Integrin α7 knockdown suppresses cell proliferation, migration, invasion and EMT in hepatocellular carcinoma

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Abstract. The present study aimed to investigate the effects of integrin α7 (ITGA7) on regulating hepatocellular carcinoma (HCC) progression and endothelial-mesenchymal transition (EMT). ITGA7 mRNA and protein expression in human normal liver epithelial cells and HCC cell lines were determined by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. ITGA7 small interfering RNA [siRNA; ITGA7-knockdown (KD) group] and nonsense siRNA (control group) were transfected into Huh7 cells and SNU449 cells, respectively. ITGA7 mRNA and protein expression (RT-qPCR and western blotting, respectively), cell proliferation (Cell Counting Kit-8 assay), apoptosis (annexin V/propidium iodide assay), migration (wound scratch assay) and invasion (Transwell assay) were then detetected. E-cadherin, α-smooth muscle actin (α-SMA), vimentin and V-cadherin levels (RT-qPCR and western blotting) were also assessed. ITGA7 mRNA and protein expression levels were increased in Li7, Huh7, SKHEP1 and SNU449 cells compared with THLE-3 cells. Following transfection, ITGA7 mRNA and protein expression was lower in the ITGA7-KD group compared with that in the control group in both Huh7 and SNU449 cells, indicating successful transfection. In the ITGA7-KD group, cell proliferation decreased at 48 and 72 h, cell apoptosis rates increased at 48 h, cell migration rate was reduced at 24 h and cell invasion decreased at 24 h compared with the control group. Additionally, increased E-cadherin but decreased α-SMA, vimentin and V-cadherin mRNA and protein expression levels were observed in the ITGA7-KD group compared with the control group at 24 h.

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In conclusion, ITGA7 knockdown suppressed HCC progression and inhibited EMT in HCC *in vitro*, implying that ITGA7 might be a novel treatment target for HCC.

Introduction

Liver cancer was the sixth most common cancer and the fourth leading cause of cancer deaths worldwide in 2018, which resulted in 841,080 new cases and 781,265 deaths, accounting for 4.7% of all cancer cases and 8.2% of all cancer deaths (1). As the most common type of liver cancer, hepatocellular carcinoma (HCC) comprises 75-85% of cases of liver cancer (1). Surgical resection is an optimal modality in patients with HCC who have small solitary tumors and well-preserved liver function, and could be subjected to this invasive operation and achieve satisfactory efficacy (2). Patients who are diagnosed in advanced stages of HCC, who account for the majority of all cases, are often not suitable candidates for surgical resection (2). Liver transplantation, another curative treatment option, is most suitable for patients with HCC who are not good candidates for resection, although donor shortage and high medical costs limit its application (2). Hence, investigating the molecular mechanisms of HCC progression is required for the detection of novel and effective therapeutic targets to improve HCC prognosis.

Integrins, which are heterodimers, consist of α and β subunits, which participate in a range of cellular processes including cell survival, growth, invasion and migration (3). Integrin α7 (ITGA7), which belongs to the integrin family of adhesion molecules, plays a role in cell-cell and cell-extracellular matrix interactions in multiple cellular processes (4). According to previous studies, ITGA7 is oncogenic in the pathological processes of several carcinomas including glioblastoma, esophageal squamous cell carcinoma (OSCC) and lung cancer (4-6). For instance, ITGA7 promotes OSCC cell migration and invasion, while concurrently increasing E-cadherin and α-smooth muscle actin (α-SMA) expression, which are common markers of endothelial-mesenchymal transition (EMT), indicating that ITGA7 may promote malignant cellular function and induce EMT in OSCC cells (5). Hence, it was hypothesized that ITGA7 may also have promotive effects on cellular function and EMT regulation in HCC. However, to the best of our knowledge, little is known about the role of ITGA7 in HCC. Thus, the present study aimed to

investigate the effects of ITGA7 on regulating HCC progression and EMT.

Materials and methods

Cell culture. Human normal liver epithelial cells (THLE-3) and HCC cell lines SKHEP1 and SNU449 were purchased from American Type Culture Collection. HCC cell lines Li7 and Huh7 were purchased from RIKEN BioResource Center. THLE-3 cells were cultured in 90% bronchial epithelial growth medium (Lonza Group, Ltd.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Li7 cells and SNU449 cells were cultured in 90% RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. Huh7 cells were cultured in 90% DMEM (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. SKHEP1 cells were cultured in 90% Eagle's minimum essential medium (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. All medium was added 100 U/ml penicillin and 100 lg/ml treptomycin (Sigma-Aldrich; Merck KGaA). Cells were maintained in a humid incubator at 37°C.

