

Original Article

Cytohesin-3 is upregulated in hepatocellular carcinoma and contributes to tumor growth and vascular invasion

Ying Fu¹, Jun Li¹, Ming-Xuan Feng², Xiao-Mei Yang¹, Ya-Hui Wang¹, Yan-Li Zhang¹, Wenxin Qin¹, Qiang Xia², Zhi-Gang Zhang¹

¹State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; ²Department of Liver Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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Abstract: Hepatocellular carcinoma (HCC) is a malignant tumor with high morbidity and mortality, and is characterized by high potential for metastasis and recurrence. The outcome of it is still poor due to lacking of targeted therapeutic strategies. There is an urgent need to find new therapeutic targets for interventions against HCC metastasis and recurrence. In the present study, we found cytohesin-3, a member of the cytohesin family, was upregulated in HCC tissues, and its expression was negatively correlated with the overall survival and relapse-free survival of HCC patients. Further clinicopathological correlation analysis revealed that cytohesin-3 expression was related with tumor size and vascular invasion. And *in vitro* studies revealed that knock-down of cytohesin-3 suppressed HCC cells proliferation and migration. These results suggest that cytohesin-3 may act as a novel prognostic factor of HCC, and it might also be useful to exploit targeted therapeutic drugs against HCC growth and metastasis.

Keywords: Cytohesin-3, hepatocellular carcinoma, prognosis, cell proliferation, vascular invasion

Introduction

Hepatocellular carcinoma (HCC) ranks the fifth most common cancer and the third leading cause of cancer-related death worldwide [1]. As a highly malignant solid tumor, HCC is primarily characterized by early metastasis and poor prognosis. Presently, hepatic resection and liver transplantation are still the most curative therapeutic choices for HCC patients. However, the long-term survival rate remains frustrating due to frequent recurrence and metastasis after operation [2]. Therefore, it is of great significance to explore new prognosis markers and therapeutic targets for HCC.

Cytohesins, as cytoplasmic multidomain proteins, are guanine nucleotide exchange factors (GEFs) for ADP ribosylation factors (ARFs) which belong to the small Ras-like GTPase family. The cytohesin family consists of four highly homologous members: cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (GRP1) and cytohesin-4 [3, 4]. All the family members have similar structural

organization, including an N-terminal coiled-coil motif, a central Sec7 domain, and a C-terminal pleckstrin homology (PH) domain [5]. It has been identified that the coiled-coil motif is involved in homodimerization, PH domain is responsible for mediating membrane localization through interaction with specific polyphosphoinositides and the Sec7 domain mainly catalyzes the exchange of guanosine-5'-diphosphate (GDP) for guanosine-5'-triphosphate (GTP), which activates Arf proteins like Arf1 and Arf6 [4].

In recent years, accumulating evidences have demonstrated that cytohesins play important roles during some physiological and pathological processes. For example, cytohesin-1 is confirmed to regulate leukocyte function-associated antigen-1 (LFA-1)-mediated leukocyte adhesion to endothelial cells and modulate cell migration across endothelial cells [6]. Cytohesin-2 and cytohesin-3 contribute to β 1 integrin recycling [7, 8]. Most importantly, cytohesins are supported to be cytoplasmic activators of receptor tyrosine kinase signaling by the epider-

mal growth factor receptor (EGFR) [9] and insulin receptor (IR), respectively [10, 11]. It is well known that the EGFR and IGFR signaling pathways play pivotal roles in carcinogenesis and cancer progression, especially in HCC [12-14]. Meanwhile, the functions of cytohesins in cancers are being paid more and more attentions. It has been demonstrated that inhibition of cytohesin-1 leads to reduced IGFR signaling in prostate cancer [15], targeting cytohesin can decrease gefitinib-resistant lung cancer cells proliferation [16] and cytohesins may also be involved in EGFR signaling pathway in colorectal cancer [17]. Cytohesin-2 is proved to correlate with prognosis for HCC patients [18]. However, the roles of cytohesin-3 in HCC have not been reported yet.

In this study, we first examined the expression status of cytohesin-3 in 18 paired tumor and their corresponding non-tumor liver tissues, and found cytohesin-3 was significantly overexpressed in tumor tissues compared to non-tumor liver tissues. Subsequently, we evaluated cytohesin-3 expression in tissues of 202 HCC patients by immunohistochemical staining and analyzed the relevance of cytohesin-3 expression with patient's prognosis. The results showed that cytohesin-3 expression had a close relationship with the overall survival (OS) and relapse-free survival (RFS) of HCC patients. Furthermore, we detected the cytohesin-3 expression in HCC cell lines and knockdown it in MHCC-97H and Hep3B cells which were high expression of cytohesin-3. Silencing of cytohesin-3 suppressed MHCC-97H and Hep3B cells proliferation and migration *in vitro*. All these together proposed us that cytohesin-3 may act as a novel prognostic factor and therapeutic target for HCC.

