

Knockdown of integrin subunit $\alpha 7$ inhibits cell proliferation, invasion and promotes apoptosis via downregulating FAK/PI3K signaling pathway in hepatocellular carcinoma

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Abstract

Background The study aimed to explore the effect of integrin subunit α 7 (ITGA7) knockdown on cell proliferation, apoptosis, invasion, and regulation of FAK/PI3K pathway in hepatocellular carcinoma (HCC).

Methods ITGA7 mRNA and protein expressions were detected in HCC tumor tissues, adjacent tissues, HCC cell lines (including: Hep G2, BEL-7402, SMMC-7721, Huh-7 cell lines) and human liver epithelial cell line (THLE-3). The effect of ITGA7 knockdown on cell proliferation, apoptosis, invasion, expressions of FAK and PI3K were detected after transfection of ITGA7 small interfering RNA (siRNA) and control siRNA into Huh-7 cells.

Results ITGA7 was upregulated in HCC tumor tissues compared with adjacent tissues, and increased in Hep G2, SMMC-7721 and Huh-7 cell lines while unchanged in BEL-7402 cell line compared with THLE-3 cell line. As for cell activities, ITGA7 knockdown inhibited cell proliferation, invasion but promoted apoptosis in Huh-7 cells. In addition, ITGA7 knockdown decreased the expressions of FAK and PI3K in Huh-7 cells, implying that ITGA7 knockdown might reduce HCC progression via inhibition of FAK/PI3K pathway in HCC.

Conclusions ITGA7 is upregulated, and its knockdown inhibits cell proliferation, invasion but promotes apoptosis via suppression of FAK/PI3K signaling pathway in HCC, which implies that ITGA7 might serve as a potential treatment target for HCC.

Backgrounds

Hepatocellular carcinoma (HCC), as the primary type of liver cancer, comprises 75%–85% of all liver cancer cases and is one of the most common causes of cancer-related mortality worldwide [1]. Several therapeutic options including surgical resection, liver transplantation, radiofrequency ablation etc., are available for HCC patients depending on HCC stage, liver function and comorbidities [2]. However, there are still a large number of HCC patients suffering from tumor recurrence and metastasis, which leads to the poor prognosis in HCC patients and enormous pressure to medical resources [3, 4]. Therefore, it is essential to explore more potential therapeutic targets to improve the treatment efficacy for HCC patients.

Integrins are a family of transmembrane proteins expressed in almost all types of cells and are composed of an α and a β subunit as heterodimers on the cell surface [5, 6]. Integrin subunit α 7 (ITGA7), as one of the integrin family members, is shown to be an important laminin receptor in the development of skeletal muscle and forms heterodimer with integrin β 1 [7]. Recent studies suggest that ITGA7 is dysregulated and involved in the development and progression in several cancers [8, 9]. For example, ITGA7 overexpression is revealed to be closely correlated with aggressive tumor behavior and poor clinical outcomes in oesophageal squamous cell carcinoma (OSCC) [9]. In addition, evidence reveals that ITGA7 activates FAK, and induces the activation of the downstream PI3K-mediated signaling pathway in OSCC, and FAK/PI3K is also shown to regulate epithelial-mesenchymal transition (EMT) as well as tumor

progression in HCC [9–11]. Taken together, we speculated that ITGA7 might be dysregulated in HCC and affect the cell activities by regulating FAK/PI3K signaling pathway. Therefore, we designed the cellular experiments in this present study to explore the role of ITGA7 and its potential molecular regulatory mechanisms in HCC.

Methods

Tissue samples collection and ITGA7 detection

HCC tumor tissue and adjacent tissue were collected from our sample storage room. All tissue specimens were obtained by surgery removal. For the snap-frozen specimen stored at -80°C , it was used for the detection of ITGA7 mRNA expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR); as for the specimen fixed with formalin and embedded in paraffin wax, it was used for the determination of the ITGA7 protein expression using immunohistochemistry (IHC) assay.

IHC assay

IHC assay was used to detect the ITGA7 protein expression in the specimens. The specimens were cut into $4\ \mu\text{m}$ sections, deparaffinized with xylene and rehydrated by ethanol. Then antigen retrieval was performed with retrieval buffers through microwave heating. To block endogenous peroxidase, the sections were incubated with H_2O_2 , then were blocked using normal goat serum. Afterwards, the sections were incubated with Rabbit polyclonal to ITGA7 antibody (1:1000; Abcam, USA) at 4°C overnight. Next day, the sections were incubated with horseradish peroxidase-conjugated goat-anti-rabbit Immunoglobulin G antibody (1:1000; Abcam, USA). Subsequently, the sections were stained with diaminobenzidine (DAB) (Dako, Denmark) and counterstained by hematoxylin, then were dehydrated by gradient ethanol. Finally, the sections were sealed and observed on an Inverted microscope (Olympus, Japan).

