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ORIGINAL ARTICLE

Is Integrin Subunit Alpha 2 Expression a Prognostic Factor for Liver Carcinoma? A Validation Experiment Based on Bioinformatics Analysis

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Abstract

ITGA2 (Integrin alpha-2) has been detected to be over-expressed in a number of cancers and has been suggested to be involved in cell adhesion and cell-surface mediated signaling. Our previous study using bioinformatic analyses has shown that ITGA2 might be a key gene being involved in the Cadmium-induced malignant transformation of liver cells. In the present study, we firstly aimed to learn the possible functions of ITGA2 via bioinformatics analysis, and then test its expression and clinical significance in liver carcinoma specimens through laboratory experiments. Gene ontology (GO) and pathway enrichment analysis, as well as protein-protein interaction (PPI) analysis has been conducted in Genecards. Then, a tissue microarray containing 90 cases of liver cancer and 90 paired adjacent non-cancerous samples was used for detection of ITGA2 expression by immunohistochemistry assay. Consequently, ITGA2 may be enriched in pathways regarding cell adhesion and migration. PPI analysis suggests that ITGA1, ITGB2, FLT4, LAMB1 and AGRN may have a close relationship with ITGA2. No association between ITGA2 expression and clinical parameters was observed. However, the data showed that ITGA2 might be an independent prognostic factor for liver cancer patients. In conclusion, the data suggest that ITGA2 over-expression might be a potential unfavorable prognostic factor and a potential therapeutic target for liver carcinoma.

Keywords Liver carcinoma · ITGA2 · Tissue microarray · Immunohistochemistry · Prognosis

Introduction

Cadmium (Cd) is a non-essential trace element for people, and is widespread in water, soil and air, with the development of industry. Thus, people in different area can usually be exposed to Cd through food intake, drinking water, and air [1]. Once it is taken and absorbed, it will accumulated in the body because the lack of mechanisms by which Cd is excreted. The accumulated Cd may bind to metallothionein and is stored in solid organs such as liver, kidney and prostate [2]. Chronic or acute exposure to Cd might exert toxic effect on cells, resulting in different extent of malignant transformation or damage of the cells [3]. Hence, Cd is classified as a human carcinogen by the International Agency for Research on Cancer [4], and its exposure has been suggested to be a risk factor for a number of cancers, particularly liver carcinoma [5].

Cd exposure may lead to cell malignant transformation via complicated molecular mechanisms. However, little has been elucidated to date. To learn possible genes that might be critical in this process, we have recently screened out several key genes through analysis of high throughput data [6]. ITGA2 (Integrin Subunit Alpha 2) is one of the key genes that have been shown to be up-regulated in liver carcinoma cells compared to the normal controls by Oncomine database. However, using the data based on a TCGA cohort, we failed to find an association between ITGA2 expression and clinical parameters of liver carcinoma. In addition, the survival analysis also failed to reveal ITGA2 as a prognostic factor for liver cancer [6]. Whether ITGA2 plays a role in the development of liver carcinoma remains largely uncertain.

Since the results of bioinformatic analyses usually indicate a tendency, not precise estimations. Validation experiments are required to get a more confident result. To our knowledge, little evidence concerning the role of ITGA2 in

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liver carcinoma has been published. Therefore, in the present study, we firstly aimed to learn the possible functions of ITGA2 in cancer progression by bioinformatic analysis. Then, the expression of ITGA2 protein was tested by immunohistochemistry (IHC) assay by using a specific tissue microarray. Afterwards, the roles and the prognostic values of ITGA2 in liver carcinoma were further evaluated.

Materials and Methods

Bioinformatics Analysis

The biological functions of ITGA2, comprising gene ontology (GO) function analysis, pathway analysis and protein–protein interaction (PPI) network, were evaluated by using Genecards database [7].

A Tissue Chip Containing Liver Cancer Specimens

A liver cancer tissue microarray (Hliv-HCC180Sur-03) was obtained from Shanghai Outdo Biotech Co., Ltd., which contained 90 HCC tissues and 90 paired adjacent non-cancer tissues. The operations were performed between Jan 2010 and Sep 2011. The last follow-up time was Sep 2013. All patients were clinicopathologically diagnosed as liver cancer (hepatocellular cell carcinoma) and received no extra treatment before surgery.