ITGA7 small interfering RNA (siRNA) construction and transfection. siRNA was used to knock down ITGA7 expression. ITGA7 siRNA and nonsense siRNA were designed and synthesized by Guangzhou RiboBio Co., Ltd. ITGA7 siRNA (80 nM) and nonsense siRNA were transfected into Huh7 cells and SNU449 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions for 6 h at 37°C. Subsequently, cells transfected with ITAG7 siRNA were considered ITGA7-knockdown (KD) cells, and cells transfected with nonsense siRNA were marked as control cells. At 24 h after transfection, lTGA7 mRNA and protein expression levels were determined by RT-qPCR and western blotting; cell apoptosis was detected by an annexin V/propidium iodide (AV/PI) assay at 48 h, expression of apoptosis-related protein cleaved caspase 3 was detected by western blotting, and cell migration and invasion abilities were assessed by wound scratch and Transwell assays, respectively. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay at 0, 24, 48 and 72 h. Additionally, whether the mRNA and protein expression of E-cadherin, vimentin, N-cadherin and α-SMA were regulated by ITGA7 was determined by RT-qPCR and western blotting at 24 h after transfection. In addition, the sequences of ITGA7 siRNA were as follows: Forward, 5'-GCAUCAAGAGCUUCGGCUATT-3' and reverse, 5'-UAGCCGAAGCUCUUGAUGCTT-3'.

RT-qPCR. ITGA7 mRNA expression was assessed in THLE-3, Li7, Huh7, SKHEP1 and SNU449 cells. E-cadherin, vimentin, N-cadherin and α-SMA mRNA expression was assessed in Huh7 and SNU449 cells. Following cell dissociation using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.), TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA. Subsequently, 1 μg RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) with following thermocycling condition: 42°C for 15 min and 85°C for 5 sec. TB Green™ Fast qPCR mix (Takara, Bio, Inc.) was used for qPCR. The following thermocycling conditions were used: 95°C for 5 min; 40 cycles

of 95°C for 5 sec and 61°C for 30 sec. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (7). Primer sequences are shown in Table I. GAPDH was used as the internal reference gene. In addition, the sequences of ITGA7 siRNA were as follows: Forward, 5'-GCAUCAAGAGCUUCGGCUATT-3' and reverse, 5'-UAGCCGAAGCUCUUGAUGCTT-3'.

Western blotting. Total protein was extracted using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Inc.). The Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) was used to measure protein concentration. Subsequently, 20 μg protein sample was fractionated using NuPAGE™ 4-20% Tris-Acetate Midi Protein Gels (Thermo Fisher Scientific, Inc.), and transferred to PVDF membranes. Membranes were blocked using 5% skim milk for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. Membranes were incubated with a secondary antibody for 1 h at room temperature. The chemiluminescence of blots was detected using Pierce™ ECL Plus Western Blotting substrate (Invitrogen; Thermo Fisher Scientific, Inc.) and then exposed to X-ray film (Kodak) following treatment. GAPDH was used as the internal reference protein. Antibodies used for western blotting are listed in Table II.

CCK-8 assay. Cells were plated at a density of $3x10^4$ the 96-well plates for 24 h. Following the addition of 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) and 90 μl RPMI-1640 medium to each plate, cells were incubated at 37°C with 5% CO_2. Optical density values were detected using a microplate reader (Biotek Instruments, Inc.).

AV/PI. Cells were digested with pancreatin and washed with PBS. Following suspension in 100 μl binding buffer and addition of 5 μl AV and 5 μl PI, cells were incubated in the dark with a Annexin V-FITC Apoptosis Detection kit according to the manufacturer's protocol (Sigma-Aldrich; Merck KGaA).

Wound scratch assay. Cells, which were pre-culture in medium containing 1% FBS for 24 h, were cultured until 80% confluence and scraped with a sterile pipette tip to create adherent cell gaps. Subsequently, the cells were incubated and then observed at 0 and 24 h by inverted fluorescence microscopy (Nikon Corporation). The migration rate was calculated as follows: Migration rate = (scraped area- residual area)/scraped area.

Transwell assay. After coating Matrigel basement membrane matrix (BD Biosciences) on the upper Transwell chamber (Costar; Corning, Inc) at 37°C for 1 h. Cells ($3x10^4$) in FBS-free medium (DMEM for Huh-7 cells and RPMI-1640 for SNU-449 cells) were seeded in the upper chamber, and lower chamer was filled with 500 μl 10% FBS containng-medim (DMEM for Huh-7 cells and RPMI-1640 for SNU-449 cells). Following incubation for 24 h at 37°C and wiping of the upper cells, cells in the lower chamber were fixed with formaldehyde (Sigma-Aldrich; Merck KGaA). After staining with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature, the invasive cell count of each well was calculated by the averaging the invasive cell count

Table I. Primers used for reverse transcription-quantitative PCR.