Cell culture

HCC cell lines including Hep G2, BEL-7402, SMMC-7721, and Huh-7 and human liver epithelial cell line THLE-3 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Hep G2 cell line was cultured in 90% Minimum Essential Medium (MEM) (Gibco, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, USA). BEL-7402 and SMMC-7721 cell lines were cultured in 90% Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, USA) with 10% FBS (Gibco, USA). Huh-7 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% FBS. THLE-3 was cultured in 90% BEGM Bullet Kit (Lonza, USA) with 10% FBS (Gibco, USA). All cell lines were cultured at the condition of 37°C and 5% CO_2 .

ITGA7 expression detection in cells

RT-qPCR and western blot were performed to detect the ITGA7 mRNA and protein expressions in human HCC cell lines. And the mRNA and protein expressions of ITGA7 were also detected in THLE-3 cell line which was used as control.

Small interfering RNA (siRNA) synthesis

ITGA7 siRNA was designed by Gene Link siRNA Design Guidelines (website: <http://www.genelink.com/sirna/RNAicustomorder.asp>) and was synthesized by Shanghai Qeejen Bio-Tech Co., Ltd (Shanghai, China). In addition, the normal control (NC) siRNA was also synthesized by Shanghai Qeejen Bio-Tech Co., Ltd (Shanghai, China).

SiRNA transfection and subsequent detections

ITGA7 siRNA and NC siRNA were transfected into Huh-7 cells by HilyMax (Dojindo, Japan). The cells transfected with ITGA7 siRNA were named as Si-ITGA7 group, and the cells transfected with NC siRNA were named as Si-NC group, respectively. The mRNA and protein expressions of ITGA7 in cells were determined by RT-qPCR and western blot at 24h after transfection. Cell proliferation was detected by Cell Counting Kit-8 (CCK-8) assay at 0h, 24h, 48h, 72h after transfection. Cell apoptosis was detected by Annexin V (AV)/ Propidium Iodide (PI) assay at 48h after transfection. Cell invasion was evaluated by transwell assay at 24h after transfection.

FAK and PI3K detection

GeneCards (<https://www.genecards.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.jp/kegg/pathway.html) were applied to predict the downstream gene of ITGA7. And it was found that FAK and PI3K were downstream genes of ITGA7, that is, FAK/PI3K might be regulated by ITGA7. Consequently, the mRNA and protein expressions of FAK and PI3K were further determined by RT-qPCR and western blot at 24h after transfection as well.

RT-qPCR

Total RNA was extracted from tissues or cells using TRIzol™ Reagent (Invitrogen, USA) and then reversely transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad, USA). Following that, qPCR was performed using SYBR® Advantage® qPCR Premix (Clontech, USA) to quantify ITGA7, FAK and PI3K expressions. And the results were calculated using $2^{-\Delta\Delta C_t}$ method with GAPDH as an internal reference. The instrument used was ABI 7900HT Real-Time PCR System (Applied Biosystems, USA). Primers were listed in *Table 1*.

Western blot

Total protein was extracted with RIPA buffer (Sigma, USA). After that, the protein concentration in each sample was measured using the Bicinchoninic Acid Kit for Protein Determination (Sigma, USA). 20 ug protein was loaded to NuPAGE™ 4–12% Bis-Tris Protein Gels (Thermo, USA) and transferred onto nitrocellulose membrane (Thermo, USA). After blocking with 5% BSA (Thermo, USA) for 2 h, the membranes were incubated with the primary antibodies overnight at 4 °C. Then, the membranes were incubated with the secondary antibody for 90 min at 37 °C. Pierce™ ECL Plus western Blotting Substrate (Thermo, USA) and X-ray film (Kodak, USA) was used to illuminate and visualize the bands. The antibodies used in the western blot were summarized in *Table 2*.

CCK-8 assay

Cell proliferation of Huh-7 cells was detected at 0 h, 24 h, 48 h and 72 h after transfection using CCK-8 (Dojindo, Japan). The detailed process were as follows: 10 ul CCK-8 and 90 ul serum-free medium were added to each group of Huh-7 cells, then the cells were incubated under the environment of 5% CO₂ and at 37 °C. Optical density (OD) value was measured by Microplate reader (BioTek, USA).

AV/PI assay

Cell apoptosis rate of Huh-7 cells was detected at 48h after transfection using Annexin V-FITC Apoptosis Detection Kit (Sigma, USA). In brief, Huh-7 cells in each group were collected and washed with phosphate buffer solution (PBS), then suspended in 100 ul binding buffer. Then 5 ul Annexin V (AV) and 5 ul propidium iodide (PI) were added to the cells, followed with cells incubated in darkness for 15 min. Subsequently, 400ul binding buffer was added and apoptosis rate was detected using CytoFLEX Flow cytometer (Beckman Coulte, USA) and analyzed using Software, FlowJo Software 7.6 (FlowJo-LLC, USA).