IHC Staining

ITGA2 protein expressions were tested by using the two-step method of IHC. The sections were deparaffinized and rehydrated. Antigen retrieval was conducted by autoclaving the slides in 10 mM citric acid buffer. The sections were rinsed with distilled water and saturated in phosphate buffered saline (PBS) for 5 min and then were incubated with a 1: 200 dilution of rabbit anti-monoclonal antibody (primary antibody; Abcam) overnight at 4 °C. The staining was visualized using DAB solution and counterstained with hematoxylin.

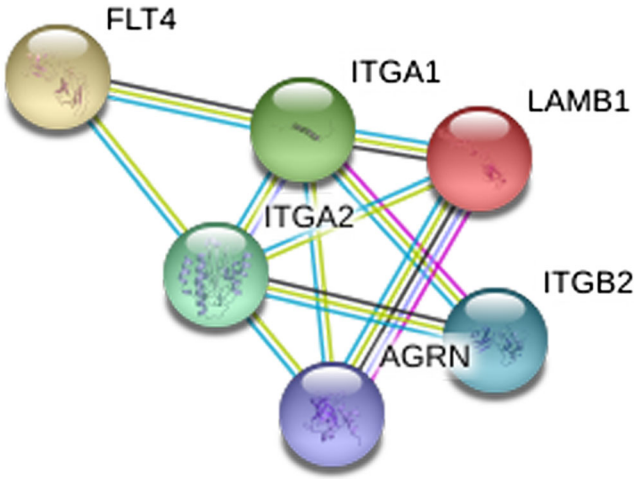

Fig. 1 Protein-Protein interaction network for ITGA2

Evaluation of IHC Staining

The IHC stain results were identified by integrated scoring. The scoring method that combined intensity and percentage of positivity was previously described [8] . In brief, the staining intensities of ITGA2 were scored from 0 percentages of positively stained cells were scored in scales of 0 to 4, in which 1 represents (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%).

The results were evaluated and scored independently by two pathologists without knowledge of the clinical parameters of the cases. The proportion and intensity scores were then multiplied to gain a total score, with a range from 0 to 12. Cut-off levels for the scoring were presented as follows: scores of >6 were classified as high expression; conversely, scores of ≤6 were classified as low expression.

Table 1 Results of GO analysis and pathway enrichment analysis for ITGA2 (top 5)

GO ID	Qualified GO term	Evidence
GO:0001666	response to hypoxia	Inferred from electronic annotation
GO:0002687	positive regulation of leukocyte migration	Inferred from electronic annotation
GO:0006929	substrate-dependent cell migration	Inferred from Mutant Phenotype
GO:0006971	hypotonic response	Inferred from electronic annotation
GO:0007155	cell adhesion	Inferred from electronic annotation; Traceable Author Statement
Pathway enrichment		
1	Apoptotic Pathways in Synovial Fibroblasts	
2	Integrin Pathway	
3	Focal Adhesion	
4	Blood-Brain Barrier and Immune Cell Transmigration: Pathways Overview	
5	MAPK-Erk Pathway	

Statistical Analysis

For continuous variables, data were expressed as mean value ± SD. Differences between groups were analyzed with Analysis of Variances (ANOVA) or a *t*-test. A chi-squared (χ^2) test was used to differentiate the rates of different groups. The Kaplan-Meier method was used to calculate the overall survival curves. A log-rank test was used to determine differences in the survival rates. COX multivariate regression survival analysis was conducted involving all the potential predict factors. These analyses were performed by utilizing SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

