissues. In addition, FAK, integrin β1, and TAZ were concordantly down-regulated in simple steatosis, and may have been involve in the steatosis development.

Keywords: Non-alcoholic fatty liver disease, Primary sclerosing cholangitis, Hepatitis B, Nonalcoholic steatohepatitis

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Background

Cirrhosis is the twelfth most common cause of mortality in the world (1). Hepatitis B virus (HBV), hepatitis C virus (HCV), cholestatic disease, and alcoholic disease are the major causes of cirrhosis. However, nonalcoholic fatty liver diseases (NAFLDs) as causes of cirrhosis have been becoming more common in most countries in the recent decade (2). NAFLD is traditionally defined as hepatic simple steatosis that progresses to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (3). However, the progression from simple steatosis to NASH has been questioned by the results from recent studies (4).

Integrins are transmembrane proteins that mediate the signal transduction from the extracellular matrix to the inside of the cell in order for controlling the shape, motility, and cell cycle (5). In other words, the binding of ligands to the integrins results in conformational changes in the integrins and cluster of them on the cell surface (6). The expressions of integrins are up-regulated in different type of chronic liver disease such as infection with HBV or HCV, primary sclerosing cholangitis (PSC), and primary biliary cholangitis (7). It has been shown that integrin β1 plays a critical role in the progression of insulin resistance, a common feature of NASH and NAFLD (8). Clustered integrins (a complex lacking kinase activity) can recruit and activate kinases including focal adhesion kinase (FAK) and Src family kinases (5,6). FAK is a cytoplasmic tyrosine kinase that localizes to the sites of cell membrane where integrins are clustered at the time of its activation (9). Activation of FAK through its autophosphorylation results in the initiation of a kinase cascade and reorganization of the cytoskeleton (9). Therefore, FAK may be considered as a component of mechanical signal transductions that regulates the cellular responses to external mechanical stimuluses (10). The upregulation of FAK has been

reported in prostate, breast, and colon cancer (11). Furthermore, recent studies have documented the role of FAK in fibrogenesis in humans and mice, although there is still lack of detailed information about the mechanisms by which FAK contributes to fibrosis signaling (12). FAK is recruited and activated by integrin β1. The activation of FAK is involved in migration of human lung fibroblasts (13). Yes-associated protein and transcriptional coactivator with PDZ-binding motif (YAP/TAZ) are transducers of the hippo pathway and are predominantly inactive and located in the cytoplasm, which also inhibit cellular proliferation (14). It has been reported that nuclear accumulation of YAP occurs during FAK activation in MCF-10A cells on fibronectin (15). However, it has been suggested that YAP/TAZ and FAK pathways act independently in the regulation of immunomodulatory properties of adipose-derived mesenchymal stem cells (10).

In the current study, therefore, the integrin β1 and FAK mRNA and TAZ protein expressions in human liver tissues from patients with NASH, PSC, viral, alcoholic hepatitis (ALH), and autoimmune hepatitis (AIH) cirrhosis were investigated and compared with those in control liver tissues. Furthermore, integrin β1 and FAK gene expression as well as protein expression of TAZ in liver tissues with simple steatosis were measured and compared with those in liver tissues with NASH cirrhosis and control.

Materials and Methods

Study Population

To conduct the present study, 30 cirrhotic liver tissue with different etiologies (six samples per group) were collected from the patients who had undergone orthotopic liver transplantation in the Namazi Transplant Center, Shiraz University of Medical Sciences, Shiraz, Iran. The subjects aged over 18 years and afflicted with NASH, AIH, PSC, ALH, and HBV/HCV cirrhosis were included in the study. The liver tissue samples with mixed etiology or etiologies other than the above-mentioned ones such as Wilson disease and Budd-Chiari syndrome were excluded from the study. Fresh liver tissue samples were taken and frozen in liquid nitrogen immediately after detaching the whole liver tissue from the patients undergoing the orthotopic liver transplantation. Pieces of liver tissue samples were also cut and stored in 4% formalin solution for later histopathology investigations. In addition, liver samples of simple steatosis (n = 6) and control (n = 9) were obtained from livers exhibiting no characteristics for liver transplantation. The livers belonged to those patients who aged over 18 years and had no history or signs of chronic liver disease, alcohol consumption, HBV/HCV infection, and HCC. Liver tissue was fixed with formalin and embedded in paraffin. Then the samples were sectioned (4–5 μm of thickness), stained with hematoxylin and eosin dyes, and analyzed histopathologically by a skilled pathologist. Liver samples with > 5% of steatosis without fibrosis and inflammation were considered as simple steatosis (16).