Transwell assay

Cell invasion of Huh-7 cells was detected at 24h after transfection. In brief, transfected Huh-7 cells were seeded into chamber (Cornig, USA) with 8 um Polycarbonate film coated with Matrigel basement membrane matrix (BD, USA). Cells in the upper chamber were cultured in serum-free media, while medium containing 10% FBS was placed in the lower chamber. After incubating for 24h, cells on the upper membrane were removed with cotton wool and chamber was fixed with 4% formaldehyde and stained in 0.1% crystal violet. The stained cells were visualized using an inverted microscope (Olympus, Japan) and the number of stained cells was quantified.

Statistical analysis

Statistical analysis and graph plotting were performed by GraphPad Prism 7.00 software (GraphPad Int, USA). Data were mainly presented as mean±standard deviation (SD). Comparison among groups was detected using one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test, and comparison between two groups was detected using unpaired, parametric *t* test. All tests were two-tailed. *P* value < 0.05 was considered as significant in this study.

Results

ITGA7 expression in HCC tissue and cell lines

ITGA7 mRNA relative expression was increased in tumor tissue (N = 10) compared with adjacent tissue (N = 10) (*P*<0.001) (*Figure 1A*). ITGA7 protein expression was also elevated in tumor tissue compared with adjacent tissue (*Figure 1B*). Cellular experiments displayed that ITGA7 mRNA relative expression was increased in Hep G2 (*P*<0.01), SMMC-7721 (*P*<0.05) and Huh-7 (*P*<0.001) cell lines, but unchanged in BEL-7402 cell line (*P*>0.05) compared with human liver epithelial cell line THLE-3 (*Figure 1C*). And western blot visually revealed that ITGA7 protein expression was increased in Hep G2, SMMC-7721 and Huh-7 cell lines but was similar in BEL-7402 cell line compared with human liver epithelial cell line THLE-3 (*Figure 1D*). These data suggested that ITGA7 was upregulated in HCC tissue and cell lines.

ITGA7 expression after transfection in Huh-7 cells

As the highest expression of ITGA7 was shown in Huh-7 cells, it was chosen for the following cellular experiments. After transfection, ITGA7 mRNA relative expression was decreased in Si-ITGA7 group compared with Si-NC group (*P*<0.01) (*Figure 2A*). And western blot also visually revealed that ITGA7 protein expression was downregulated in Si-ITGA7 group compared with Si-NC group (*Figure 2B*). These data indicated the successful transfection.

Effect of ITGA7 knockdown on cell proliferation and cell apoptosis in Huh-7 cells

OD value by CCK-8 was decreased in Si-ITGA7 group at 48h (*P*<0.05) and at 72h (*P*<0.01) but unchanged at 0h and 24h (both *P*>0.05) after transfection compared with Si-NC group in Huh-7 cells (*Figure 3A*). And cell apoptosis rate was increased in Si-ITGA7 group compared with Si-NC group at 48h after transfection in Huh-7 cells (*P*<0.01) (*Figure 3B, 3D*). These data indicated that ITGA7 knockdown inhibited cell proliferation but promoted cell apoptosis in HCC.

Effect of ITGA7 knockdown on cell invasion in Huh-7 cells

Invasive cell count was reduced in Si-ITGA7 group compared with Si-NC group at 24h after transfection in Huh-7 cells ($P < 0.01$) (Figure 4A, 4B).. These data suggested that ITGA7 knockdown inhibited cell invasion in HCC.

Effect of ITGA7 knockdown on FAK and PI3K expressions in Huh-7 cells

The expressions of FAK and PI3K were detected, which observed that FAK (Figure 5A) and PI3K (Figure 5B) (both $P < 0.05$) mRNA relative expression were decreased in Si-ITGA7 group compared with Si-NC group at 24h after transfection in Huh-7 cells. And western blot also illustrated that FAK and PI3K protein expressions were reduced in Si-ITGA7 group compared with Si-NC group at 24h after transfection in Huh-7 cells (Figure 5C).. Data above indicated that ITGA7 knockdown decreased expressions of FAK and PI3K in HCC.

Discussion

In the present study, we found that (1) ITGA7 was upregulated in HCC tissue and HCC cell lines (including: Hep G2, SMMC-7721 and Huh-7 cell lines), and its knockdown inhibited cell proliferation, invasion but promoted apoptosis in HCC. (2) ITGA7 knockdown decreased the expressions of FAK and PI3K in HCC.

