Integrin α9 Suppresses Hepatocellular Carcinoma Metastasis by Rho GTPase Signaling

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1. Introduction

Hepatocellular carcinoma (HCC) is a highly malignant solid tumor which results in chronic inflammation in the liver [1]. Until now, there is no effective drug for the treatment with advanced HCC patients [2]. The principal character of HCC is early metastasis and poor prognosis. A series of changes in the tumor microenvironment (TME) are involved in the progression of HCC [3]. The adaptation of cancer cells to its surrounding microenvironment depends on the interaction between the extracellular matrix (ECM) with membrane receptors [4]. Many molecules in TME have been reported to influence tumor development by regulating tumor cell proliferation, apoptosis, and motility [5–7]. It has been shown that integrin receptors and its downstream signal molecules, including Src, FAK, and p130Cas, have a remarkable influence on tumor progression and metastasis [8].

Integrins are heterodimeric integral membrane glycoproteins composed of noncovalently associated α- and β-subunits forming 24 heterodimers that recognize distinct but overlapping ligands, which can mediate cell adhesion, migration, and proliferation [9–11]. Different integrins are involved in different cellular processes, such as cell attachment to ECM, cell proliferation, and cell motility, which can be used as therapeutic targets in cancer [12].

Integrin α9 subunit, which pairs only with integrin β1 subunit, mediates the binding to a large number of ECM components to affect cell adhesion and motility. There is a key role of integrin α9β1 in lymphangiogenesis and angiogenesis [13, 14]. Integrin α9β1 is expressed not only by several human normal cells but also by different kinds of human cancer cells and closely correlated with tumor grade [15, 16]. It has been reported that integrin α9β1 in colon carcinoma is linked to tumor cell proliferation and migration by enhanced epithelial-mesenchymal transition (EMT) [17].
However, the biological functions of ITGA9 in HCC and the underlying molecular mechanisms have not been studied yet, therefore placing the restrictions on developing novel anticancer-targeted therapies. In this study, we investigated the role of ITGA9 in HCC and the underlying mechanisms involved in its function, trying to provide a new potential target for HCC treatment.

2. Materials and Methods

2.1. Cell Cultures. Normal human liver cell line THLE-2 was from American Type Culture Collection (ATCC). HCC cell lines HuH7, Hep3B, HepG2, SMMC-7721, MHCC-LM3, MHCC-97L, and MHCC-97H have been described previously [4, 18]. All of these cells were cultured in a specific medium according to ATCC instructions, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, and incubated in a humidified incubator under 5% CO2 at 37°C.

2.2. Clinical Samples. HCC tissue microarrays containing 202 HCC samples, 131 pairs of primary HCC, and their corresponding noncancerous liver (CNL) tissues were obtained from HCC patients treated at the Department of Transplantation and Hepatic Surgery, Renji Hospital (RJH). All human specimens were received from patients who underwent surgical resection and signed informed consent before their operations. The research was approved by the Research Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine.

2.3. Immunohistochemical and H&E Staining. Immunohistochemical staining and H&E staining were performed as described previously [19]. Anti-ITGA9 (ab140599; Abcam) antibody was used. To quantify the level of ITGA9 protein expression, each tumor was assigned with a score according to the intensity of cell membrane staining and the proportion of stained tumor cells (score 0 = 0–5%, score 1 = 6–30%, score 2 = 31–70%, and score 3 = 71–100%). Two pathologists quantified ITGA9 protein level independently in a blinded manner (low expression group: score 0-1, high expression group: score 2-3).

2.4. Lentivirus Production and Cell Transduction. The human ITGA9 ORF (NM_002207.2) was subcloned into the pEZ-lv105 vector (GeneCopoeia, China) to generate pEZ-lv105-ITGA9 plasmid. Virus packaging and cell transduction were performed as previously reported [19].

2.5. Cell Apoptosis Assay. Apoptotic cells were analyzed by annexin V-FITC staining and PI labeling as previously described [20]. SMMC-7721 and MHCC-LM3 cells with Lenti-vector or Lenti-ITGA9 were cultured and used in this assay.

2.6. Quantitative Real-Time PCR (qPCR). Total RNA from HCC cell lines was extracted by using TRizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed as previously described [21]. β-Actin was used as internal control for quantification. The data were analyzed using the 2−ΔΔCt approach. For ITGA9 mRNA level in HCC cell lines, ΔCt value of THLE-2 cell line was used as the reference. Primer sequences used in our study were as follows: ITGA9-F, 5′-CCCAGAAGAGGTACGGG-3′; ITGA9-R, 5′-GCAGCAGGAAGATGAGGA-3′; β-actin-F, 5′-TGTTGGGCGCCCGAGGCACC-3′; and β-actin-R, 5′-CTCC TTAAATGCAGCACGATTT-3′.