Target gene	Primer sequence (5'-3')	
	Forward	Reverse
ITGA7	GCCACTCTGCCTGTCCAATG	GGAGGTGCTAAGGATGAGGTAGA
E-cadherin	TGATTCTGCTGCTCTTGCTGTT	CCTCTTCTCCGCCTCCTTCTT
α-SMA	CATTCACGAGACCACCTACAACAG	CGCCGATCCACACCGAGTAT
GAPDH	GACCACAGTCCATGCCATCAC	ACGCCTGCTTCACCACCTT

ITGA7, integrin α7; α-SMA, α-smooth muscle actin.

Table II. Antibodies used for western blotting.

Antibody	Manufacturer	Catalog number	Dilution
Primary antibodies			
ITGA7 mouse mAb	Santa Cruz Biotechnology, Inc.	sc-51576	1:1,000
E-cadherin mouse mAb	Santa Cruz Biotechnology, Inc.	sc-8426	1:1,000
Vimentin mouse mAb	Santa Cruz Biotechnology, Inc.	sc-6260	1:500
N-cadherin mouse mAb	Santa Cruz Biotechnology, Inc.	sc-393933	1:1,000
α-SMA mouse mAb	Santa Cruz Biotechnology, Inc.	sc-53142	1:1,000
Cleaved caspase 3 mouse mAb	Cell Signaling Technology, Inc.	9664S	1:1,000
GAPDH mouse mAb	Santa Cruz Biotechnology, Inc.	sc-47724	1:1,000
Secondary antibodies			
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology, Inc.	sc-2005	1:5,000

ITGA7, integrin α7; α-SMA, α-smooth muscle actin.

of five fields of view in each well, which was observed using an inverted fluorescence microscopy (Nikon Corporation) at a magnification of x200.

Statistical analysis. Statistical analysis and graph plotting were performed using GraphPad Prism 7.02 (GraphPad Software, Inc.). All assays were repeated in triplicate. Data are presented as the mean ± SD. Comparison between two groups were performed using unpaired t-test, while multiple comparisons were performed using Dunnett's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

ITGA7 is highly expressed in HCC cell lines compared with human normal liver epithelial cells. ITGA7 mRNA expression was higher in HCC cell lines [including Li7 (P<0.01), Huh7 (P<0.001), SKHEP1 (P<0.01) and SNU449 (P<0.05) cells] compared with human normal liver epithelial cells (THLE-3 cells) (Fig. 1A). In addition, ITGA7 protein expression was elevated in HCC cell lines [Li7 (P<0.01), Huh7 (P<0.001), SKHEP1 (P<0.001) and SNU449 (P<0.05) cells] compared with human normal liver epithelial cells (THLE-3 cells; Fig. 1B and C). ITGA7 expression was the highest in

Huh7 cells and the lowest in SNU449 cells. Hence, Huh7 and SNU449 cells were selected for subsequent experiments to assess the effects of ITGA7 knockdown on cell proliferation, migration, invasion and EMT in HCC cells.

ITGA7 expression is attenuated in the ITGA7-KD group compared with the control group after transfection. ITGA7 mRNA (P<0.001; Fig. 2A) and protein (Fig. 2C) expression decreased in the ITGA7-KD group compared with the control group in Huh7 cells. ITGA7 mRNA (P<0.001; Fig. 2B) and protein (Fig. 2D) expression levels were also lower in the ITGA7-KD group compared with the control group in SNU449 cells.