Integrins are heterodimeric cell surface receptors, establishing a physical connection between extracellular matrix (ECM) and the actin cytoskeleton as well as controlling the activation of several intracellular signaling pathways [5, 6]. Meanwhile, numerous evidences indicate that integrins are involved in EMT and function as a key regulator of solid tumor growth and metastasis with the consideration of its functions in regulating pro-survival, proliferative signaling and cell migration [5]. ITGA7 belongs to integrin α chain family and participates in the formation of mesenchymal tissues including skeletal and vascular smooth muscle [7]. It has been reported to be dysregulated and play important roles in development and progression of several cancers [9, 10, 12]. For example, ITGA7 is upregulated and its overexpression promotes cell migration and invasion ability in OSCC [9]. In another study, ITGA7 is reported to be upregulated on the surface of primary tumorigenic glioblastoma cells, and cellular studies reveal that ITGA7 knockdown contributes to the decrease in cell proliferation and invasion [10]. According to the previous studies, we proposed that ITGA7 might affect cell proliferation, apoptosis and invasion in HCC. To explore the possibility, we conducted the present study and found that ITGA7 was upregulated in HCC tissue compared with adjacent tissue. And we did the cellular experiments, which exhibited that ITGA7 expression was increased in HCC cell lines (including: Hep G2, SMMC-7721 and Huh-7 cell lines) compared with human liver epithelial cell line THLE-3, and meanwhile its knockdown

inhibited cell proliferation, cell invasion but promoted cell apoptosis in HCC. Data above revealed that ITGA7 served as a tumor promotor and contributed to the aggressive tumor progression in HCC. The possible explanations might include that: (1) Considering that function of integrins in cell metabolism, ITGA7 knockdown might lead to a reduction of glucose uptake, decreased ATP levels, elevated generation of reactive oxygen species and reduced fatty acid oxidation which are crucial for cell metabolism and biosynthetic production, thus, ITGA7 knockdown might contribute to the promotion of cell apoptosis and inhibition of cell proliferation. (2) According to the previous study, ITGA7 interacted with integrin $\beta 1$ which regulated lactate transportation and was of invasive ability, therefore the ITGA7 knockdown might inactive integrin $\beta 1$, decreasing the ability of cell invasion in HCC [5]. (3) ITGA7 knockdown might impair normal activation of the epidermal growth factor receptor, decreasing the expressions of some oncogenes on the oncogenic signaling pathway in HCC, such as: FAK/PI3K signaling pathway which was a pathway that mediated cell survival, thus contributing to the reduced cell proliferation, invasion and elevated apoptosis in HCC, which was further verified in our following cellular experiments.

Existing evidences indicate that ITGA7 activates FAK, which elicits the activation of downstream PI3K-mediated signaling pathway, thereby regulates tumor progression in several cancers other than HCC [9, 10]. For example, ITGA7 overexpression enhances expressions of stemness-related genes and EMT features as well as increased abilities to self-renew through the activation of the FAK-mediated and FAK downstream PI3K/Akt signaling pathway in OSCC [9]. In another study, the upregulation of integrin $\beta 1$, which ITGA7 forms the heterodimer with, mediates the FAK expression and leads to the activation of PI3K-AKT signaling pathway, which is responsible for continued cell motility as well as cell survival in metastatic triple negative breast cancer [13]. In addition, FAK has been reported to be downstream effectors to activate PI3K-mediated signaling pathway in EMT progression, and the suppression of FAK and PI3K contributes to the reduced invasion in HCC [12, 14]. According to the aforementioned evidence, we speculated that ITGA7 knockdown might reduce HCC progression via inhibition of FAK/PI3K pathway in HCC. Thus, we detected the expressions of FAK and PI3K after siRNA transfections and observed that FAK and PI3K were downregulated by ITGA7 knockdown, which suggested that ITGA7 knockdown might inhibit tumor progression by blocking FAK/PI3K signaling pathway in HCC. The possible reasons included that: (1) According to the previous study, ITGA7 is reported to interact with integrin $\beta 1$ to form $\alpha 7\beta 1$ heterodimer, which can increase PI3K expression, therefore ITGA7 knockdown might lead to the decreased expression of $\alpha 7\beta 1$ heterodimer, contributing to the inactivation of FAK/PI3K signaling pathway [15]. (2) Given that integrin membrane traffic was important for cell metabolism, ITGA7 knockdown might accompany with the metabolic stress for tumor cells, which prevented the growth factors expression and ECM engagement, thus inactivating the pro-survival signaling FAK/PI3K in HCC. However, the detailed underlying mechanism needed further cellular experiments for validation.