Materials and methods

Clinical samples

All paired samples of primary HCC, their corresponding non-tumorous liver tissues and samples in tissue microarray (TMA) were obtained during surgical resection at Department of Liver Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine. 202 HCC samples of TMA were collected from 2004 to 2010 and follow-up ended in December 2012. Patient age ranged from 17 to 73 years, with a median age of 50 years. All samples were obtained with informed consent and use of

human samples was approved by the ethical review committee of the World Health Organization Collaborating Center for Research in Human Production (authorized by the Shanghai Municipal Government).

Immunohistochemistry

Tissue samples embedded in paraffin were cut into 5 μm sections and stained according to standard immunohistochemistry protocols. After deparaffinized and rehydrated, tissue sections were performed antigen retrieval using citric acid (pH 6.0) at 97°C for 30 minutes and followed by treatment with 0.3% H_2O_2 for 10 minutes. Then, the slides were blocked with 10% BSA (Sangon, Shanghai, China) for 1 hour at room temperature. Primary antibody for cytohesin-3 (Abcam, Cambridge, UK) was incubated overnight at 4°C and then the slides were incubated with suitable peroxidase-conjugated secondary antibody at room temperature for 1 hour. Next, the sections were treatment with diaminobenzidine and counterstained by haematoxylin. All the sections were observed and photographed with a microscope (Carl Zeiss) and the cytohesin-3 expression level was scored independently by 2 pathologists. The score was evaluated depending on the ratio and intensity of stained cells: 0-5% scored 0; 6%-35% scored 1; 36%-70% scored 2; 70%-100% scored 3. The final score was designated as low expression (score 0-1) and high expression (score 2-3) group.

Clinicopathological correlation and survival analysis

To assess the clinical relevance of cytohesin-3 expression in HCC, available clinicopathological data of patients including age, sex, tumor size, whether liver cirrhosis, serum alpha-feto-protein (AFP) level, local infiltration status, number of tumor nodules, tumor microsatellite formation, tumor encapsulation, vascular invasion, thrombosis, tumor differentiation and TNM stage were subjected to analyses using SPSS 19.0 for windows (IBM). Correlation of cytohesin-3 expression with the overall survival and relapse-free survival of HCC patients were analyzed by the Kaplan-Meier method.

Cell culture

HCC cell lines were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum

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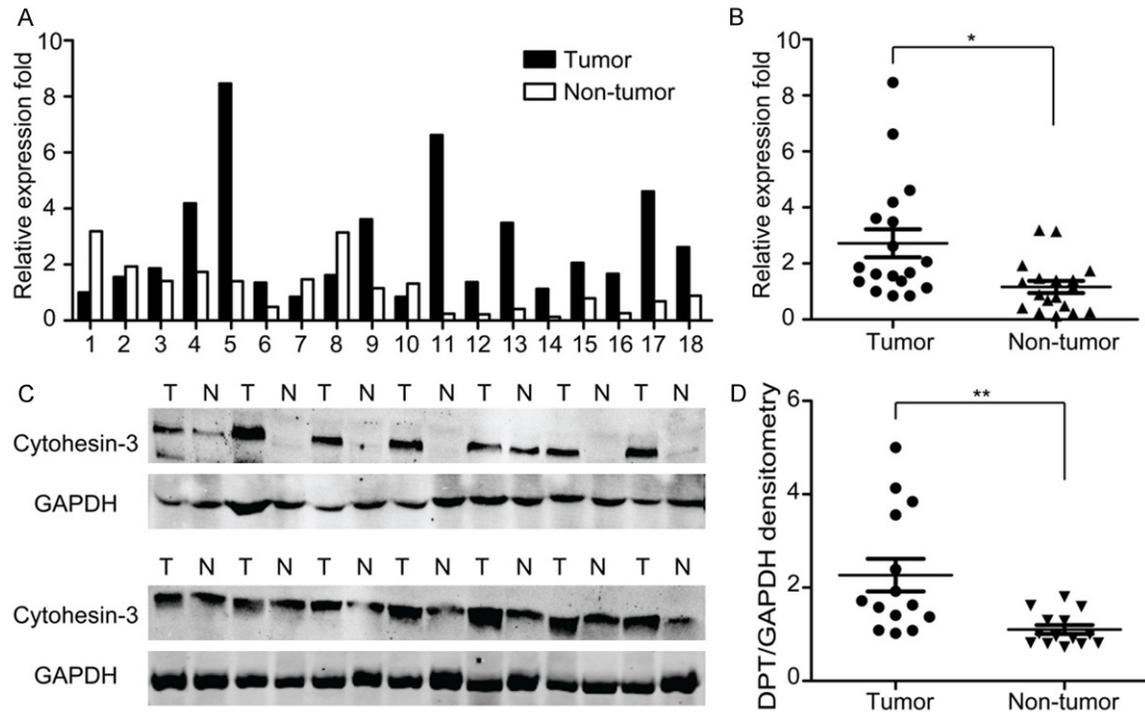