ITGA7 knockdown decreases cell proliferation but increases cell apoptosis. The effects of ITGA7 on regulating cell proliferation and apoptosis in Huh7 and SNU449 cells were then investigated. In Huh7 cells, cell proliferation decreased in the ITGA7-KD group at 48 h (P<0.05) and 72 h (P<0.01) compared with the control group (Fig. 3A). Meanwhile, the cell apoptosis rate at 48 h was increased in the ITGA7-KD group compared with the control group (P<0.01; Fig. 3C and E), and cleaved caspase 3 protein expression was increased in the ITGA7-KD group compared with the control group (Fig. 3G). In SNU449 cells, cell proliferation was reduced
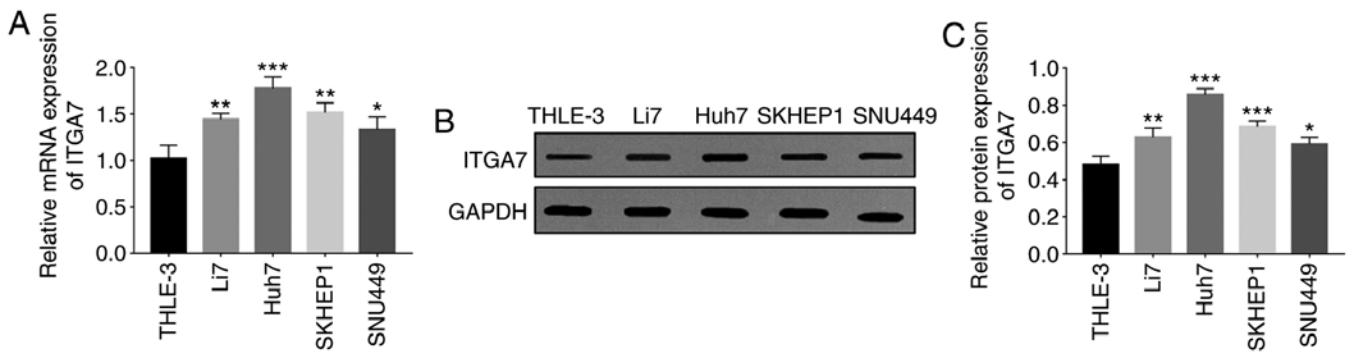
Figure 1. ITGA7 expression levels in cell lines. (A) ITGA7 mRNA expression in HCC cell lines and human normal liver epithelial cell line. (B and C) ITGA7 protein expression in HCC cell lines and human normal liver epithelial cell line. ITGA7, integrin α7; HCC, hepatocellular carcinoma. *P<0.05, **P<0.01 and ***P<0.001 vs. THLE-3 cells.
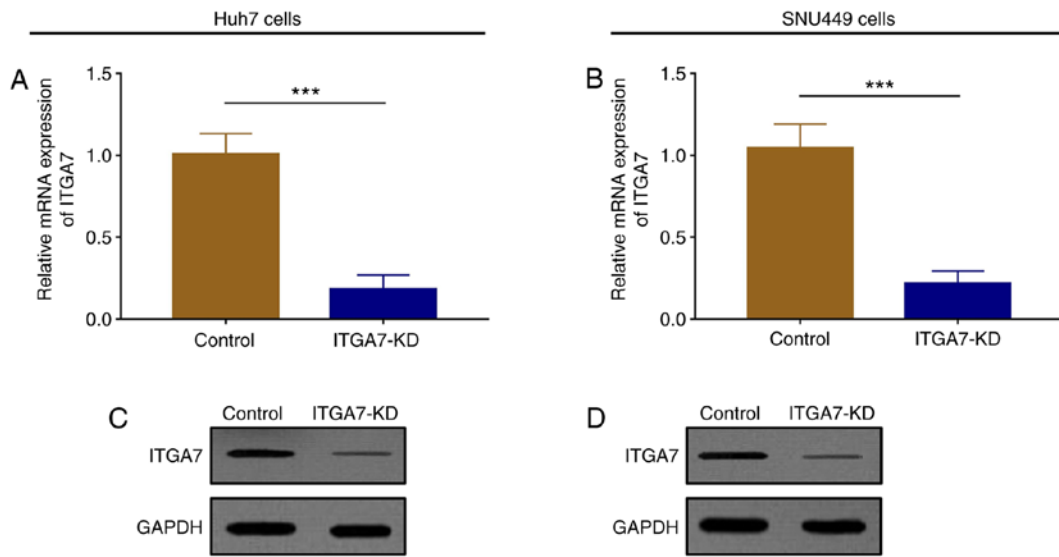

Figure 2. ITGA7 expression following transfection with ITGA7 small interfering RNA. ITGA7 mRNA expression in (A) Huh7 and (B) SNU449 cells. ITGA7 protein expression in (C) Huh7 and (D) SNU449 cells. ITGA7, integrin α7; KD, knockdown. ***P<0.001.

in the ITGA7-KD group at 48 h (P<0.05) and 72 h (P<0.05) compared with the control group (Fig. 3B). The cell apoptosis rate at 48 h was enhanced in the ITGA7-KD group compared with the control group (P<0.01; Fig. 3D and F), and cleaved caspase 3 protein expression increased in the ITGA7-KD group compared with the control group (Fig. 3H).

ITGA7 knockdown represses cell migration. The effects of ITGA7 on regulating cell migration in Huh7 and SNU449 cells were assessed. In Huh7 cells, the wound scratch assay showed that the migration rate at 24 h after transfection was lower in the ITGA7-KD group compared with the control group (P<0.01; Fig. 4A and C). In SNU449 cells, the migration rate at 24 h after transfection was also attenuated in the ITGA7-KD group compared with the control group (P<0.001; Fig. 4B and D).

ITGA7 knockdown suppresses cell invasion. Transwell assays were performed to investigate the effects of ITGA7 on regulating Huh7 and SNU449 cell invasion. The number of invasive Huh7 cells was decreased in the ITGA7-KD group compared

with the control group (P<0.01) at 24 h (Fig. 5A and C). The number of invasive SNU449 cells was also lower in the ITGA7-KD group compared with the control group (P<0.01) at 24 h (Fig. 5B and D).