Isolation of RNA and Quantitative Real-Time PCR Analysis

Total RNA from liver samples was isolated using the RNeasy kit (Qiagen, Germany). The cDNA synthesis kit (Thermo Fisher Scientific) was used to synthesis complementary DNA (cDNA) from total RNA. Then it was included in a quantitative real-time polymerase chain reaction (qRT-PCR) using specific forward and reverse primers for integrin β1 gene (ITGB1), FAK (Table 1) and LightCycler® 96 Real-Time PCR System (Roche, Germany). The housekeeping gene ACTB was used as internal control for normalizing the data and $2^{-\Delta CT}$ method was adopted for analyzing the relative gene expression of ITGB1 and FAK.

Western Blot Analysis

Hepatic protein expression of TAZ was determined conducting the western blot analysis and a procedure described previously (17). In sum, frozen liver specimens (10–15 mg) were homogenized using 600 μL radioimmunoprecipitation assay buffer supplemented with a protease inhibitor (Catalog no. P8340; Sigma-Aldrich, St. Louis, Missouri). Then the total protein concentrations of lysates were detected by implementing the bicinchoninic acid protein assay method. After separation of lysate proteins on a polyacrylamide gel with SDS and transblotting onto nitrocellulose membranes, anti-TAZ primary antibody with dilution 1:5000 (ab110239; Abcam, Cambridge, UK) was employed to detect TAZ protein. Horseradish peroxidase–conjugated goat anti-rabbit IgG with dilution 1:3000 (ab6721; Abcam, Cambridge, UK) was used as secondary antibody. For internal control the β-actin protein was applied.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's test was adopted to assess the differences between the study groups. Moreover, the Spearman correlation coefficient test was used to find the correlation between variables. All results are shown as mean ± standard error of mean (SEM). $P < 0.05$ was considered statistically significant for all analysis.

Results

Study Subjects

Overall, 9 control liver samples, 6 liver samples with simple steatosis, and 30 liver tissue samples with cirrhosis caused by HBV/ HCV infection, PSC, AIH, ALH, and NASH (six

Table 1. List of Primers

Gene ID			
β1 integrin	Human	Forward	5'-TTCAAGGGCAAACGTGTGAG-3'
		Reverse	5'-GGACACAGGATCAGGTTGGA-3'
FAK	Human	Forward	5'-TGGAATAGATGAAGCCAGGGAT-3'
		Reverse	5'-CTCTCTCACGCTGTCCGAAG-3'
ACTB	Human	Forward	5'-GAGCCTCGCCTTTGCCGATCC-3'
		Reverse	5'-ACATGCCGGAGCCGTTGTCG-3'

Table 2. Demographic Features of the Patients With NASH-, PSC-, Alcoholic-, AIH-, and HBV/HCV-Related Cirrhosis, Simple Steatosis, and Control

	Control Subjects	Simple Steatosis	NASH Cirrhosis	PSC Cirrhosis	AIH Cirrhosis	Alcoholic Cirrhosis	HBV/HCV Cirrhosis
No. of subjects	9	6	6	6	6	6	6
Gender (male/female)	6/3	2/4	4/2	1/5	2/4	6/0	5/1
Age (y)	45.11 ± 2.89	51.8 ± 3.79	52.62 ± 4.28	30.33 ± 3.94	32.33 ± 5.59	36.01 ± 5.11	54.62 ± 2.77
BMI (kg/m^2)	23.65 ± 1.07	26.42 ± 1.89	28.54 ± 1.56	26.72 ± 1.79	23.76 ± 1.64	28.30 ± 2.51	23.09 ± 1.29
MELD score	7.44 ± 0.37	12.5 ± 0.96	21.83 ± 1.25	17.33 ± 2.60	23.5 ± 1.20	26.75 ± 4.88	22.66 ± 3.79

Abbreviations: NASH, nonalcoholic steatohepatitis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; MELD, model for end-stage liver disease; BMI, body mass index.

samples per group) were collected for conducting this study. Table 2 summarizes the demographic data of the study subjects. The mean age of patients with NASH- and HBV/ HCV-related cirrhosis was higher than that of the subjects from other three cirrhotic groups. Furthermore, patients with NASH and ALH cirrhosis had higher BMI than the subjects in other cirrhotic groups. However, the MELD score for cirrhotic patients with different etiologies was not different.