Figure 1. Cytohesin-3 is upregulated in hepatocellular carcinoma (HCC) tissues. A: Relative mRNA expression folds of cytohesin-3 which was normalized to human 18s in 18 pairs of HCC tissues and non-tumor liver tissues. B: Statistical results of relative mRNA expression folds. Values are means \pm SD ($*P < 0.05$). C: Western blotting analysis of cytohesin-3 expression in 14 pairs of HCC and corresponding non-tumor liver tissues. T represents tumor tissues and N represents non-tumor liver tissues. D: The densitometric analysis of the cytohesin-3 protein expression was shown and GAPDH was included as a loading control. Values are means \pm SD ($**P < 0.01$).

(FBS) and 1% antibiotics in a humidified incubator.

Quantitative real-time PCR

Total RNA was extracted from tissues and HCC cell lines using Trizol reagent (Takara, Dalian, China) and reverse transcribed by PrimeScript RT-PCR reagent (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative real-time PCR was subsequently performed with SYBR Premix Ex Taq (Takara) using an ABI7500 instrument (Applied Biosystems Inc). The primers used for cytohesin-3 are as follows: forward 5'-GACCGATAACTGCCTCTATT-3'; reverse 5'-TTCATCCACTCCTCCTTCT-3'. The relative expression levels of cytohesin-3 were normalized to human 18s (forward: 5'-TGCGAGTACTCAACACCAACA-3'; reverse: 5'-GCATATCTTCGGCCACA-3').

Western blot

The total proteins of HCC tissue samples were extracted using T-Per tissue protein extraction reagent (Thermo Scientific) according to its pro-

tol and total proteins of HCC cells were obtained by IP lysis buffer (Beyotime). For Western blot analysis, protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Millipore). After blocked with 5% no-fat milk (BD), the membrane was incubated with primary antibody for cytohesin-3 (Abcam, Cambridge, UK) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech Group, Chicago IL) at 4°C overnight and followed by incubation of secondary antibodies IRDye 800 anti-rabbit (LI-COR, Lincoln, NE) or IRDye 680 anti-mouse (LI-COR, Lincoln, NE) for 1 hour at room temperature. Bands were detected by an Odyssey infrared imaging system (LI-COR, Lincoln, NE) and quantification was analyzed using Image J software.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplexes targeted cytohesin-3 (siRNA-1: forward 5'-GUCGCCAGUUCUUUUUATT-3', reverse 5'-UAUAAAAGG AACUGGGCGACTT-3'; siRNA-2: forward 5'-CAGCAGAGAUCUUUCUAUTT-3', reverse 5'-AUAG-