ITGA7 knockdown represses EMT. To assess the effects of ITGA7 on EMT underlying HCC pathogenesis, the expression levels of EMT markers, including E-cadherin, vimentin, N-cadherin and α-SMA, were detected in Huh7 and SNU449 cells after transfection. In Huh7 cells, mRNA (P<0.05) and protein expression of E-cadherin increased (Fig. 6A and I); however, mRNA (all P<0.05) and protein expression levels of vimentin (Fig. 6C and I), N-cadherin (Fig. 6E and I) and α-SMA (Fig. 6G and I) were decreased in the ITGA7-KD group compared with the control group at 24 h. In SNU449 cells, mRNA (P<0.01) and protein expression levels of E-cadherin (Fig. 6B and J) increased, while mRNA (all P<0.05) and protein expression levels of vimentin (Fig. 6D and J), N-cadherin (Fig. 6F and J) and α-SMA (Fig. 6H and J) were decreased in the ITGA7-KD group compared with the control group at 24 h.
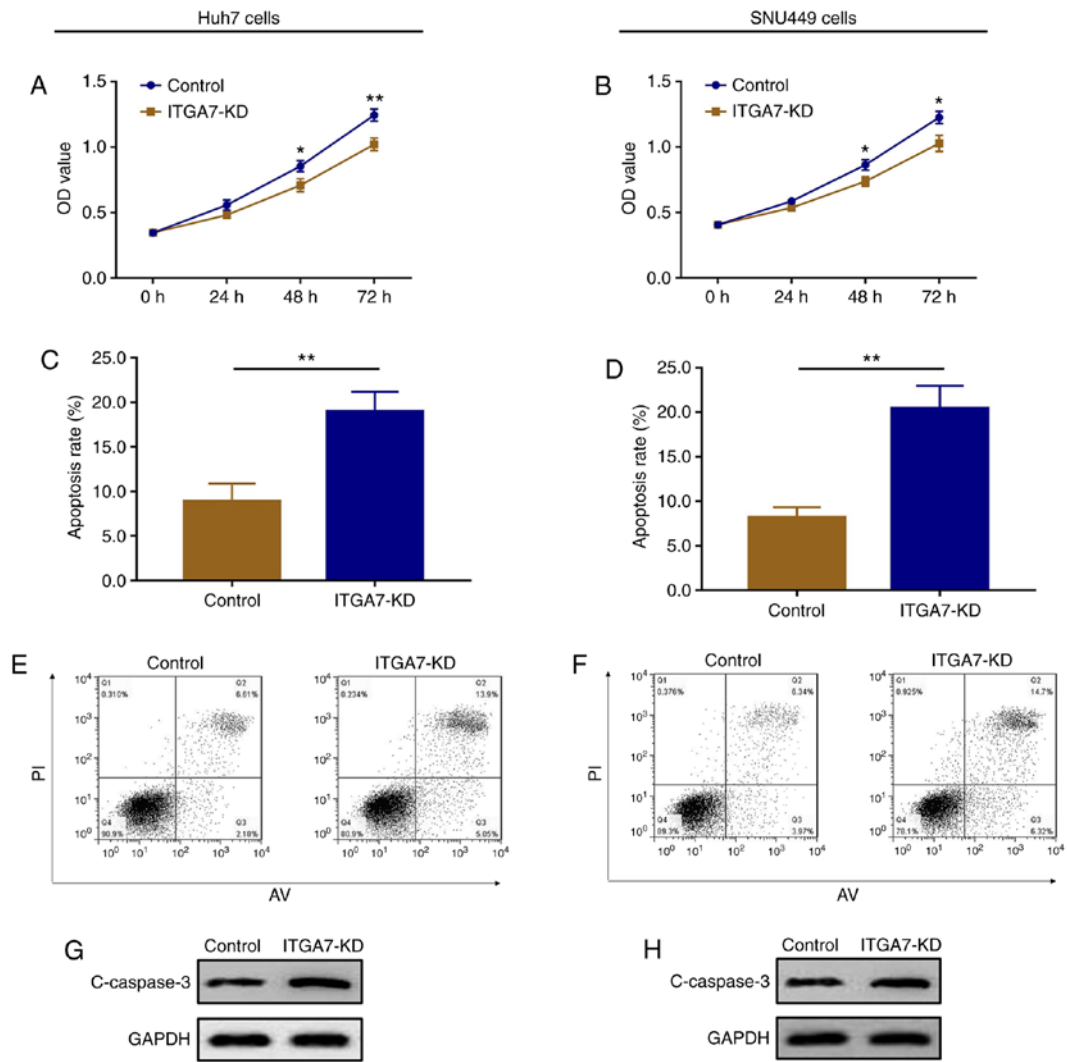
Figure 3. Cell proliferation and apoptosis. Proliferation of (A) Huh7 and (B) SNU449 cells following transfection. *P<0.05 and **P<0.01 vs. the control group. Apoptosis rate of (C) Huh7 and (D) SNU449 cells following transfection. **P<0.01. Representative flow cytometry plots used to determine apoptosis rates in (E) Huh7 and (F) SNU449 cells. Cleaved caspase 3 and GAPDH protein expression following transfection in (G) Huh7 and (H) SNU449 cells. ITGA7, integrin α7; C-caspase 3; cleaved caspase 3; KD, knockdown; AV, annexin V; PI, propidium iodide.
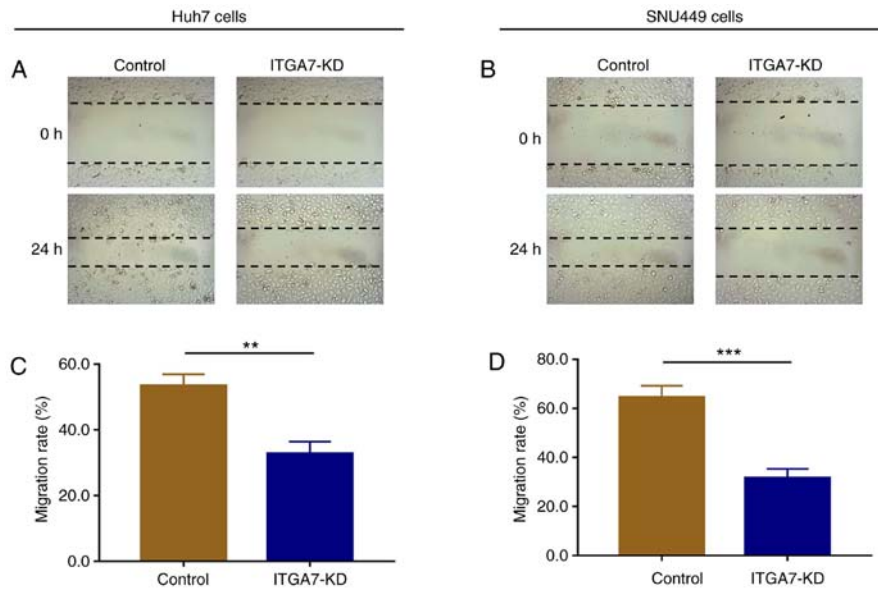