Conclusions

In summary, ITGA7 is upregulated, and its knockdown inhibits cell proliferation, invasion but promotes apoptosis via suppression of FAK/PI3K pathway in HCC, which implies that ITGA7 might serve as a potential treatment target for HCC.

Declarations

Ethics approval and consent to participate

Our study did not require an ethical board approval because it did not contain human or animal trials.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

JS and CY designed and coordinated the study, made substantial contributions to the analysis, and drafted the manuscript. JS, CY and XZ performed statistical analysis. JS, CY and XZ participated in data collection and interpretation. CC helped to draft the manuscript. JS, CY and CC reviewed the manuscript and exerted a major impact on the interpretation of data and critical appraisal of the manuscript. All authors have read and approved the final manuscript.

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Not applicable.

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Tables

Table 1. Primers applied in RT-qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ITGA7	GCCACTCTGCCTGTCCAATG	CGGAGGTGCTAAGGATGAGGTA
FAK	GGTGAAGGAAGTCGGCTTGG	TCTTGCTGGAGGCTGGTCAT
PI3K	GCCTCCACGACCATCATCAG	TTCTTCACGGTTGCCTACTGG
GAPDH	GACCACAGTCCATGCCATCAC	ACGCCTGCTTCACCACCTT

ITGA7, integrin subunit alpha 7; FAK, focal adhesion kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2. Antibodies applied in western blot

Antibody	Company	Dilution
Primary Antibody		
Anti-ITGA7 antibody (rabbit polyclonal)	Abcam (UK)	1:1000
Anti-FAK antibody (rabbit monoclonal)	Abcam (UK)	1:2000
Anti-GAPDH antibody (rabbit monoclonal)	Abcam (UK)	1:5000
Secondary Antibody		
Goat Anti-Rabbit IgG H&L (HRP)	Abcam (UK)	1:10000

Figures

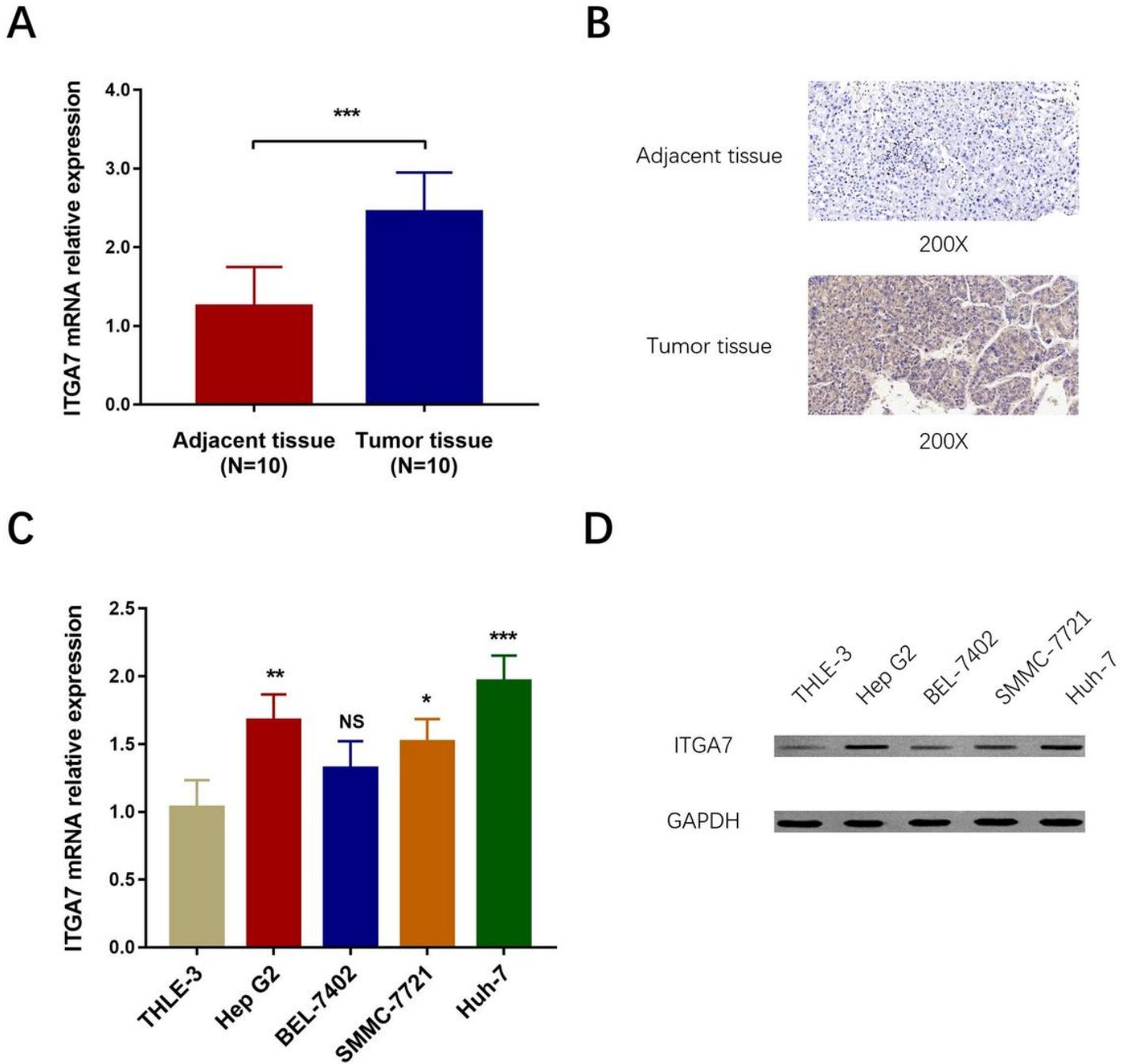
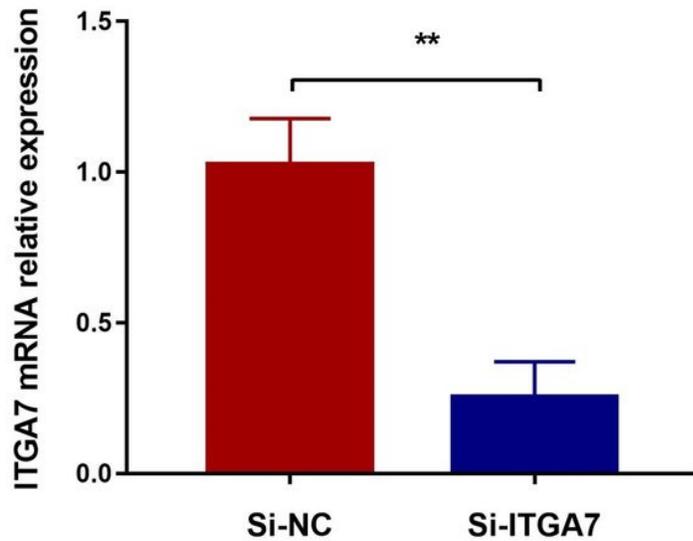