2.7. Western Blot. Western blots were performed as described previously [7]. The primary antibodies against ITGA9 (ab140599; Abcam), FAK (ab81293; Abcam), p-FAK Tyr397 (ab81298; Abcam), Src (2108; Cell Signaling Technology), p-Src Tyr527 (2105; Cell Signaling Technology), and α-tubulin (T6199; Sigma-Aldrich) were incubated for overnight at 4°C, followed by incubating with species-specific antibodies (926–32213; 926–68051; LI-COR, Lincoln, NE) for 1 h. The signals were detected by Odyssey infrared imaging system (LI-COR, Lincoln, NE) and further quantified by ImageJ software.

2.8. Cell Viability and Colony Formation Assay. The cell viability and colony formation were determined as previously described [22]. To determine cell viability, 2000 cells/well were seeded into a 96-well plate and detected by Cell Counting Kit-8 (CCK8, Dojindo, Japan) after 0, 1, 2, 3, and 4 days, respectively. For flat plate clone formation, 1000 cells/well were seeded into a 6-well plate and grown for 14 days followed by staining with 0.1% crystal violet solution in 20% methanol. The experiments were done in triplicate and repeated twice.

2.9. Transwell Assay. The ability of cell motility was detected by using transwells with 12μm pores (Merck Millipore) as previously described [23]. 2×104 HCC cells in 200μl culture medium without FBS were seeded on the upper chamber, and 600μl medium with 5% FBS was injected into the lower chamber. For invasion assay, 100μl Matrigel (BD Bioscience, USA) was placed on the upper chamber. Cell numbers were scored from six random areas of each well.

2.10. Xenograft Studies. Xenograft studies were performed as previously described [4]. All mice were sacrificed after 6 weeks, and the xenograft was stripped out and weighed for further analysis.

2.11. Immunofluorescence Staining. Cells were seeded at 12-well U-Chamber (Ibidi, Germany), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.05% Triton X-100 for 1 min at room temperature. Primary antibodies used in immunofluorescence staining were vinculin (EPR8185; Epitomics) and paxillin (ab32084; Abcam). The nucleus was stained with DAPI (Sigma-Aldrich, USA). Images were acquired by confocal microscopy (LSM 510, META Laser Scanning Microscope, Zeiss). The raw density was assessed using ImageJ.

2.12. Pull-Down Assay. Cells were serum-starved overnight and stimulated with LPA. Active small G-proteins were detected by pull-down with GST-RBD and GST-CRIB.
Anti-RhoA (2117; Cell Signaling Technology), anti-Rac1 (2320346; Merck Millipore), and anti-Cdc42 (2466; Cell Signaling Technology) against the corresponding small G-protein were used for immunoblotting in this assay. The active and total GTPases were subsequently detected with horseradish peroxidase- (HRP-) conjugated secondary antibody according to the manufacturer’s recommendations.

2.13. Statistical Analysis. Statistical analysis was performed using Student’s t-test for two groups or ANOVA for multiple groups. All quantitative data presented are mean ± standard error of mean (SEM) of at least three experiments. P < 0.05 was considered statistically significant. Graphical representation was created by GraphPad Prism 5 software (San Diego, USA).

3. Results

3.1. ITGA9 Is Significantly Downregulated in HCC and Correlates with Vascular Invasion and Prognosis. To explore biological functions of ITGA9 in HCC, we first analyzed ITGA9 expression using the TCGA and the GEO databases. We found that ITGA9 mRNA level was downregulated in HCC compared to CNL tissues (Figure 1(a)). For validation,
we next investigated the expression of ITGA9 in HCC tissue microarray by qPCR and immunohistochemical staining. Consistently, HCC tissues showed significantly decreased ITGA9 expression compared to normal-matched tissues (Figures 1(b) and 1(c)). Statistical analysis showed the decreased ITGA9 level in 72.55% of HCC patients compared to the paired CNL (Figure 1(d)).

Furthermore, ITGA9 protein level associated well with alpha-fetoprotein, vascular invasion, tumor thrombosis, tumor size, and TNM stage (Table 1). Similar results were also obtained from GSE14520 microarray datasets. ITGA9 mRNA and protein levels were closely correlated with ALT, TNM staging, BCLC staging, and CLIP staging in the HCC tissues (Table 2).