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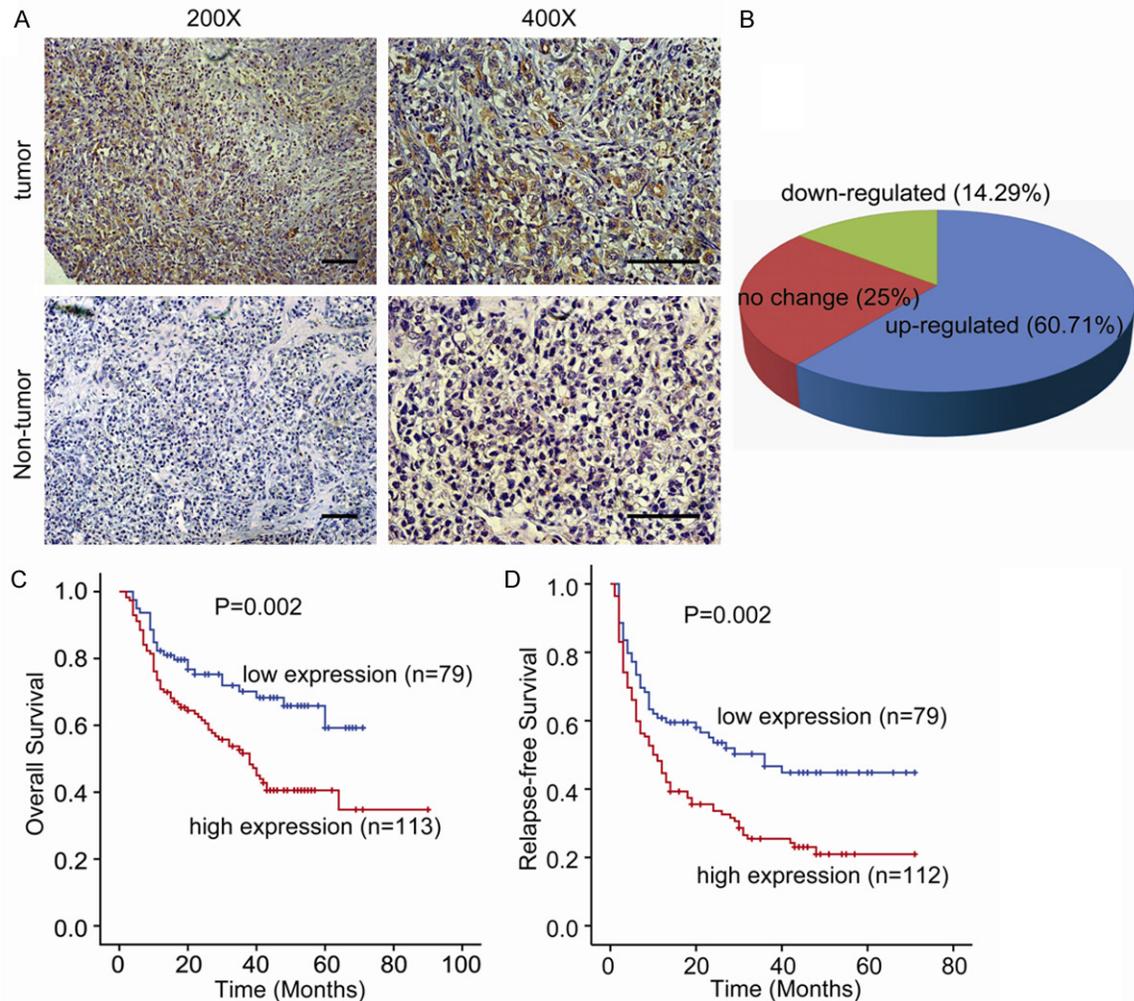


Figure 2. Cytohesin-3 high expression is closely related to patient prognosis. A: Immunohistochemical staining of cytohesin-3 in HCC and corresponding non-tumor liver tissues. 200 × and 400 × represent original magnification. Scale bars, 20 μm. B: The expression of cytohesin-3 was upregulated in 60.71% of HCC patients. n = 168. C: Kaplan-Meier analysis of overall survival for the expression of cytohesin-3 (P = 0.002). D: Kaplan-Meier analysis of relapse-free survival for the expression of cytohesin-3 (P = 0.002).

AAGGGAUCUCUGCUGTT-3') and negative control (NC) siRNA duplex (forward: 5'-UUCUCCGAACGUGUCACGUTT-3'; reverse: 5'-ACGUGACACGUUCGGAGAATT-3') were purchased from Genepharma (Shanghai, China). Transfection was performed using the Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 reagent (CCK-8, Dojindo, Japan). Cells were seeded into a 96-well plate at 2×10^3 cells per well with 100 μl complete medium and cultured at 37°C. 10 μl CCK-8 solution was

added to each well after 0, 24, 48, 72, 96 and 120 hours, respectively. In viable cells, WST-8 was metabolized to produce a colorimetric dye that is detected at 450 nm using a microplate reader (BIO-TEK).