Figure 1

Detection of ITGA7 expression in HCC tissue and cell lines. Comparison of ITGA7 mRNA relative expression between adjacent tissue and tumor tissue (A). Comparison of ITGA7 protein expression between adjacent tissues and tumor tissues by IHC assay (B). Comparison of ITGA7 mRNA (C) and protein (D) relative expression between HCC cell lines (including Hep G2, BEL-7402, SMMC-7721, and Huh-7 cell lines) and human liver epithelial cell line (THLE-3 cell line). Comparison among groups was detected using one-way analysis of variance followed by Turkey's multiple comparison test, and comparison between two groups was detected using unpaired, parametric t test. $P < 0.05$ was considered

significant. NS, non-significant; *P <0.05, **P <0.01, ***P <0.001. IHC, immunohistochemistry; ITGA7, integrin subunit α 7; HCC, hepatocellular carcinoma.

A



B

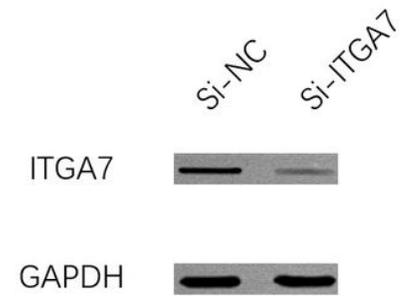


Figure 2

ITGA7 expression in Huh-7 cells after transfection. Comparison of ITGA7 mRNA (A) and protein (B) expression between Si-NC group and Si-ITGA7 group in Huh-7 cells at 24h after transfection. Comparison between two groups was detected using unpaired, parametric t test. P <0.05 was considered significant. **P <0.01. ITGA7, integrin subunit α 7; Si-NC, Huh-7 cells transfected with normal control small interfering RNA; Si-ITGA7, Huh-7 cells transfected with ITGA7 small interfering RNA.