3.2. ITGA9 Affects HCC Cell Growth Both In Vitro and In Vivo. To elucidate the relevance of ITGA9 and HCC progression, we first analyzed ITGA9 level in HCC cell lines. Compared with immortalized normal liver cell THLE-2, ITGA9 mRNA and protein levels were expressed lower in most of the examined HCC cell lines (Figure 2(a)). Then, we stably overexpressed ITGA9 expression in SMMC-7721 and MHCC-LM3 cells (Figures 2(b) and 2(c)). In CCK8 assay, ITGA9 overexpression significantly decreased viability of HCC cells (Figure 3(a)). In plate clone formation assay, ITGA9 overexpression dramatically reduced colony formation of the HCC cells, manifested in the number of clones (Figure 3(b)). Moreover, cell apoptosis was obviously increased in both ITGA9-overexpressing SMMC-7721 and MHCC-LM3 cells (Figure 3(c)). Furthermore, the effect of ITGA9 overexpression on tumorigenesis was evaluated in xenografts in vivo. The volume and weight of the tumors from Lenti-ITGA9 cells were clearly attenuated compared to the tumors from control cells (Figure 3(d)). Taken together, these results show an inhibitory function of ITGA9 in HCC growth.

3.3. ITGA9 Inhibits HCC Cell Metastasis Both In Vitro and In Vivo. Then, we examined ITGA9 functions in cell migration and invasiveness in vitro. HCC cells with ITGA9 overexpression migrated obviously slower, and their invasion efficiency significantly decreased compared with control cells (Figures 4(a) and 4(b)). Additionally, ITGA9 overexpression or control cells were orthotopically injected into nude mice to evaluate the ability of metastases in vivo. Metastatic modules from Lenti-ITGA9 cells displayed less than those from Lenti-vector cells (Figure 4(c)). And histological staining indicated that intrahepatic metastasis was strongly inhibited by ITGA9 overexpression in HCC cells (Figure 4(d)). These results demonstrate that ITGA9 plays a crucial role in HCC cell invasiveness and metastasis.

3.4. ITGA9 Overexpression Disrupts Focal Adhesion Assembly, Inactivates Rac1/RhoA, and Reduces FAK/Src Phosphorylation. To uncover the underlying mechanisms of integrin α9-mediated suppression of HCC progression, we firstly explored the related pathway by analyzing the TCGA database. ITGA9 expression was closely associated with the pathways involved in cancer and regulation of actin cytoskeleton and focal adhesion, which was shown by KEGG pathway analysis (Table 3). It has been reported that integrin-mediated focal adhesion plays a curial role in

| Variable                        | High | Low | P value |
|---------------------------------|------|-----|---------|
| Age                             |      |     |         |
| ≤50 years                       | 63   | 40  | 0.081   |
| >50 years                       | 72   | 27  |         |
| Gender                          |      |     |         |
| Female                          | 14   | 14  | 0.042   |
| Male                            | 121  | 53  |         |
| Alpha-fetoprotein               |      |     |         |
| ≤20 ng/ml                       | 53   | 16  | 0.025   |
| >20 ng/ml                       | 80   | 51  |         |
| Gamma-glutamyltransferase       |      |     |         |
| ≤50 (U/l)                       | 48   | 27  | 0.536   |
| >50 (U/l)                       | 86   | 40  |         |
| Liver cirrhosis                 |      |     |         |
| Yes                             | 116  | 60  | 0.649   |
| No                              | 19   | 7   |         |
| Tumor multiplicity              |      |     |         |
| Single                          | 109  | 59  | 0.191   |
| Multiple                        | 26   | 8   |         |
| Tumor satellite                 |      |     |         |
| Yes                             | 42   | 19  | 0.688   |
| No                              | 93   | 48  |         |
| Tumor encapsulation             |      |     |         |
| Incomplete                      | 87   | 50  | 0.186   |
| Complete                        | 46   | 17  |         |
| Tumor thrombosis                |      |     |         |
| Yes                             | 22   | 22  |         |
| No                              | 113  | 45  |         |
| Tumor differentiation           |      |     |         |
| I                               | 3    | 0   | 0.056   |
| II                              | 55   | 17  |         |
| III                             | 76   | 50  |         |
| Vascular invasion               |      |     |         |
| Yes                             | 32   | 25  | 0.043   |
| No                              | 103  | 42  |         |
| Tumor size                      |      |     |         |
| ≤5 cm                           | 76   | 25  | 0.011   |
| >5 cm                           | 59   | 42  |         |
| TNM stage                       |      |     |         |
| I                               | 83   | 35  | 0.027   |
| II                              | 17   | 6   |         |
| III                             | 34   | 25  |         |

*P < 0.05 (n = 202; Pearson’s χ² test).
Figure 2: Analysis of ITGA9 expression in cell lines. (a) ITGA9 levels in 7 HCC cell lines and the immortalized human liver cell line THLE-2 as measured by qPCR and Western blot. (b and c) Expression of ITGA9 in SMMC-7721 and MHCC-LM3 cells with Lenti-vector or Lenti-ITGA9. Tubulin was used as an internal control. Values are means ± SEM. *P < 0.05.