In vitro migration assay

Cell migration assays were performed using Transwell chambers (Millipore). 5×10^4 cells in 200 μl serum-free DMEM were seeded in the upper chamber of a transwell and 700 μl medium supplemented with 10% FBS was added to the lower chamber. Migrated cells were fixed and stained with 0.1% (w/v) crystal violet 24 hours later. Five randomly selected fields per

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Table 1. Correlation of Cytohesin-3 expression with clinicopathological parameters of 202 HCC patients by Pearson's χ^2 test

Variable		Cytohesin-3 (n = 202)		
		High	Low	P
Age	≤ 50 years	65	39	0.225
	> 50 years	53	45	
Gender	Female	13	15	0.166
	Male	105	69	
Tumor size	≤ 5 cm	53	49	0.041
	> 5 cm	65	35	
Liver Cirrhosis	Yes	103	74	0.864
	No	15	10	
Serum AFP	≤ 20 ng/ml	40	29	0.995
	> 20 ng/ml	76	55	
Local	Yes	17	5	0.052
	No	99	79	
Tumor multiplicity	Single	96	72	0.415
	Multiple	22	12	
Tumor satellite	Yes	23	9	0.087
	No	94	75	
Tumor encapsulation	None	68	41	0.191
	Complete	49	43	
Vascular invasion	Yes	42	15	0.006
	No	76	69	
Thrombi	Yes	31	13	0.067
	No	87	71	
Tumor differentiation	I	0	3	0.056
	II	37	35	
	III	80	46	
TNM stage	I	63	56	0.087
	II	12	11	
	III	43	17	

well were photographed and the numbers of migrated cells were counted.

Statistical analysis

Statistic differences were calculated using two-tailed Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Cytohesin-3 expression is elevated in HCC tissues

To evaluate the expression status of cytohesin-3 in HCC, we first performed quantitative real-time polymerase chain reaction (qRT-PCR)

for 18 paired of HCC and corresponding non-tumor liver tissues. The results showed that the cytohesin-3 expression level was significantly higher in HCC tissues than that in the non-tumor liver tissues (**Figure 1A** and **1B**). Subsequently, we validated our primary results by Western Blotting for 14 matched pairs of HCC and non-tumor liver tissues, protein expression level of cytohesin-3 was consistent with mRNA level in the same samples (**Figure 1C** and **1D**).

Cytohesin-3 expression is closely related to tumor size, vascular invasion and patient prognosis

To further investigate the clinical significance of cytohesin-3 in HCC, we examined cytohesin-3 expression in another independent 202 HCC samples on a tissue microarray (TMA) by immunohistochemical staining. Majority positive staining was detected in HCC tissues (**Figure 2A**). Statistical analysis of 168 available paired tissues revealed that the cytohesin-3 was elevated in 60.71% (102/168) of HCC patients, whereas it was down-regulated in 14.29% (24/168) of HCC patients (**Figure 2B**).

Next, we analyzed the relevance of cytohesin-3 expression with patients clinicopathological parameters and found that the expression level of cytohesin-3 was closely related with tumor size ($P = 0.041$) and vascular invasion ($P = 0.006$) (**Table 1**). The results indicated that cytohesin-3 may play important roles in HCC progression and metastasis. Furthermore, Kaplan-Meier survival analysis demonstrated that patients with higher cytohesin-3 expression had lower rates of overall survival (OS) ($P = 0.002$) and relapse-free survival (RFS) ($P = 0.002$) than those with lower cytohesin-3 expression (**Figure 2C** and **2D**). These data strongly suggested that cytohesin-3 may act as a novel prognostic marker for HCC.

Cytohesin-3 expression is variable in HCC cell lines

We also assessed the cytohesin-3 protein expression in 12 HCC cell lines and 2 non-HCC cell lines and found cytohesin-3 was highly expressed in most of HCC cell lines: MHCC-LM3, SK-Hep1, HepG2, Hep3B, MHCC-97H, SNU423, SNU449 and PVTT, and 2 non-HCC