Figure 4. Cell migration. Representative wound scratch assay images of (A) Huh7 and (B) SNU44 cells. (C) Huh7 and (D) SNU44 cell migration rates. Magnification, x200. **P<0.01 and ***P<0.001. ITGA7, integrin α7; KD, knockdown.
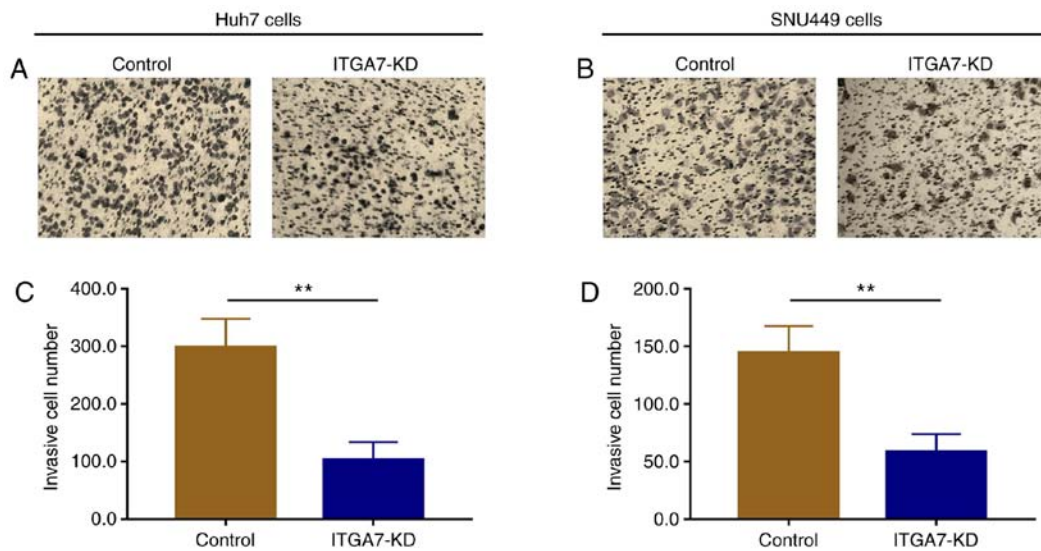
Figure 5. Cell invasion. Representative Transwell assay images of (A) Huh7 and (B) SNU44 cells. (C) Huh7 and (D) SNU44 cell invasion. Magnification, x200. **P<0.01. ITGA7, integrin α7; KD, knockdown.

Discussion

Integrins are transmembrane protein receptors that attach cells to the extracellular matrix and bind ligands secreted by other cells (8,9). As one of the integrins, ITGA7 is proposed to serve as a key regulator in tumor propagation and cancer stem cell properties (5,10). Previous studies revealed that ITGA7 is highly expressed in various cancer cells, including OSCC and mesothelioma (5,11). Although these previous studies detected an upregulation of ITGA7 in various cancer cells, to the best of our knowledge, its role in HCC is still unclear. The present study revealed that ITGA7 was overexpressed in HCC cell lines, including Li7, Huh7, SKHEP1 and SNU449 cells, compared with human normal liver epithelial THLE-3 cells. ITGA7 regulates several genes and pathways, including the focal adhesion kinase (FAK)/AKT-zinc finger E-box Binding Homeobox 1 (ZEB1) signaling pathway, to promote cell proliferation and metastasis, subsequently contributing to the malignant transformation of HCC (5,12). Thus, ITGA7 was overexpressed in HCC cells compared with healthy control cells.

