

Original Article

Expression of Girdin in primary hepatocellular carcinoma and its effect on cell proliferation and invasion

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Abstract: Girdin has been proven to play a vital role in the process of proliferation, apoptosis, and invasion in various cancer cells, yet the underlying molecular mechanism in primary hepatocellular carcinoma (HCC) has not yet been clarified. Thereafter, we performed immunohistochemistry to detect the expression of Girdin in 40 primary HCC tissues and 30 matched adjacent tissues using hepatic carcinoma tissue microarray. Our data showed that the positive expression rate of Girdin in hepatocellular carcinoma tissues was 67.5%, higher than that found in adjacent tissues of 16.7% ($P < 0.05$). It closely correlates to tumor size, T stage, TNM stage and Edmondson-Steiner stage ($P < 0.05$) of HCC patients. After specific small interfering RNA of Girdin was transfected into HepG2 and Huh7.5.1 cells, the proliferation and invasion ability of tumor cells were significantly inhibited. In summary, we suggest that the oncogenic role of Girdin could provide new molecular target for the treatment of HCC.

Keywords: Girdin, hepatocellular carcinoma (HCC), proliferation, invasion

Introduction

HCC is one of the more common tumors, which is characterized by high grade malignancy, progressive, clinical difficulty and poor curative effect, as well as a continuous increase in morbidity. The cancer statistics of America in 2012 [1] showed that morbidity has significantly increased by 3.9% for men and 1.9% for women over the past decade, among which, Asians were a high risk population with morbidity and mortality being twice as high as that of white people. The survival rate of HCC patients after 5 years is only 14%.

Girdin is a new intracellular macromolecule protein which was discovered in 2005 [2-6], and it regulates many signal transduction pathways through interacting with a number of important signal molecules, such as Akt/PKB [7, 8], Gαi/s [9], EGFR [10], therefore playing an important role in the process of tumor growth, apoptosis, infiltration and migration [2, 11, 12]. Many researchers hold a view that Girdin protein can be

regarded as molecular markers for a number of malignant tumors and is closely related to various prognoses, namely NSCLC [13], breast cancer [14], esophagus carcinoma [15], colorectal cancer [7] and glioblastoma [16]; however, its specific molecular mechanism in primary hepatic carcinoma has not been clarified. Firstly, immunohistochemical methods were used to detect the expression of Girdin in HCC and adjacent tissues, then its relationship with clinico-pathological characteristics was analyzed; then the effects of Girdin-specific siRNA on cell proliferation and invasion ability in HCC cell lines was explored, HepG2 and Huh7.5.1, so as to understand the function and significance of Girdin in HCC and provide a scientific basis and new ideas for the diagnosis and individual treatment of HCC.

Materials and methods

Tissue microarray

The tissue microarray was purchased from Aijia Biological Technology Co., Ltd. (Changsha,

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China). This microarray included 40 cases of HCC as well as 30 matched adjacent tissues, which were normal liver tissues 2 cm away from cancer tissues. 28 of the cases were above 50 before surgery, 12 cases were less than 50. 35 cases were male, and 5 cases were female. Pathological grading was 16 cases of grade I-II, and 2 cases of grade III-IV. According to the Clinical Guidelines for Primary Hepatocellular Carcinoma, TMN clinical staging was carried out as follows: 21 cases of stage I-II, 19 cases of stage III-IV. All patients underwent surgery and diagnosis from August 2006 until September 2008.

The detection and scoring were performed respectively by two experienced pathologists via the Immunohistochemical SP method. Girdin expression was evaluated according to the ratio of positive cells and staining intensity. The mean percentage of positive tumor cells was determined in at least five random fields at 400 × magnification for each section. 0 point for positive cell percentage less than 5%, 1 point for positive cell percentage between 5% and 24%; 2 points for that between 25% and 49%; 3 points for that between 50% and 74%, and 4 points for that no less than 75%. The intensity of the Girdin immunoreaction was scored as follows: 0 point for no staining in the cell, 1 point for light yellow particles inside the cell, 2 points for dark brown orange particles inside the cell; 3 points for dark brown particles inside the cell. The percentage of positive tumor cells and the staining intensity then were multiplied to produce an immunohistochemical staining score. This was deemed negative (-) for points less than 2, positive (+) for that no less than 2; and low expression for points between 2 and 4, while high expression was defined as being that between 5 and 7 points.