Table 2: Correlation of clinicopathological features with ITGA9 expression in GSE14520 microarray data.

| Variable                        | ITGA9 (n) | P value |
|---------------------------------|-----------|---------|
|                                | High      | Low     |       |
| Age                             |           |         |       |
| ≤50 years                       | 51        | 74      | 0.180 |
| >50 years                       | 38        | 79      |       |
| Gender                          |           |         |       |
| Female                          | 12        | 19      | 0.124 |
| Male                            | 77        | 134     |       |
| HBV status                      |           |         |       |
| AVR-CC                          | 19        | 39      | 0.164 |
| CC                              | 57        | 103     |       |
| N                               | 2         | 4       |       |
| *ALT                            |           |         |       |
| >50 (U/l)                       | 28        | 72      | 0.017 |
| ≤50 (U/l)                       | 61        | 81      |       |
| AFP                             |           |         |       |
| >300 ng/ml                      | 49        | 61      | 0.052 |
| ≤300 ng/ml                      | 38        | 90      |       |
| Main tumor size                 |           |         |       |
| >5 cm                           | 35        | 53      | 0.307 |
| ≤5 cm                           | 53        | 100     |       |
| Multinodular                    |           |         |       |
| No                              | 67        | 123     | 0.351 |
| Yes                             | 22        | 30      |       |
| Cirrhosis                       |           |         |       |
| No                              | 7         | 12      | 0.995 |
| Yes                             | 82        | 141     |       |
| *TNM staging                    |           |         |       |
| 0                               | 11        | 6       | 0.043 |
| I                               | 31        | 65      |       |
| II                              | 25        | 53      |       |
| III                             | 89        | 29      |       |
| *BCLC staging                   |           |         |       |
| 0                               | 5         | 15      | 0.035 |
| A                               | 49        | 103     |       |
| B                               | 12        | 12      |       |
| C                               | 12        | 17      |       |
| *CLIP staging                   |           |         |       |
| 0                               | 26        | 72      | 0.016 |
| 1                               | 28        | 51      |       |
| 2                               | 19        | 16      |       |
| 3                               | 4         | 5       |       |
| 4                               | 1         | 2       |       |
| 5                               | 0         | 1       |       |
| Predicted risk metastasis signature |       |         |       |
| High                            | 47        | 74      | 0.505 |
| Low                             | 42        | 79      |       |
| CGH_survival_group              |           |         |       |
| G1                              | 11        | 8       | 0.063 |
| G2                              | 19        | 25      |       |

*P < 0.05 (n = 242; Pearson’s χ² test).
Figure 3: ITGA9 prevents HCC growth in vitro and in vivo. (a) Analysis of HCC cell viability with ITGA9 overexpression or control by CCK8. \( n = 6 \). (b) Analysis of HCC cell proliferation with ITGA9 overexpression or control by colony formation. (c) Annexin V/PI staining was used to measure apoptosis in HCC cells. Numbers indicated the percentage of each quadrant. \( n = 3 \). (d) In vivo orthotopic growth of ITGA9-overexpressed versus control HCC cells. \( n = 6 \). Values are means ± SEM. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \).
controlling cell motility [24]. Since paxillin and vinculin represent newly formed and mature focal adhesion, respectively, we investigated these two adapter proteins in ITGA9-overexpressed and control cells. Compared with the control cells, ITGA9 overexpression cells showed no obvious difference in paxillin-positive adhesion plaque, whereas displaying more vinculin-positive adhesion plaque (Figures 5(a) and 5(b)).

It is well known that cytoskeleton rearrangement and focal adhesion formation are orchestrated by small G-proteins, which play key roles in the motility of cancer cells. By pull-down assay, we found the activity of Rac1 and RhoA decreased significantly in ITGA9 overexpression cells. However, there was no significant difference detected in Cdc42 activity between ITGA9 overexpression and control cells (Figure 5(c)). The mechanism for ITGA9-mediated...
dysregulation of focal adhesion could also be related to FAK and Src, which are key adaptor molecules in adhesions. Indeed, the phosphorylation levels of FAK and Src were decreased in ITGA9 overexpression HCC cells compared to control cells (Figure 5(d)).