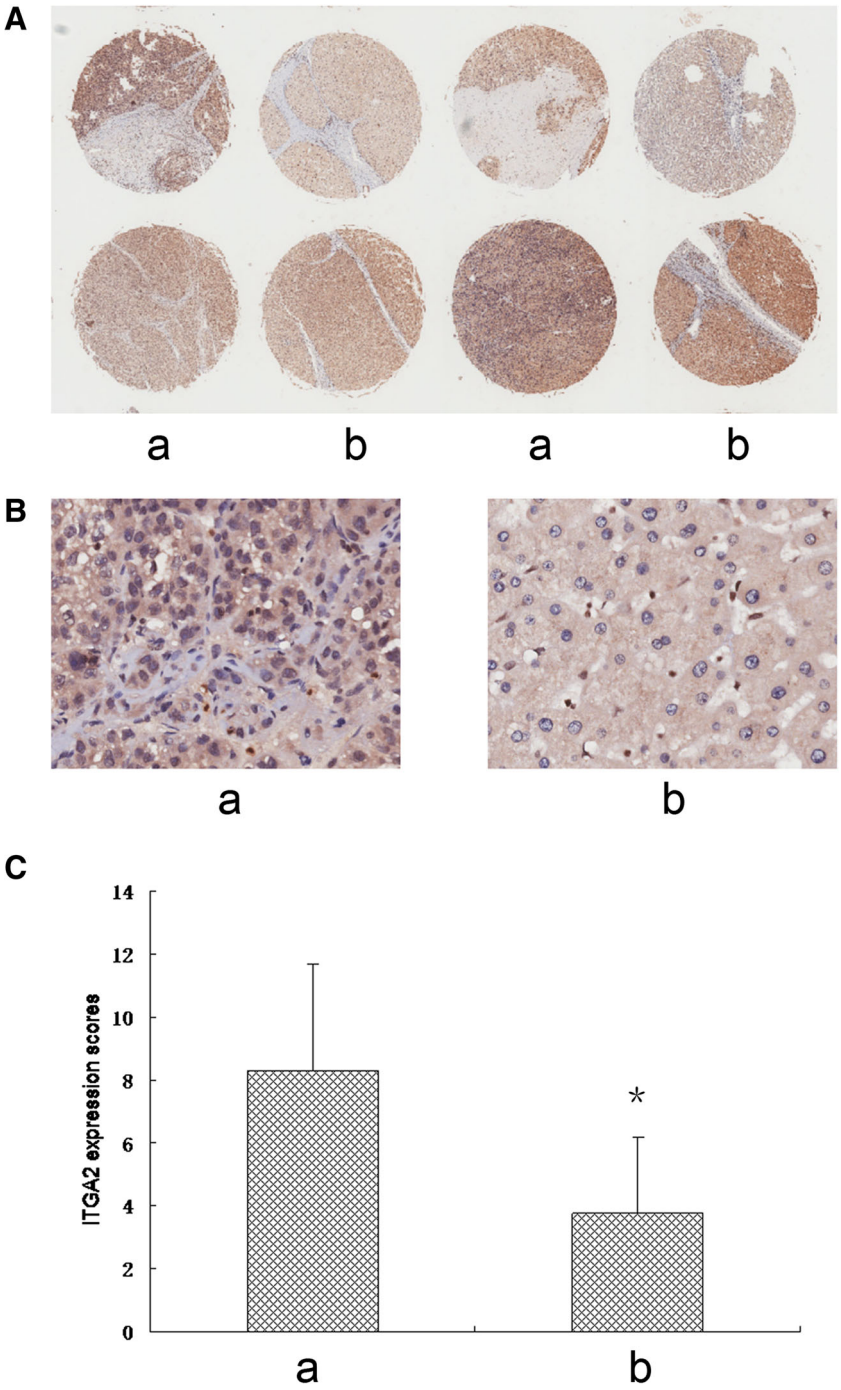

Fig. 2 ITGA2 is up-regulated in liver carcinoma tissues. **A** ITGA2 protein expressions in tissue microarray (four paired liver cancer tissues and adjacent normal tissues) were measured with IHC (×10); **B** Representative examples of ITGA2 expression in liver cancer tissues and adjacent normal tissues (×200); **C** The ITGA2 expression was higher in liver cancer than that in the normal tissues (*$P < 0.05$ vs a). (*a*) Liver carcinoma tissues; (*b*) Normal adjacent liver tissues

Results

Functional Annotation and Pathway Enrichment of ITGA2

To learn the possible functions of ITGA2, the GO and pathway enrichment analysis were assessed. As shown in Table 1, the top 5 GO and pathway items, respectively, were presented.

GO analysis showed that ITGA2 has an association with items such as response to hypoxia, positive regulation of leukocyte migration, substrate-dependent cell migration, hypotonic response and cell adhesion.

Pathway enrichment analysis showed that ITGA2 might be enriched in pathways such as Apoptotic Pathways in Synovial Fibroblasts, Integrin Pathway, Integrin Pathway, Blood-Brain Barrier and Immune Cell Transmigration and MAPK-Erk Pathway.

Protein–Protein Interaction (PPI) Network Construction

A network was constructed to reveal the proteins that have a close relation with ITGA2 by using Genecards and STRING database, with a confidence of 0.40. As a result, the most connected proteins to ITGA2 were ITGA1, ITGB2, FLT4, LAMB1 and AGRN (Fig. 1).