Main reagents

Girdin monoclonal antibody against human (MABT100) was purchased from the MLLIPORE Company. As for the Immunostain SP kit, concentrated DAB kit, phosphate buffer (PBS, 0.01 M, pH7.2-7.4), citrate buffer (CB, 0.01 M, pH6.0), hematoxylin dye liquor and trypsin-EDTA solution, they were all purchased from Changsha Aijia Biotechnology Co., Ltd. The Specificity siRNA and reference sequence were synthesized by Shanghai GenePharma Co., Ltd.

The DMEM medium was purchased from the American company, Hyclone. Fetal calf serum was purchased from Hangzhou Evergreen Co., Ltd. The TRIZOL reagent and Lipofectamine 2000 TM were purchased from the American company, Invitrogen. SYBR Green qPCR Mix was purchased from the Japanese company, TOYOBO. The inverse transcription kit was purchased from the American company, Fermentans, and the PCR was purchased from BGI Tech Solutions Co., Ltd.

Cell culture

The human HCC cell lines, HepG2 and Huh7.5.1 were purchased from the Cell Biology Laboratory of Xiangya Medical College, Central South University. Cells were cultured in DMEM medium containing 10% FBS at 37°C with 5% CO₂.

Cell transfection

HepG2 and Huh7.5.1 were divided into three groups, in the following manner: Girdin siRNA group, Negative control (NC) group and blank control (Con) group; the NC group had a transfected reference sequence while the blank control group did not. The siRNA sequence was designed (5'-GAAGGAGAGGCAACUGGAUTT-3', 4166-4184 nucleotide) aiming at Girdin and reference fragment of siRNA according to the human Girdin gene sequence provided by GenBank and siRNA sequence with a higher transfection rate, as published in the bibliography. Transient transfection was applied to cells in each group using Lipofectamine 2000 TM transfection reagent, using real-time PCR to detect the expression of Girdin mRNA of cells in each group.

Real-time PCR test

The Trizol method was employed to extract RNA of the cells of each group after being transfected for 24 h and the purity and completeness of RNA was detected. The cDNA sequence of human Girdin was searched for in the Genbank database, primers were designed and the specificity tested. The primer sequence of Girdin gene is as follows: 5'-GACCAACTAGAGGGAACTCG-3' for the upstream, 5'-TACTTTGTTTCTGTGCCATT-3' for the downstream, 175 bp for size of fragment. The reaction conditions for PCR are: pre-degeneration happens at 95°C, it ta-