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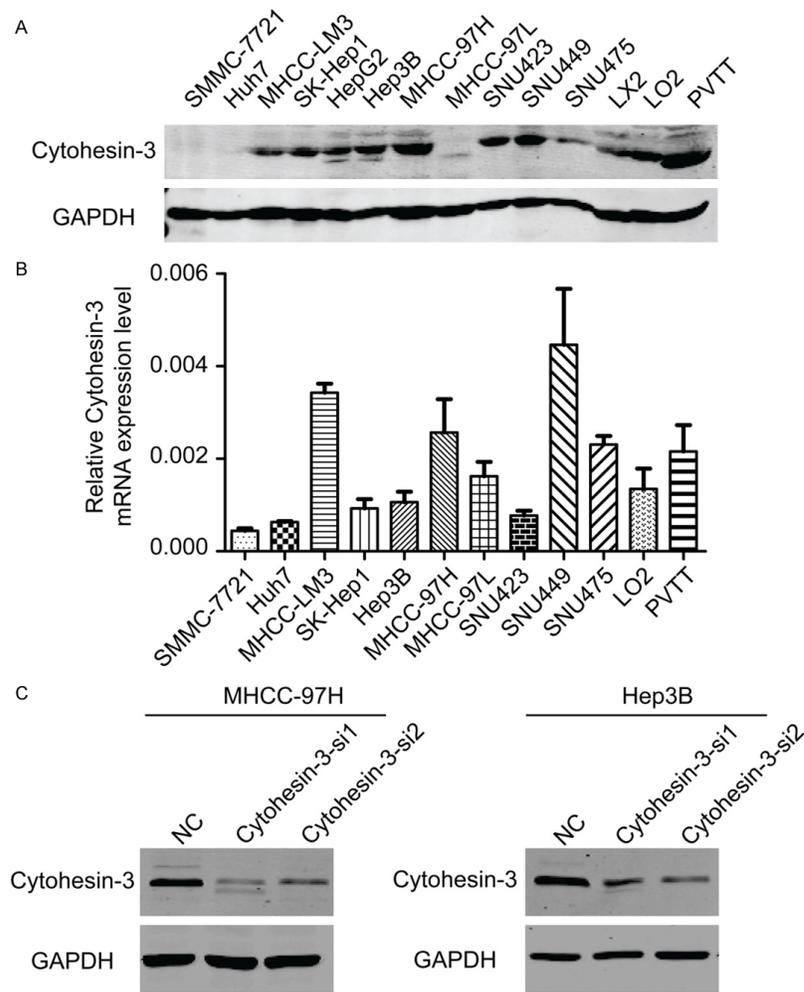


Figure 3. Cytohesin-3 is widely expression in HCC cell lines. A: Western blotting analysis of cytohesin-3 expression in 12 HCC cell lines and 2 non-HCC cell lines. B: Relative mRNA expression of cytohesin-3 in 12 cell lines. C: Western blotting analysis of cytohesin-3 expression in MHCC-97H and Hep3B cells at 48 h after transfection with siRNA targeted cytohesin-3 or negative control (NC).

cell lines: LX2 (hepatic stellate cell) and LO2 (immortal liver cell) (Figure 3A). Interestingly, PVTT, a special HCC cell line derived from portal vein tumor thrombus had a high cytohesin-3 expression, indicating a potential role of cytohesin-3 in HCC metastasis. Moreover, the mRNA level of cytohesin-3 was detected therein and the expression status was generally consistent with protein level (Figure 3B).

To further explore the function of cytohesin-3 in HCC, cytohesin-3 was silenced in MHCC-97H and Hep3B cells which have relatively higher expression level of cytohesin-3 by using small interfering RNA (siRNA). The results showed that cytohesin-3 protein expression levels were

significantly decreased by siRNA at 48 hours of post-transfection (Figure 3C).

Silencing of cytohesin-3 suppresses HCC cell proliferation in vitro

Since cytohesin-3 expression was closely correlated with tumor size of HCC patients, we speculated that cytohesin-3 might be involved in HCC cell growth. To validate our supposition, we performed cell proliferation assays for MHCC-97H and Hep3B cells transfected with siRNA targeted cytohesin-3 or negative control (NC). The results showed that silencing of cytohesin-3 significantly inhibited HCC cell proliferation (Figure 4A and 4B).

Knockdown of cytohesin-3 inhibits HCC cell migration

In our preliminary analysis of clinicopathological relevance, cytohesin-3 expression was identified to have striking relationship with vascular invasion of HCC patients. Furthermore, we found that cytohesin-3 was highly expressed in PVTT

cell which is derived from the metastatic thrombus. These data indicated that cytohesin-3 may play a role in HCC invasion or metastasis. Therefore, we further explored the functional role of cytohesin-3 in HCC cell migration. By *in vitro* cell migration assay, we found that silencing of cytohesin-3 significantly reduced the migration rates of both MHCC-97H and Hep3B cells (Figure 5A and 5B).

Discussion

The poor outcome of HCC is ascribed to its high metastatic potential to a great extent [19, 20]. HCC metastasis is a very complex process which is far from being clearly elucidated [21].