Previous studies indicated that ITGA7 is involved in the pathological progression of different carcinomas through affecting cell activities such as cell migration and invasion (5,6,9,13-15). For example, the interaction between ITGA7 and laminin-induced outside-in signaling contributed to glioblastoma stem-like cell growth and invasion (12). Furthermore, the interaction between ITGA7 and S100P activated the FAK/AKT-ZEB1 signaling pathway, which induced lung cancer cell migration and invasion (6). Furthermore, ITGA7 is associated with cancer cell stemness. In another study, ITGA7 promoted the stemness of OSCC cells via FAK/MAPK/ERK signaling, which subsequently induced the tumorigenicity and metastasis of OSCC (5). In addition, ITGB7 knockdown enhanced cell apoptosis but inhibited cell proliferation and invasion in breast cancer (14). Although a few studies have been performed to explore the role of ITGA7 in different types of carcinoma, there remain certain

contradictions. Several lines of evidence revealed the role of ITGA7 as a tumor suppressor in various malignancies. For example, ITGA7 appears to activate cyclin-dependent kinase inhibitor 3 (CDKN3) and Rac GTPase-activating protein 1 (RACGAP1) expression to inhibit cell motility and metastasis of HCC cells (16). Another study revealed that ITGA7 may be a tumor suppressor that impedes tumor growth and inhibits migration in prostate cancer (9). Additionally, ITGB7 interacts with high temperature requirement A2 to promote prostate cancer cell death (13). To the best of our knowledge, little is known about the role of ITGA7 in HCC. The present study investigated the effect of ITGA7 on regulating HCC cell activities. It was found that ITGA7 knockdown decreased cell proliferation, migration and invasion, but increased apoptosis of HCC cells, which suggested that ITGA7 knockdown might suppress the function of HCC cells. There are a few possible explanations for this. Similar to its cancerogenic effect on tumor progression in lung cancer, ITGA7 might interact with S100P to trigger FAK/AKT-ZEB1 signaling to enhance HCC cell proliferation, migration and invasion, thereby contributing to HCC tumor progression (6). Similar to the promotive effects of ITGA7 on tumor progression in glioblastoma, ITGA7 might accelerate HCC cell growth and invasion via interacting with laminin-induced outside-in signaling, thereby leading to tumor progression of HCC (12). ITGA7 may attach cells to the extracellular matrix and interact with ligands secreted by other cells to activate HCC cell invasion and migration, which subsequently promotes tumor progression of HCC (8,9). ITGA7 may also regulate CDKN3, which dephosphorylates tyrosine residues of different cyclin-dependent kinases and represses cell cycle progression in yeast and mammalian cells, to increase HCC cell invasion and motility, subsequently accelerating tumor progression in HCC (16-18). ITGA7 also may modulate RACGAP1 to increase cell growth, enhance cell motility and promote tumor metastasis. Taken together, it may be hypothesized that ITGA7 knockdown suppresses tumor progression of HCC (16,19). In addition, the discrepancies in results between the present study and previous studies
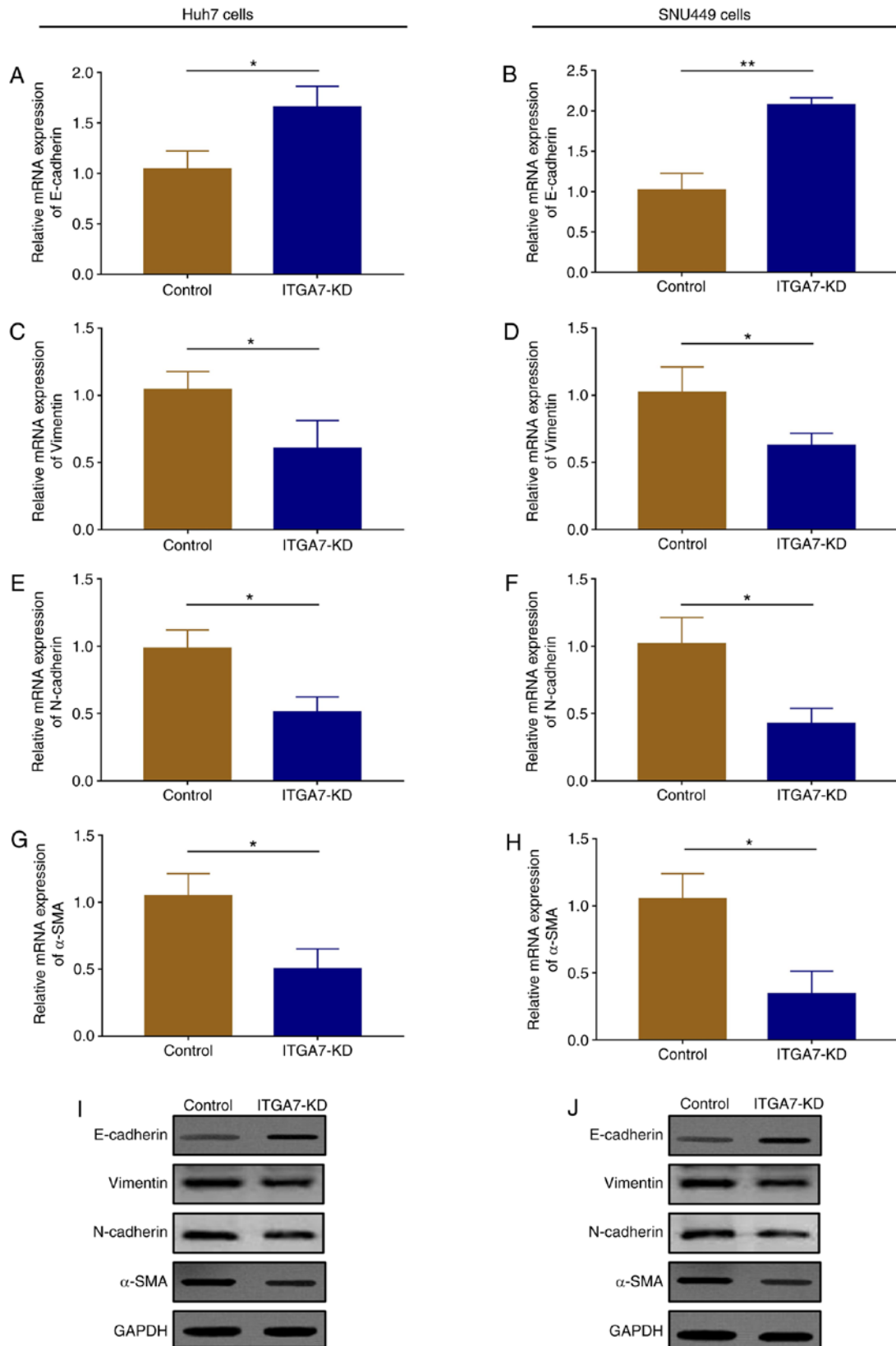
Figure 6. Effects of ITGA7 on endothelial-mesenchymal transition. (A and B) E-cadherin, (C and D) vimentin, (E and F) N-cadherin and (G and H) α-SMA mRNA expression after transfection in Huh7 cells and SNU449 cells. Protein expression of E-cadherin, vimentin, N-cadherin and α-SMA after transfection in (J) Huh7 and (I) SNU449 cells. *P<0.05 and **P<0.01. ITGA7, integrin α7; α-SMA, α-smooth muscle actin; KD, knockdown.