Expression of ITGA2 Protein Assessed by IHC

The samples on the tissue chip were detected for ITGA2 protein expression by IHC with the specific antibodies. There were originally a total of 90 cancer and 90 pared non-cancer samples on the slice. However, two cancer points and one non-cancer point were missed during the staining process. Therefore, there were 88 cancer and 89 non-cancer points stained on the slice (Fig. 2A). The basic characteristics of the 88 liver cancer cases were presented in Table 2. In this tissue chip, no lymph node metastasis and distant metastasis could be observed in all cancer cases. Thus, these two parameters were not evaluated in the present study.

Specific staining was mainly found in the cytomenbrane and cytoplasm of both the cancer and normal cells (Fig. 2B). The expression scores of ITGA2 was higher in liver cancer tissues than those in para-carcinoma tissues ($P = 0.000$; Fig. 2C).

Relationship between Clinicopathologic Parameters and Expression of ITGA2 Proteins

The expression levels of ITGA2 in liver cancer tissues were divided into two groups as high and low groups according to the scores as mentioned above (Fig. 3A). As shown in Table 3, the relationship between ITGA2 expression level and clinicopathologic parameters was explored. No associations were observed between ITGA2 high expression and the parameters such as age, gender, T and clinical stage.

Association of ITGA2 Protein Expression with the Prognosis of Liver Carcinoma

To learn the prognostic value of ITGA2 expression in liver cancer, a survival curve containing the information of overall survival time was created. As shown in Fig. 3B, the log-rank test showed that patients with high expressions of ITGA2 had a shorter overall survival time than those of patients with low expressions ($P = 0.01$). Then, multivariate Cox regression analysis was also conducted. The data showed that high expression of ITGA2 protein might be an independent prognostic factor for liver carcinoma patients (Hazard Ratio: 2.965; 95% Confidence Interval: 1.280–6.868) (Table 4).

Table 2 Patient characteristics of this study

Characteristic		No. of patients
Age (year)	88	
Median (range)		55 (28–76)
< 55		43
≧55		45
Gender	88	
Male		73
Female		15
Pathology diagnosis	88	
With cirrhosis		16
Without cirrhosis		72
Pathology grade	88	
I		3
I-II		14
II		48
II-III		14
III		9
T stage	81	
T1		7
T2		43
T3		29
T4		2
Clinical stage	81	
1		7
2		43
3		31