Levels of Integrin β1, FAK and TAZ in Liver Tissues of Cirrhotic Patients

The results of the qRT-PCR analysis revealed that the gene expression of integrin β1 was significantly increased in liver tissues from cirrhotic patients with different etiologies in comparison with those from control, with the exception of AIH cirrhosis (Figure 1a). In other words, patients with PSC cirrhosis had the highest gene expression of integrin β1, while integrin β1 in patients with AIH cirrhosis did not differ with that of control. Previous studies have shown that clustered integrins can recruit and activate FAK protein (5,6). For this reason, FAK gene expressions in cirrhotic groups and control were analyzed in the study. As shown in Figure 1b, the hepatic gene expressions of FAK in cirrhotic groups were not significantly different from that of control, although the hepatic gene expression of FAK was higher in patient with PSC cirrhosis compared to those in the subjects from other cirrhotic groups. In addition, no correlation was observed between integrin β1 and FAK gene expression ($P > 0.05$). On the other hand, protein expression of TAZ, a protein which has been associated with FAK protein, was measured using western blotting

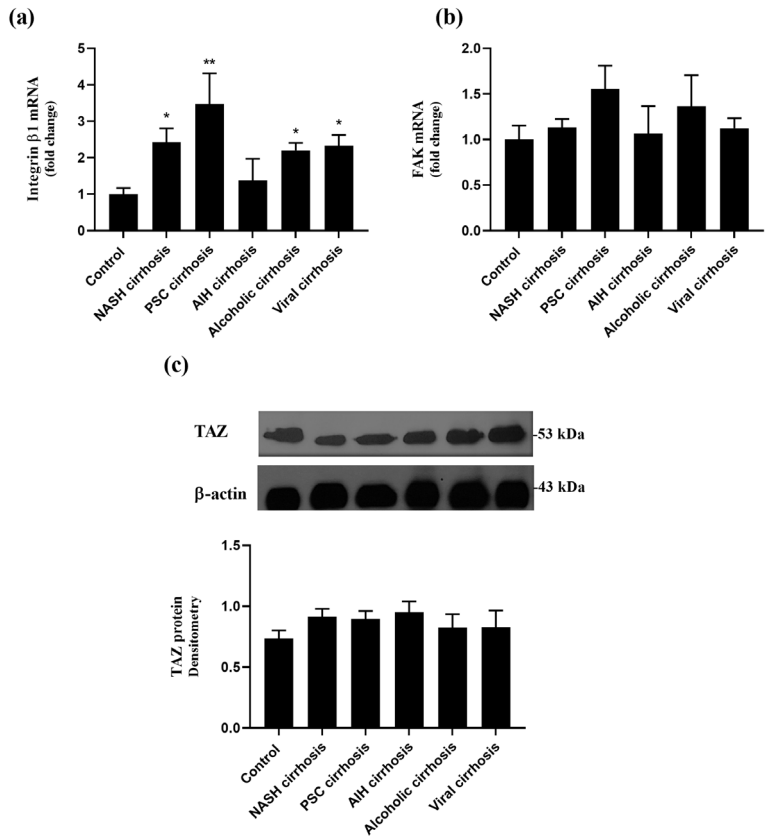

Figure 1. Integrin β1 and FAK Gene Expressions (a and b) and TAZ Protein Level (c) in Liver Tissues With NASH- (n = 6), PSC- (n = 6), Alcoholic- (n = 6), AIH- (n = 6), and HBV/HCV-Related Cirrhosis (n = 6) and Control (n = 9). Western blotting bands are representative of one sample per group, and graphs are representative of four samples per group. All results were normalized to β-actin and are displayed as the mean ± SEM. (*$P < 0.05$ and **$P < 0.01$ vs. control). Abbreviations: FAK, Focal adhesion kinase; AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; PSC, primary sclerosing cholangitis

when studying the cirrhotic groups and control. Similar to the FAK gene expression, TAZ protein expressions in cirrhotic groups were not significantly different from that of control (Figure 1c).