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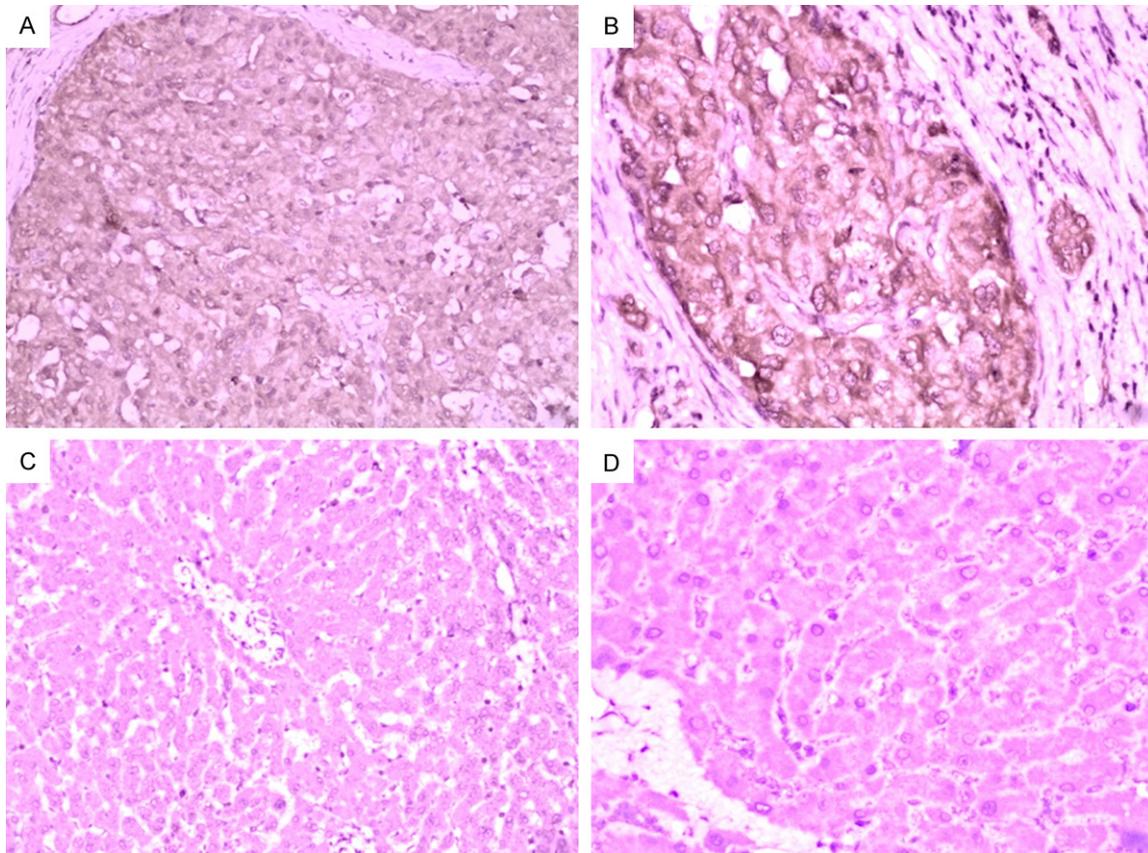


Figure 1. Girdin protein expression (Immunohistochemistry). A. Expression in HCC tissues (original magnification $\times 100$). B. Expression in HCC tissues (original magnification $\times 200$). C. Expression in adjacent tissues of HCC (original magnification $\times 100$). D. Expression in adjacent tissues of HCC (original magnification $\times 200$).

kes 3 min to go into circulation; each degeneration happens at 95°C and lasts for 10 sec, it will anneal at 58°C and last for 30 sec; there will be 35 circulations in total.

Cell proliferation assay

Transfected HepG2 and Huh7.5.1 cells of each group were placed onto a 96-well cell culture plate, setting up 5 complex wells (5×10^3 cells/well). A routine culture was carried out, and MTT detection was performed on a 96-well cell culture plate at 0 h, 24 h, 48 h and 72 h, respectively. The optical density (OD) was detected for each well at the wave length of 570 nm with a Microplate Reader (Bio-Rad, USA). Each assay was performed in triplicate wells and repeated three times.

Cell Invasion assay

Cells in each group were collected and re-suspended in serum-free H-DMEM at a concentra-

tion of 5×10^4 cells/ml, respectively. Then, 500 μl cell suspensions were added into the upper chamber, and the bottom chamber was filled with 750 μl H-DMEM containing 10% FBS. After incubation for 24 h at 37°C , 5% CO_2 , a cotton bud was used to remove the cells that had not permeated through the polycarbonate membrane. Then, the cells that transmigrated through the polycarbonate membrane and adhered to the bottom were stained with Trypan blue for 15 min and were then photographed and counted. After that, 500 μl 10% ethanol was added to each well so as to dissolve the dye on the polycarbonate membrane. Then, cells were transferred to a 96-well plate to measure their absorbency at 570 nm, using a Microplate Reader. Each assay was performed in triplicate wells.