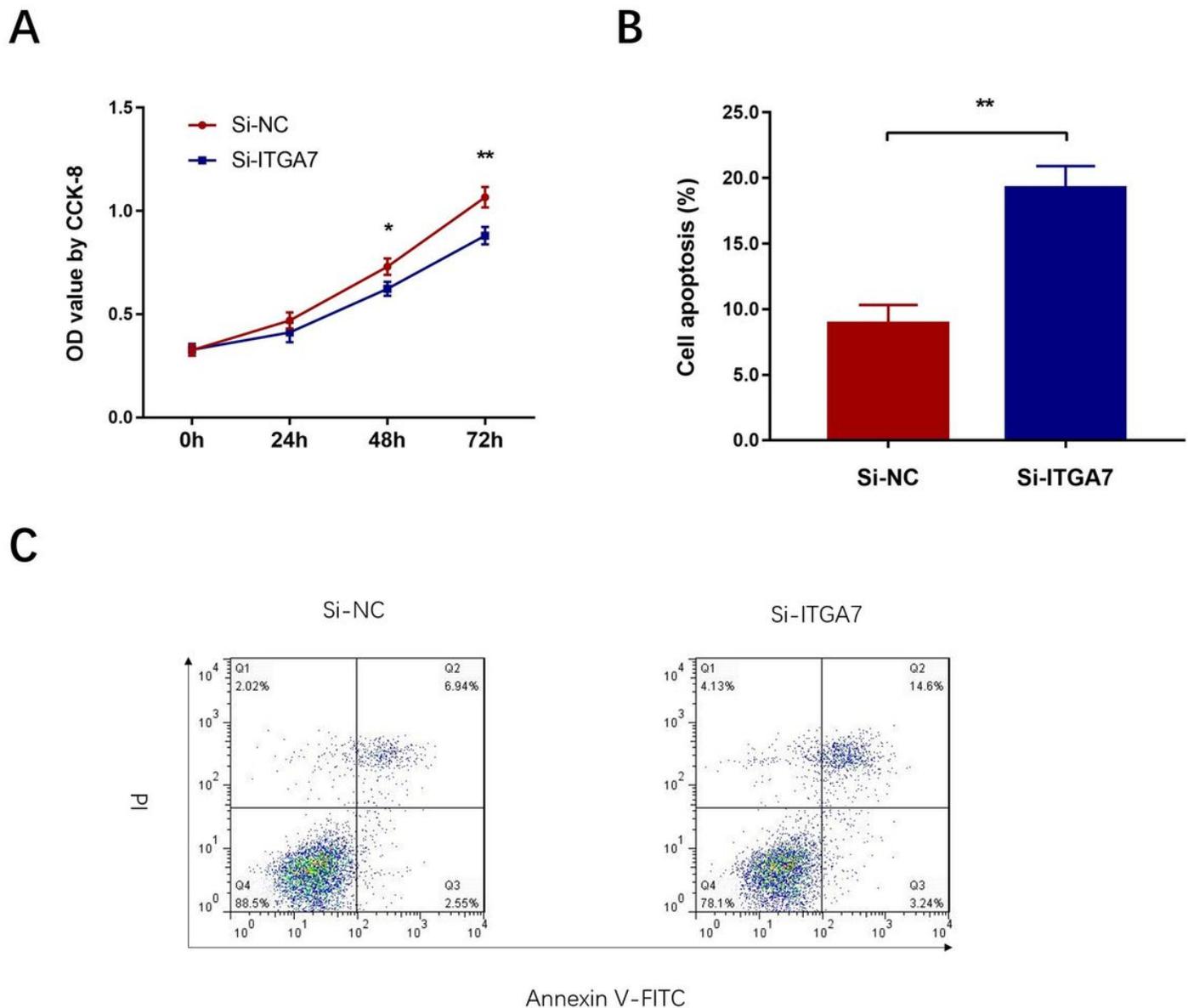


Figure 3

Cell proliferation and cell apoptosis in Huh-7 cells after transfection. Comparison of OD value by CCK-8 between Si-NC group and Si-ITGA7 group in Huh-7 cells at 0h, 24h, 48h and 72h after transfection (A). Comparison of cell apoptosis rate between Si-NC group and Si-ITGA7 group in Huh-7 cells at 48h after transfection (B, C). Comparison between two groups was detected using unpaired, parametric t test. $P < 0.05$ was considered significant. * $P < 0.05$, ** $P < 0.01$. OD, optical density; ITGA7, integrin subunit $\alpha 7$; Si-NC, Huh-7 cells transfected with normal control small interfering RNA; Si-ITGA7, Huh-7 cells transfected with ITGA7 small interfering RNA.