Levels of Integrin β1, FAK, and TAZ in Liver Tissues of Simple Steatosis Patients

Previous studies have documented the important role of integrin β1 in the progression of insulin resistance, a common feature of NAFLD. Simple steatosis is the mildest form of NAFLD (18). In the current study, therefore, the gene expression of integrin β1 was determined in liver tissues with simple steatosis and was compared to that in tissues with NASH cirrhosis and control. Surprisingly, the integrin β1 gene expression in simple steatosis was significantly lower than that in NASH cirrhosis and control (Figure 2a). Like the integrin β1 gene expression, FAK gene expression in simple steatosis was significantly different from that of liver tissue samples with NASH cirrhosis and control (Figure 2b).

Moreover, TAZ protein expression in simple steatosis was significantly lower than that in NASH cirrhosis and control (Figure 2c). The correlation between FAK and integrin β1 gene expressions was also calculated in the present study. The results of the spearman correlation coefficient test showed a significant and positive correlation between FAK and integrin β1 gene expressions in NAFLD patients (Figure 2).

Discussion

Chronic liver diseases are critical public health concerns with high mortality rates due to their cirrhosis and complications (19). Various chronic liver diseases with different nature such as viral hepatitis, PSC, AIH, ALH, and NAFLD can cause cirrhosis. Given its increasing prevalence, NAFLD has been becoming one of the most common chronic liver diseases, placing major burdens on the health systems in the world.

Integrins are extracellular matrix receptors involved in mechanical signal transduction. It has been reported that extracellular matrix mechanical stiffness has positive correlation with integrin β1 expression in HCC patients (20). Previous studies have indicated that matrix stiffness of cirrhotic livers is significantly higher than that of the ones from the control (21). In the current study, it was shown that the gene expression of integrin β1 was up-regulated in patients with NASH, ALH, HBV/HCV and, more specifically, PSC cirrhosis, with the exception of AIH cirrhosis. Our results in this regard were in line with the findings from several studies reporting the critical role of integrins in the different types of liver injuries. A study, for instance, had shown that integrins had an intriguing correlation with HCV infection (22). Another study had found an enhanced expression of integrin β1, α1, α5 and α6 in patients with chronic hepatitis C, which was correlated with the stage of fibrosis (23). Integrin β1 is overexpressed on hepatocytes membranes of patients

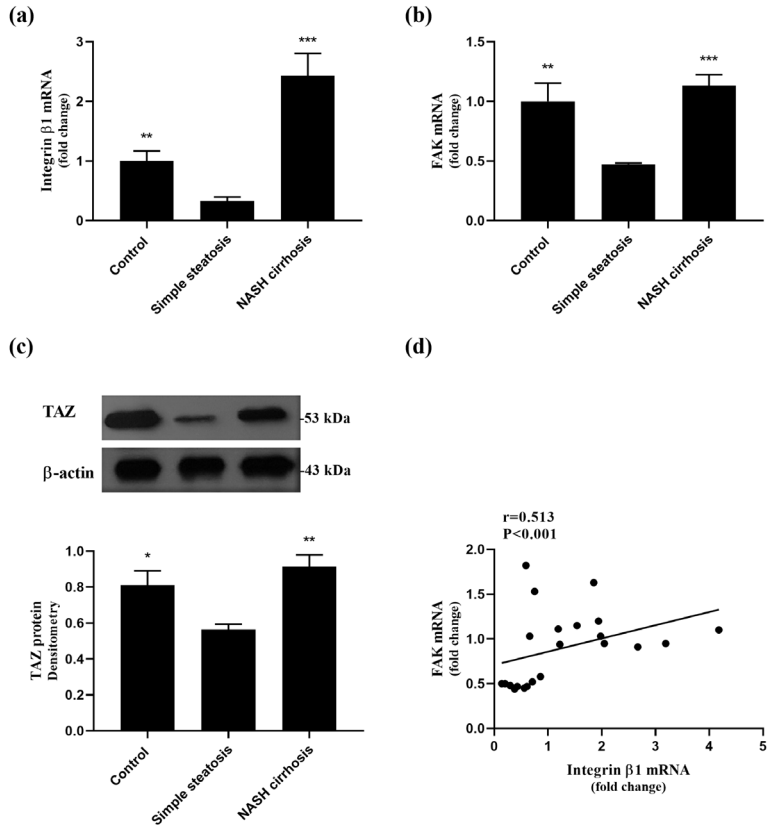

Figure 2. Integrin β1 and FAK Gene Expressions (a and b) and TAZ Protein Level (c) in Liver Tissues With NASH Cirrhosis (n=6), Simple Steatosis (n=6), and Control (n=9). Western blotting bands are representative of one sample per group and graphs are representative of four samples per group. All results were normalized to β-actin and are displayed as the mean±SEM. (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. control). (d) Correlation of FAK mRNA fold change with integrin β1 fold change in NAFLD patients. Abbreviations: FAK, Focal adhesion kinase; NASH, nonalcoholic steatohepatitis