Statistical analysis

Data was processed using SPSS16.0 statistical analysis software. Enumeration data was rep-

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Table 1. Expression of Girdin protein in HCC and adjacent tissue (No. %)

Group	Cases	Negative expression	Positive expression		
			Low	High	Total
HCC Tissues	40	13 (32.5)	17 (42.5)	10 (25)	27 (67.5)
Adjacent Tissues	30	25 (83.3)	4 (13.3)	1 (3.3)	5 (16.7)

Table 2. Correlations of Girdin expression with the clinicopathological features of HCC (No. %)

Clinicopathological feature	Case	Girdin protein expression		P value
		Negative (n = 13)	Positive (n = 27)	
Age (years)				0.941
≥ 50	28	9 (32.14)	19 (67.86)	
< 50	12	4 (33.33)	8 (66.67)	
Gender				0.898
male	35	12 (34.29)	23 (65.71)	
female	5	1 (20.00)	4 (80.00)	
Tumor location				0.316
left liver	11	5 (45.45)	6 (54.55)	
right liver	21	7 (33.33)	14 (66.67)	
bilateral liver	8	1 (12.50)	7 (87.50)	
Vascular invasion				0.736
no	36	12 (33.33)	24 (66.67)	
yes	4	1 (25.00)	3 (75.00)	
Hepatic cirrhosis				0.919
no	22	7 (31.82)	15 (68.18)	
yes	18	6 (33.33)	12 (66.67)	
Tumor size (cm)				0.03
< 5	27	12 (44.44)	15 (55.56)	
≥ 5	13	1 (7.69)	12 (92.31)	
T stage				0.02
T1-T2	23	11 (47.83)	12 (52.17)	
T2-T3	17	2 (11.76)	15 (88.24)	
TNM stage				0.046
I-II	21	10 (47.62)	11 (52.38)	
III-IV	19	3 (15.79)	16 (84.21)	
Edmondson-Steiner stage				0.002
I-II	16	10 (62.50)	6 (37.50)	
III-IV	24	3 (12.50)	21 (87.50)	

resented by percentage, and the chi-square test method was used to analyze the varying expression of Girdin protein and its relationship with clinico-pathological characteristics. All measurement data is represented via mean ± standard deviation. One-way analysis of variance for multi-group data comparison was used, as for the pairwise comparison of large amounts of data; the least significant differ-

ence method will be used. When *P* is less than 0.05, it means that the difference is of statistical significance.

Results

Positive expression percentage of HCC tissues was obviously higher than that of adjacent tissues

The immunohistochemical data proved that Girdin is located at cytoplasm. Among 30 cases of adjacent tissues, 25 cases (83.3%) showed negative expression, 4 cases (13.3%) presented low expression and 1 cases (3.3%) presented high expression, and the positive expression percentage accounted for 16.7%. However, among 40 cases of hepatocellular carcinoma tissues, 13 cases (32.5%) presented negative expression, 17 cases (42.5%) presented low expression and 10 cases (25%) presented high expression (**Figure 1; Table 1**). The data showed that the positive expression rate of Girdin protein for tissues was significantly higher than that of adjacent tissues (*P* < 0.05). It suggested that Girdin protein played an important role in the occurrence and development of HCC.

Correlation between girdin protein expression and clinical pathological features in patients with HCC

The chi-square test was used to analyze the correlation between the expression of Girdin and clinical pathological characteristics in HCC (**Table 2**). As shown in **Table 2**, the expression of Girdin protein in HCC closely correlates to tumor size, T stage, TNM stage and Edmondson-Steiner stage (*P* < 0.05), yet is unrelated to age, gender, status of liver cirrhosis, location of tumor and vascular invasion. In addition, as

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Table 3. Spearman rank correlation analysis

Variable	Girdin protein	
	Correlation coefficient	P value
Tumor size	0.368	0.02
T stage	0.381	0.015
TNM stage	0.339	0.032
Edmondson-Steiner stage	0.523	< 0.001

shown in **Table 3**, the data from the spearman rank correlation analysis showed that the expression of Girdin protein positively correlated to tumor size ($r = 0.368$, $P < 0.05$), T stage ($r = 0.381$, $P < 0.05$), TNM stage ($r = 0.339$, $P < 0.05$) and Edmondson-Steiner stage ($r = 0.523$, $P < 0.001$).