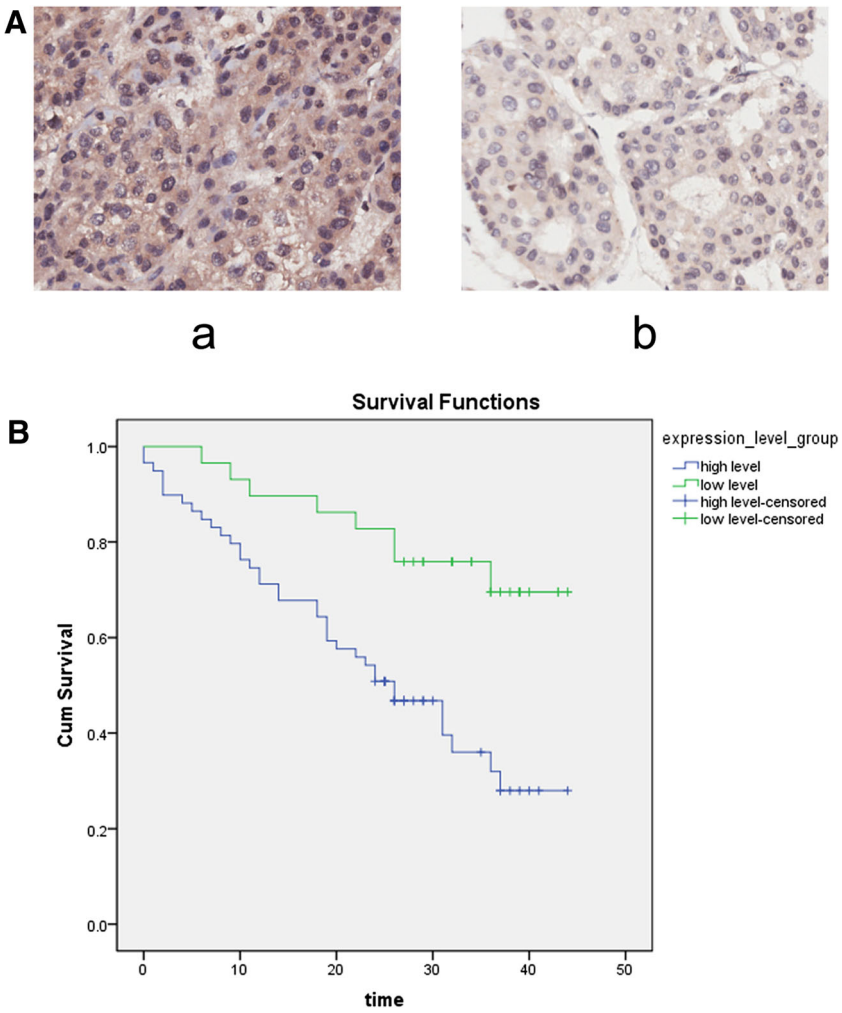

Fig. 3 ITGA2 over-expression as a prognostic factor for liver cancer patients. **A** High expression and low expression of ITGA2 in liver cancer tissues (×200); **B** Kaplan-Meier curves indicated that patients with High expression of ITGA2 have a shorter overall survival time, compared with that of patients with low ITGA2 expression ($P < 0.05$). (*a*) High expression of ITGA2; (*b*) Low expression of ITGA2

Discussion

Our previous study revealed that ITGA2 was up-regulated in liver carcinoma compared to normal liver tissues by bioinformatics analysis. However, no association of ITGA2 expression and clinical features were found. The data also failed to show a prognostic value for ITGA2 [6]. In the present validation study, using a tissue chip, we found that high expression of ITGA2 protein might be an independent prognostic factor for liver cancer.

Several reports had reported ITGA2 expression in other cancers, with conflicting results generated. For example, over-expression of ITGA2 has been found in colon cancer [9] and gastric cancer [10]. Moreover, it has been indicated to have a relation with lymph node metastasis and distant metastasis, and act as a prognostic factor for gastric cancer [10]. By contrast, low expression of ITGA2 has been detected in breast cancer tissues compared to the normal controls, which has been suggested to play a role in breast cancer cell migration and act as a prognosis factor for breast carcinoma [11]. Hence, the roles of ITGA2 may differ in various cancers. For liver carcinoma, only a few studies had been conducted on this issue. Wong et al. found that ITGA2 may contribute to liver cancer progression by analyzing a gene expression dataset [12]. Zhao et al. revealed that ITGA2 may be a target of miR-128, which mediate miR-128-caused tumor suppression [13]. Nevertheless, little evidence regarding observations on clinical cohorts could be retrieved. In our research, both the data based on bioinformatics analysis [6] and the data from validation experiments confirmed that ITGA2 protein was over-expressed in liver cancer tissues compared with that in normal tissues, and over-expression of ITGA2 may be a prognostic factor for liver cancer. To our knowledge, we for the first time assessed the roles of ITGA2 in liver cancer by using a clinical retrospective cohort.

The functions of ITGA2 are not fully understood. GO analysis showed that ITGA2 has an association with items such as response to hypoxia, positive regulation of leukocyte migration, substrate-dependent cell migration, hypotonic response and cell adhesion. Through GO analysis, we could learn that ITGA2 might have a correlation with cell adhesion, cell migration, and response to stress such as hypoxia. These

Table 3 Relationship between ITGA2 expression and clinicopathological features

Variables	Total	ITGA2 expression		P value
		High	Low	
Gender				
Male	73	52	21	0.065
Female	15	7	8	
Age (years)				
< 55	43	30	13	0.595
≧55	45	29	16	
Pathology diagnosis				
With cirrhosis	16	9	7	0.310
Without cirrhosis	72	50	22	
Pathology grade				
I + I-II + II	65	42	23	0.415
II-III + III	23	17	6	
Clinical stage				
1 + 2	50	30	20	0.192
3	31	23	8	
T stage				
T1 + T2	50	30	20	0.192
T3 + T4	31	23	8	

functions are related to cancer metastasis, recurrence and progression [14]. Moreover, pathway analysis indicated that ITGA2 might be enriched in pathways such as apoptotic pathways in Synovial Fibroblasts, integrin pathway, focal adhesion, blood-brain barrier and immune cell transmigration and MAPK-Erk pathway. We could also learn that these pathways may have an association with cell adhesion and migration, which were in accordance with the results of GO analysis.