with different stages of ALH (24). Interestingly, there is extensive scientific evidence supporting the prominent role of integrins in the context of cholestatic disease in both humans and rodents (7, 25). The correlation between integrin β6 and the stage of liver fibrosis in patients with cirrhosis has been already detected (26). Vascular endothelia and bile duct epithelium of liver tissue express integrin α3 and α6 (27). However, the role of integrins in the pathogenesis of autoimmune disease has been amply documented. Taking into account the similarity between AIH cirrhosis and control groups in terms of expression of integrin β1, it was recommended that further studies should be carried out to clarify this issue. Previous studies have demonstrated that integrins can recruit and activate FAK protein (5,6). In the present study, however, the gene expression of FAK in cirrhotic liver tissues was similar to that in control and had no correlation with integrin β1 gene expression. Among cirrhotic patients with different etiologies, FAK gene expression was higher in PSC patients compared to that in other etiologies and control. Similar to our study, the study by Jiang et al also found that the gene and protein expressions of FAK were increased in bile duct ligated rat livers, although the animals were not in the end-stage fibrosis (28). In addition to integrins, FAK protein can be activated by cytokines and growth factors, such as TGF-β1 (29). The activation of FAK signaling by TGF-β1 has been observed in lung fibroblasts (30). Therefore, it is possible that FAK expression and activation are regulated, at least partially, by the factors other than integrins in the end-stage liver disease. In a similar fashion to the FAK gene expression, the protein expression of TAZ in cirrhotic livers was found not to differ from that of control in our study. The cross link between hippo signaling and FAK has been reported by a recent study. In the given study, it has been demonstrated that the activation of FAK in MCF-10A cells cultured on fibronectin activates the YAP nuclear accumulation, and the attenuation of it blocks the YAP nuclear accumulation (15). However, independent functions of YAP/TAZ and FAK pathways have been observed in the regulation of immunomodulatory properties of adipose-derived mesenchymal stem cells (10). Nevertheless, more studies are required to exactly clarify the cross link between TAZ and FAK pathway in the liver fibrosis.

It has been also determined that integrin β1 plays a critical role in the proliferation of HCC cells and the progression of NAFLD-related HCC (31). It is noteworthy that a substantial percentage of the patients with NAFLD-related HCC has been discovered to have the mild stage of NAFLD (32). Therefore, hepatic simple steatosis may be involved in the development of HCC. In our study, the gene expression of FAK and integrin β1 in simple steatosis were down-regulated and correlated with each other. Furthermore, the protein expression of TAZ in simple steatosis was significantly lower than that in NASH cirrhosis and control. Few studies have investigated the roles of integrins and FAK in simple steatosis. However, it has been indicated that FAK is a mechanosensory protein and involves in YAP and TAZ regulation (33). A previous study has demonstrated that Integrin β1 is a key factor responsible for regulating FAK activity (9). In addition, it has been shown that lower level of FAK activation increases accumulation of lipid, and vice versa (34). Although the concordant alterations of the FAK, integrin β1, and TAZ in the simple steatosis are justifiable, more researches are required to examine the cause(s) of their down-regulation.

Conclusion

In sum, integrin β1 was up-regulated in cirrhotic liver tissues, with the highest expression being detected in PSC related cirrhosis. However, no difference was found between cirrhotic liver tissues and control regarding FAK and TAZ expressions, suggesting that FAK and TAZ activations had been regulated by, at least partially, factors other than integrins in the end-stage liver disease. It was also demonstrated that FAK, integrin β1, and TAZ were concordantly down-regulated in the simple steatosis, which may have been the underlying cause of steatosis.

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Author Contributions

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Conflict of Interests

The authors declare that there is no conflict of interests.

Ethical Issues

This study was approved by Research Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1397.340). Before initiating the study, informed consent was obtained from the study participants.

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