Girdin downregulation mediated by siRNA inhibited the proliferation ability of hepatocellular carcinoma cells

The Girdin-specific siRNA were transfected into HepG2 and Huh7.5.1 cells. The real-time fluorescence quantification PCR results showed that the relative expressions of Girdin mRNA in the Girdin siRNA group of HepG2 and Huh7.5.1 cells had obviously decreased when compared to that in the NC and Con group, and the difference was of statistical significance ($P < 0.01$), while the difference between NC group and Con group was of no statistical significance ($P > 0.05$) (**Figure 2A**). These results showed that the expression of Girdin in HepG2 and Huh7.5.1 cells of HCC had been successfully lowered. The result of further MTT experiments showed that if we make a comparison of the same cell line in each group at 24 h, 48 h and 72 h, respectively, the OD value of both HepG2 and Huh7.5.1 cells in Girdin siRNA group was significantly lower than that of the Con group and the NC group, and the difference was of statistical significance ($P < 0.01$); whereas the difference between the NC group and Con group was in direct opposition ($P > 0.05$) (**Figure 2B**), which suggested that reducing the expression of Girdin could markedly inhibit the proliferation of HepG2 and Huh7.5.1 cells.

Girdin downregulation mediated by siRNA inhibited the invasion ability of hepatocellular carcinoma cells

To further investigate the effect of Girdin on the cellular invasion of the hepatocellular carcino-

ma cell, we carried out the Transwell experiment. As a result, the OD value of the cells passing through membrane in Girdin siRNA group of HepG2 and Huh7.5.1 cells were much lower than that of NC group and Con group, which is of statistical significance ($P < 0.05$, **Figure 3**); whereas the comparison of NC group and Con group shows no statistical difference ($P > 0.05$). This result suggested that that reducing the expression of Girdin could significantly inhibit the invasion of HepG2 and Huh7.5.1 cells.

Discussion

HCC, a malignant tumor which develops in parenchymal hepatic cells or bile duct cells, is one of the more common malignant tumors in China, and its morbidity among middle-aged males is higher than for other conditions. There is no obvious specific symptom at its early stages, the majority of patients have reached the advanced or distant metastasis when diagnosed, and can no longer be operated upon surgically [17, 18]. TACE (transcatheter arterial chemoembolization) is an important nonsurgical treatment for unresectable patients with HCC [19-22], nevertheless, the overall treatment effect is poor and the five-year survival rate is very low due to HCC's high malignancy. So it is urgent to find a new potential target to treat HCC and improve the prognosis for HCC patients.

It is reported that Girdin can participate in regulating many normal physiological functions; it can participate in cell polarization [23] through interacting with Par-3 as well as participating in PI3K/Akt signal pathway to regulate the division and migration of many cells such as endothelial cells and smooth muscle cells [24, 25]; in addition, it can also interact with Gas to influence the cell proliferation at the downstream of EGFR signal [12] and participate in regulation of the mature of new nerve cells [10].

Many studies in recent years have found that the expression of Girdin increases in many malignant tumorous tissues [12, 14, 15, 17]. Its expression is related to clinico-pathological characteristics, tumor proliferation and invasion, and can also act as prognostic factor of some malignant tumors [13]. Studying of the relationship between the expression of Girdin