Taken together, ITGA9 overexpression-induced alterations, including increased vinculin-containing focal adhesions, decreased activity of Rac1 and RhoA, and reduced phosphorylation of FAK and Src, were conducive to the suppressive effects of ITGA9 on HCC cell behavior.

4. Discussion

Given that no dominant mechanism is responsible for HCC cell growth and metastasis, efforts aiming at identifying novel molecules may exert therapeutic benefits for patients suffering from HCC. Integrin receptors and associated signaling have shown to play important roles during HCC progression [25, 26]. In our current study, we demonstrated that ITGA9 expression was obviously downregulated in HCC patients. Our study is the first one to reveal that ITGA9 negatively correlated with HCC progression.

Table 3: Gene set enrichment analysis (GSEA) of ITGA9 mRNA profiling results in HCC from the TCGA database.

| Pathway                                      | Genes (n) | P value  | Q value  |
|----------------------------------------------|-----------|----------|----------|
| Pathways in cancer                           | 314       | 0.0000   | 0.0627   |
| Regulation of actin cytoskeleton             | 196       | 0.0000   | 0.0678   |
| Focal adhesion                               | 193       | 0.0000   | 0.0679   |
| Purine metabolism                            | 153       | 0.0000   | 0.0953   |
| Cell adhesion molecules cams                 | 128       | 0.0000   | 0.0574   |
| Lysosome                                     | 121       | 0.0000   | 0.0752   |
| Pyrimidine metabolism                        | 97        | 0.0000   | 0.0645   |
| ECM receptor interaction                     | 83        | 0.0000   | 0.0758   |
| Arrhythmogenic right ventricular cardiomyopathy | 68     | 0.0000   | 0.0576   |
| Non-small-cell lung cancer                   | 53        | 0.0000   | 0.0687   |
| Vasopressin-regulated water reabsorption     | 41        | 0.0000   | 0.0610   |
| Prostate cancer                              | 87        | 0.0019   | 0.0712   |
| Small-cell lung cancer                       | 84        | 0.0020   | 0.0639   |
| PPAR signaling pathway                       | 65        | 0.0020   | 0.0636   |
| Chemokine signaling pathway                  | 181       | 0.0020   | 0.0701   |
| Leukocyte transendothelial migration         | 107       | 0.0020   | 0.0583   |
| Basal cell carcinoma                         | 54        | 0.0020   | 0.0657   |
| Valine leucine and isoleucine degradation    | 44        | 0.0038   | 0.0632   |
| Hedgehog signaling pathway                   | 54        | 0.0039   | 0.0602   |
| Inositol phosphate metabolism                | 54        | 0.0040   | 0.0664   |

Studies have noted that FAK and Src promote cancer cell motility by controlling the formation and turnover of focal adhesion through multiple signal pathways [8]. It has been reported that overactive small Rho GTPases play supportive roles in tumor progression. Rac-1 mutations can drive the malignancy of melanoma [35]. Gain-of-function mutations of RhoA occur specifically in poorly differentiated adenocarcinomas [36]. In this study, we demonstrated that ITGA9 reduced the FAK and Src phosphorylation, decreased Rac1 and RhoA activation, and promoted focal adhesion maturation, leading to the suppression of HCC cell motility.

In summary, our results first demonstrated that ITGA9 suppresses HCC cell migration and invasion via FAK/Src-Rho GTPase signaling. Furthermore, our findings indicated ITGA9 might be identified as a diagnostic biomarker for HCC and provided a potential solution for the treatment of HCC.
Figure 5: ITGA9 affects focal adhesions and Rho GTPase activity. (a and b) Immunofluorescence staining of paxillin (red) and vinculin (red). F-Actin was stained by FITC phalloidin (green), and the cell nuclei were stained by DAPI (blue). Scale bars, 10 μm. (c) Pull-down assays of the activities of RhoA, Cdc42, and Rac1 in ITGA9-overexpressed or control HCC cells. (d) FAK and Src phosphorylation of ITGA9-overexpressed versus control HCC cells. Tubulin was used as an internal control. Values are means ± SEM. ns: no significance. *P < 0.05 and **P < 0.01.
Abbreviations

ITGA9: Integrin α9
HCC: Hepatocellular carcinoma
EMT: Epithelial-mesenchymal transition
CNL: Corresponding noncancerous liver
FAK: Focal adhesion kinase
Src: c-Src tyrosine kinase.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no competing financial interests.

Authors’ Contributions

Yan-Li Zhang, Xin Xing, and Li-Bo Cai contributed equally to this work.

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