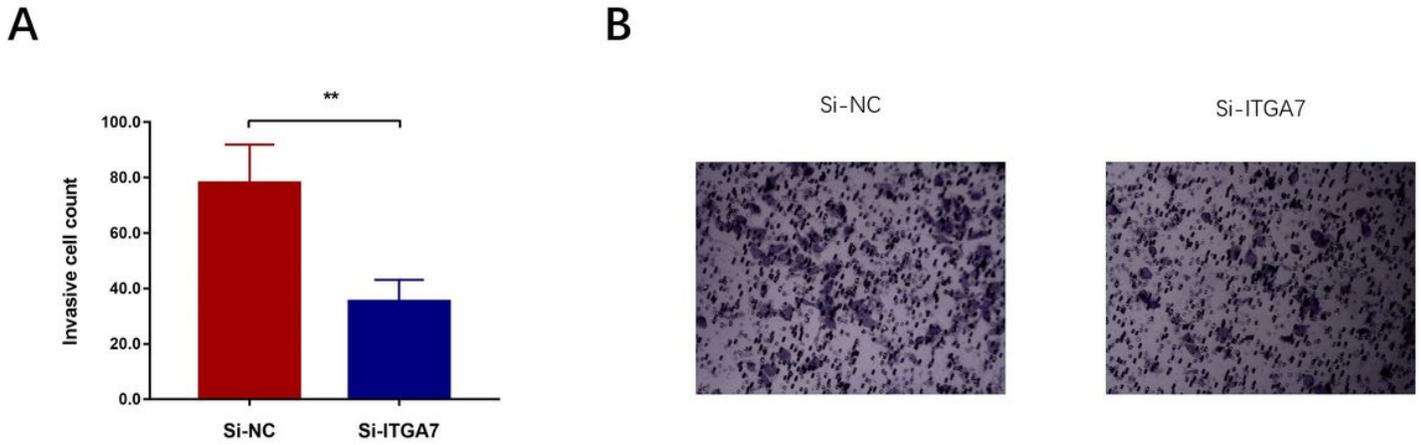


Figure 4

Cell invasion in Huh-7 cells after transfection. Comparison of invasive cell count between Si-NC group and Si-ITGA7 group in Huh-7 cells at 24h after transfection (A, B). Comparison between two groups was detected using unpaired, parametric t test. $P < 0.05$ was considered significant. $**P < 0.01$. ITGA7, integrin subunit α 7; Si-NC, Huh-7 cells transfected with normal control small interfering RNA; Si-ITGA7, Huh-7 cells transfected with ITGA7 small interfering RNA.

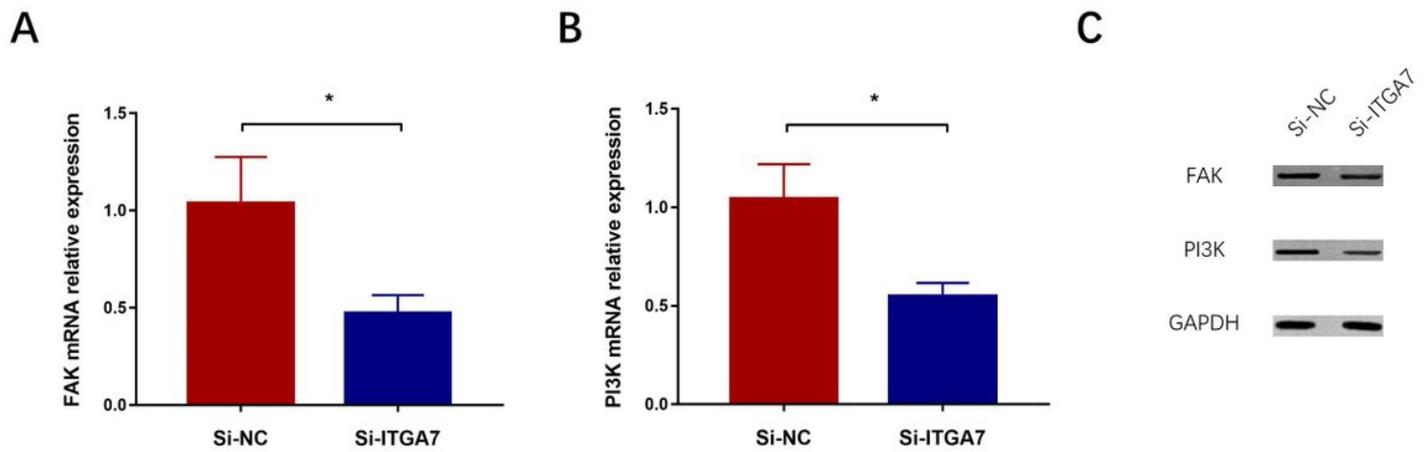


Figure 5

FAK and PI3K expressions in Huh-7 cells after transfection. Comparison of FAK mRNA (A) and protein (C) expressions between Si-NC group and Si-ITGA7 group in Huh-7 cells at 24h after transfection. Comparison of PI3K mRNA (B) and protein (C) expression between Si-NC group and Si-ITGA7 group in Huh-7 cells at 24h after transfection. Comparison between two groups was detected using unpaired, parametric t test. $P < 0.05$ was considered significant. $*P < 0.05$. ITGA7, integrin subunit α 7; Si-NC, Huh-7 cells transfected with normal control small interfering RNA; Si-ITGA7, Huh-7 cells transfected with ITGA7 small interfering RNA.