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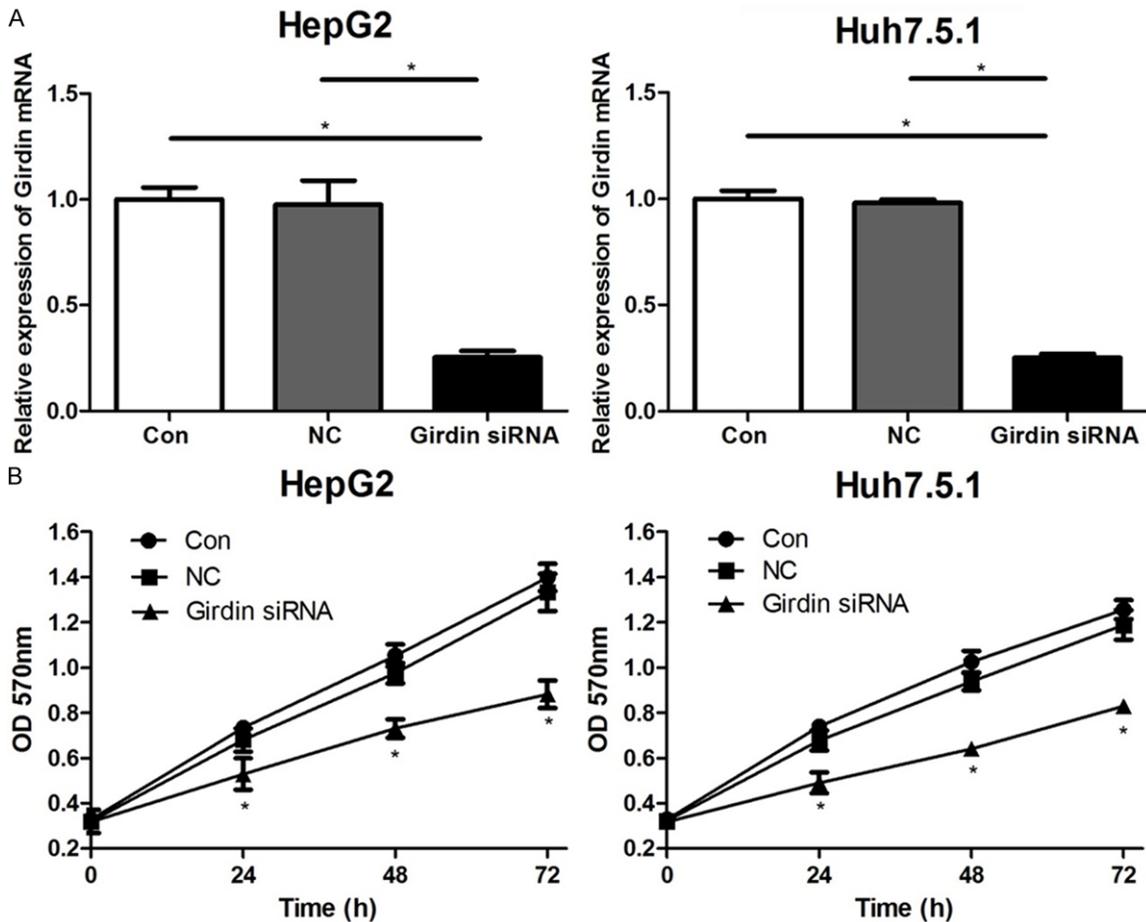


Figure 2. Cell proliferation inhibition mediated by the transfection of Girdin siRNA. A. Relative expression of Girdin mRNA in HepG2 and Huh7.5.1 cell lines. B. OD570 nm at different time in HepG2 and Huh7.5.1 cell lines which were transfected with Girdin siRNA. One-way analysis of variance was used to analyze the differences in this experiments (* P value < 0.05).

and clinico-pathological features of NSCLC patients proved that the expression of Girdin correlates with degrees of capillary infiltration/differentiation, yet is unrelated with other factors like age, gender, tumor size, pathological pattern and lymphatic infiltration [13]. Ke Cao's [15] study of Girdin in esophagus carcinoma found that inhibiting the expression of Girdin could significantly reduce the proliferation and invasion ability of ECA109 cell lines of esophagus carcinoma. After studying the relationship between the expression of Girdin and clinico-pathological features as well as the survival time of the patients with colon cancer, Garcia-Marcos [8] came to the conclusion that Girdin is a new transfer-related protein as well as an independent poor prognostic factor for colon cancer; Liu [24] also put forward the same perspective in the study of breast cancer; namely,

Girdin can also act as an independent prognostic indicator for breast cancer.