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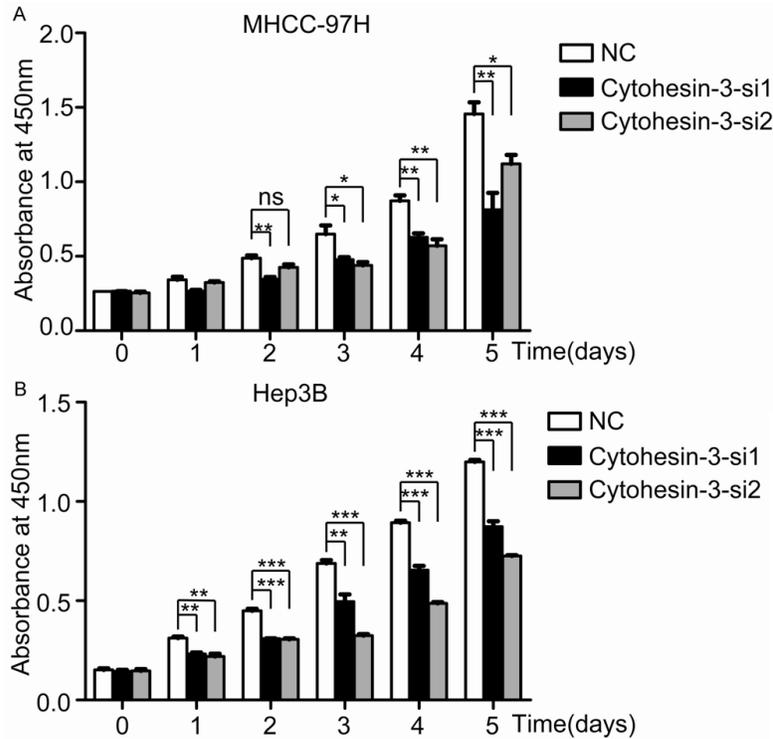


Figure 4. Knockdown of cytohesin-3 suppresses HCC cell proliferation *in vitro*. A: By CCK-8 assay, the proliferation of MHCC-97H cells which were transfected with siRNA targeted cytohesin-3 were inhibited compared with negative control (NC). Values are means \pm SD (* P < 0.05, ** P < 0.01). B: Cell proliferation analysis of Hep3B cells transfected with siRNA targeted cytohesin-3 or NC. Values are means \pm SD (** P < 0.01, *** P < 0.001).

Clinical and basic researches have revealed that both microenvironmental and internal cues of cancer cells are implicated in modulating of this complicated process [22-24]. Finding and identifying the functional roles of genes that differentially expressed between HCC tissues and corresponding non-tumor tissues is a promising way to deepen our understanding about the mysterious event [25]. Previous studies using this strategy have discovered some genes that are important in HCC growth and metastasis. For example, p28 expression is elevated in HCC tissues than in peritumoral tissues, and p28 promotes HCC invasion and metastasis via PI3K/AKT/HIF-1 α pathways [26], Rnd3 is down-regulated in HCC tissues, thus depletion of it can induce HCC cell invasion through an amoeboid-like mechanism [27]. Moreover, these differentially expressed genes might also be valuable in predicting prognosis and exploiting targeted therapeutic compounds of HCC patients [25, 28].

In the present study, we first discovered that the expression of cytohesin-3 was significantly higher in HCC tissues than those in adjacent non-tumor tissues by observing specimens from 202 HCC patients, and further analysis showed that the levels of cytohesin-3 in the HCC patients with vascular invasion were obviously higher than in those without vascular invasion. Moreover, the tumors with bigger diameters (> 5 cm) also displayed higher cytohesin-3 levels than those with smaller diameters (\leq 5 cm). Most importantly, we found high levels of cytohesin-3 in HCC tissues was correlated with poor OS and RFS. These results indicated that cytohesin-3 was not only upregulated in HCC tissues but also closely correlated with HCC vascular invasion and tumor growth.

To check whether cytohesin-3 has functional roles in HCC progression, we used siRNA to knockdown cytohesin-3 expression in two HCC cell lines, and both migration and proliferation of these two HCC cell lines were significantly suppressed by two siRNA targeted cytohesin-3, indicating important functional roles of cytohesin-3 in HCC progression and metastasis.

Tumor metastasis is a complicated process which involves cytoskeleton remodelling, cell-matrix cross talk and matrix degradation [29, 30]. Cytohesin-3 is a member of cytohesin family which has 3 common domains, including PH domain that mediates actin and cell membrane conjunction [31]. And members of cytohesin family can modulate integrin-dependent cell-matrix interaction [32, 33]. The conjunction between actin cytoskeleton and membrane is crucial for cell motility [34], and integrin-dependent cell-matrix interaction is also pivotal in cell migration [35, 36]. Therefore, further studies exploring the influences and mechanisms of cytohesin-3 on cytoskeleton and cell-matrix