The PPI analysis indicates that several proteins such as ITGA1, ITGB2, FLT4, LAMB1 and AGRN may have a relation with ITGA2 during different cell biological processes. ITGA1, ITGA2 and ITGB2 belong to the integrin family. Evidence indicated that ITGA1 promotes drug resistance and cell metastasis in pancreatic cancer [15]. Also, ITGB2 may contribute to drug resistance of leukemia cells [16].

Table 4 Multivariate analyses of ITGA2 protein expression and other clinical prognostic markers related to overall survival in liver carcinoma

Item	HR (95% CI)	P
Age (<55/≧55)	1.078 (0.544–2.134)	0.830
Sex (male/female)	2.251 (0.777–6.527)	0.135
Pathology diagnosis (Without cirrhosis/with cirrhosis)	0.960 (0.334–2.753)	0.939
Pathology grade (I + I-II + II/ II-III + III)	0.508 (0.246–1.049)	0.067
Clinical stage (1 + 2/3)	0.495 (0.241–1.015)	0.055
ITGA2 expression (high/low)	2.965 (1.280–6.868)	0.011
T stage (T1 + T2/ T3 + T4)	0.495 (0.241–1.015)	0.055

HR, hazard ratio; *CI*, confidence interval

VEGF is a well-known factor associated with angiogenesis, and FLT4 (Fms Related Tyrosine Kinase 4) is its receptor. Thus, blockade of FLT4 suppressed the tumor cell metastasis [17]. LAMB1 (laminin β1) plays a prominent role in cancer cell invasion [18]. Besides, it may be used as a biomarker discriminating colon cancer patients from controls [19]. AGRN (agrin) was up-regulated in liver cancer cells, which exerts an oncogenic role by regulating focal adhesion integrity, thus leading to cell migration and cancer progression [20]. Additionally, a report showed that AGRN can regulate the Hippo pathway effectors YAP and act as a mechanotransduction signal in the extracellular matrix [21]. Interestingly and coincidently, ITGA2 can also regulate YAP in liver cancer cells [12], implying that ITGA2 may interact with AGRN and contribute to cancer progression. Taken together, these proteins may play different roles in the development of cancers. ITGA2 might have a relationship with them during the cancer promotion processes. However, these hypothesis need to be verified by future laboratory experiments.

Our previous published report using bioinformatic appoaches based on a TCGA cohort showed that ITGA2 expression has no relationship with clinical features [6], which was in line with the results of the present validation research. However, the previous study failed to show ITGA2 as a prognostic factor for liver carcinoma [6], which was inconsistent with the present study where high ITGA2 expression was suggested to be an independent prognostic factor for liver cancer patients. The discrepancy might be due to the difference between the research object. The previous study

analyzed ITGA2 mRNA expression data from RNA-seq, while the present study focused on ITGA2 protein expression assessed by IHC assay. The results of the present study might be more convincible because mRNA expression can not always be representative of protein expression. Nevertheless, future studies with large sample sizes are needed to confirm the results.

Several limitations of the present study should be addressed. First, IHC was substantially a semi quantitative assay that may be underpowered to uncover the precise estimation of ITGA2 protein expression in liver tissues; however, the use of tissue chip as a high throughput assay may minimize the biases generated by different experiment conditions. Second, the sample size of the tissue microarray was limited. This might lead to any selection bias. In addition, third, no lymph node metastasis and distant metastasis was observed in all the cancer cases. Thus, these two important clinical parameters could not be assessed in the present validation study, though our previous bioinformatic study failed to show the association between ITGA2 mRNA expression and these two parameters. Therefore, future validation studies using large sample sizes with different detection assessments are warranted to determine the subject.

In conclusion, the results of the present study showed that over-expression of ITGA2 might be a significant independent prognostic factor for liver carcinoma and act as a potential therapeutic target for cancer research.

Author Contributions LZ and YZ designed the study and reviewed the manuscript. YH, JL and WZ performed the bioinformatics analysis and immunohistochemical staining. LZ, ZY and YL analysed the data. LZ, WL, and YZ wrote the manuscript.

Compliance with Ethical Standards

The experiments reported here were carried out according to the Declaration of Helsinki principles and the institute's ethical regulations.

Conflict of Interest The authors declare that they have no conflict of interest.

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