Firstly, immunohistochemistry technology was used to perform Girdin antibody staining of HCC tissue microarray, results showed that in positive expression of Girdin protein there existed differences between HCC tissues and adjacent tissues. These suggested that Girdin not only participates in regulating some biological behavior of normal hepatocyte, but also is involved in the occurrence and development of hepatocellular carcinoma. The analysis of the relationship between the expression of Girdin and clinico-pathological characteristics indicated that the expression of Girdin in HCC tissues positively correlated to invasion-related clinico-pathological characteristics, such as tumor size, T stage, TNM stage and Edmondson-Steiner stage, and the difference is of great signifi-

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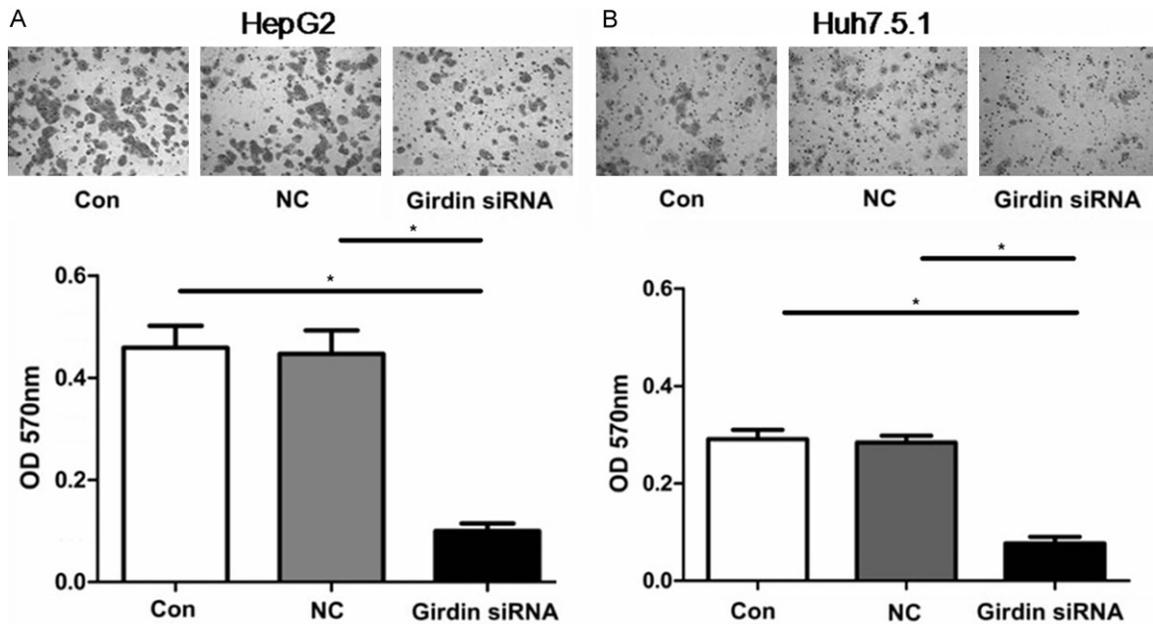


Figure 3. Cell invasion inhibition mediated by the transfection of Girdin siRNA. A. Staining of HepG2 cells ($\times 200$) and OD 570 nm of invasive cells in each group by transwell assay. B. Staining of Huh7.5.1 cells ($\times 200$) and OD 570 nm of invasive cells in each group by transwell assay. One-way analysis of variance was used to analyze the differences. (* P value < 0.05).

cance. It showed that the over-expression of Girdin may be related to the invasion and migration of hepatocellular carcinoma, which is similar to the studies in colorectal cancer [6, 7] and breast cancer [24, 26] by Jun et al. Secondly, RNA interference technology is used to inhibit the expression of Girdin protein in human HCC HepG2 and Huh7.5.1 cell lines; the reduction of Girdin can not only significantly inhibit the proliferation ability of HepG2 and Huh7.5.1 cells but also the migration and invasion ability of tumor cells. All the results showed that Girdin plays an oncogenic role in the occurrence and development of HCC, and it can accelerate the proliferation and invasion of hepatocellular carcinoma cells. Further studies are needed regarding the causes of high expression of Girdin protein in HCC and the detailed signaling of Girdin in hepatocellular carcinoma cells.

In conclusion, our study proved that Girdin protein was upregulated in HCC for the first time and preliminarily proved that it may accelerate the proliferation, migration and invasion of HCC cells. Therefore, Girdin may become a new potential target for treating HCC, and it is highly possible that further studies on Girdin can provide a new strategy for HCC molecular targeting treatments.

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Disclosure of conflict of interest

None.

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