might result from differences between the malignances studied. The present study focused on HCC, while the majority of previous studies focused on other types of cancer. Due to

the complexity of malignant pathological processes, different malignances might be distinctive in terms of pathological features. Thus, the effects of ITGA7 on cellular function and

its underlying mechanisms in other malignances may differ. Besides, different cell lines, different assay operation times and experimental procedures may have also contributed to distinctive results among different studies.

To the best of our knowledge, limited information is available regarding the role of ITGA7 in EMT and tumor metastasis (5,20,21). One previous study reported that ITGA7 promoted OSCC cell migration and invasion and induced EMT (5). EMT is not only a well-coordinated process controlled by multiple signaling pathways during embryonic development, but also a pathological characteristic in neoplasia and fibrosis (22-25). EMT has been considered as an essential regulator linked to tumor progression and tumor metastasis through accelerating cancer cell invasion and dissemination to distant organs (22-25). To assess the effects of ITGA7 on regulating EMT in HCC, E-cadherin, α-SMA, vimentin and N-cadherin levels were detected. ITGA7 knockdown increased E-cadherin expression and decreased α-SMA expression in HCC cells. To summarize, ITGA7 knockdown may repress EMT in HCC. However, how ITGA7 knockdown suppressed cell proliferation, migration, invasion and ETM in HCC remains unclear. Further experiments, such as RNA sequencing, bioinformatics and subsequent validation by RT-qPCR are required.

In conclusion, ITGA7 knockdown suppressed HCC cell proliferation, migration, invasion and EMT, and promoted apoptosis. These data indicated that ITGA7 might be a novel and effective treatment target for HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZWu and XK made substantial contributions to the design of the present study, ZWu, XK and ZW were responsible for data acquisition and interpretation. All authors read and approved the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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