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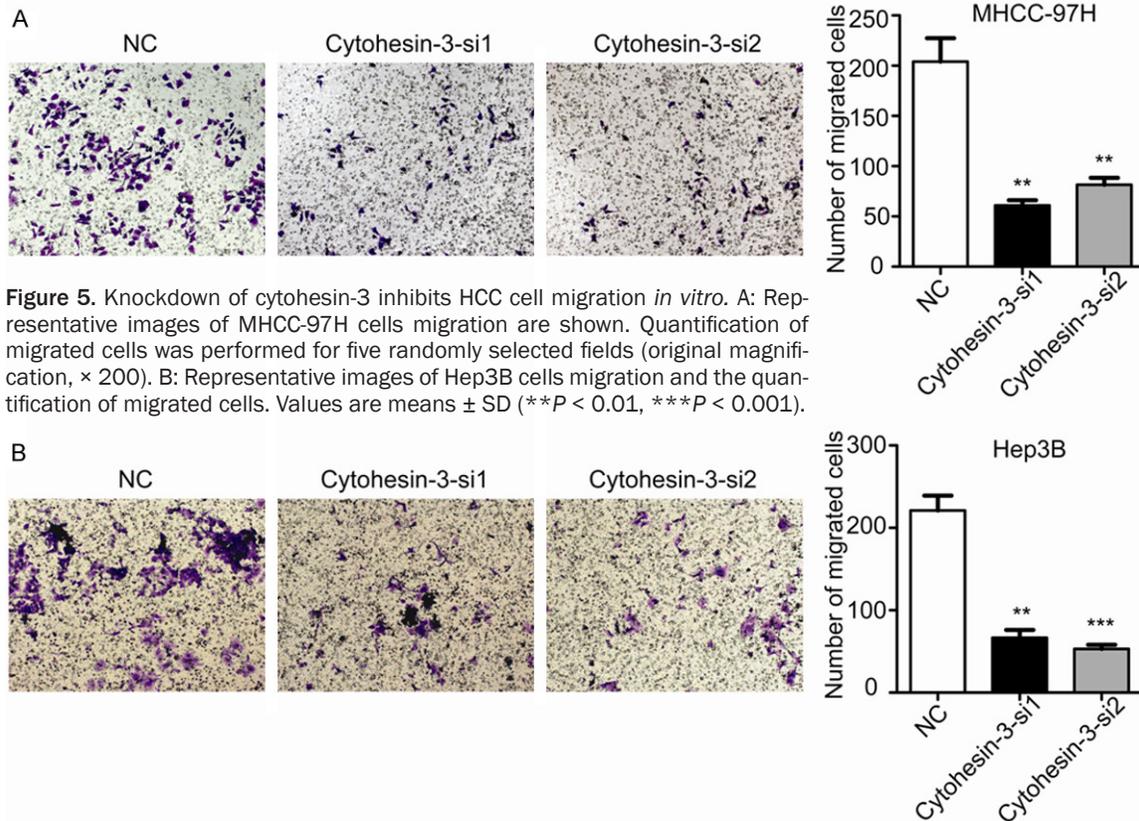


Figure 5. Knockdown of cytohesin-3 inhibits HCC cell migration *in vitro*. A: Representative images of MHCC-97H cells migration are shown. Quantification of migrated cells was performed for five randomly selected fields (original magnification, $\times 200$). B: Representative images of Hep3B cells migration and the quantification of migrated cells. Values are means \pm SD (** $P < 0.01$, *** $P < 0.001$).

interactions during HCC cells migration are still needed. Some studies had reported that cytohesin-3 was an inhibitor of Madin-Darby canine kidney epithelial cells migration [32, 37]. These diverse results of cytohesin-3 on migration indicate that the functions of cytohesin-3 in different tissues might be tissue-specific or microenvironment-dependent.

HCC is a malignant disease with extraordinarily high morbidity and mortality [1]. No satisfactory targeted drugs for all HCC patients have been reported yet, so existing basic researches are focused on identifying potential targets that might contribute to improve outcomes of special patients [38]. Our studies indicated that silencing of cytohesin-3 in its highly expressed patients might suppress HCC growth and vascular metastasis. The expression and therapeutic potential of cytohesin-3 might also be tested in other kinds of malignant tumors.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi-Gang Zhang, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 800 Dongchuan Road, Shanghai 200240, China. Tel: +86-21-34206763; Fax: +86-21-34206022; E-mail: zzhang@shsci.org; Qiang Xia, Department of Liver Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, China. Tel: +86-21-68383775; Fax: +86-21-58737232; E-mail: xiaqiang@shsmu.edu.cn

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