Loss of ARHGDIA expression is associated with poor prognosis in HCC and promotes invasion and metastasis of HCC cells

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Abstract. Rho GTPases control a wide range of cellular processes and contribute to tumor invasion and metastasis. As a regulator of Rho activity, ARHGDIA is aberrantly expressed in several types of tumors and plays different roles in the tumor process. To elucidate the role of ARHGDIA in HCC, we investigated the patterns of its expression, prognosis and clinical profiles in HCC. Functional assays were performed to investigate whether the alteration of ARHGDIA has an effect on cell growth, migration and invasion, as well as the status of Rho GTPases. We found that ARHGDIA was frequently downregulated in HCC and associated with tumor invasion and metastasis. Moreover, ARHGDIA was significantly associated with OS and TTR of HCC patients. Low level of ARHGDIA exhibited a decreased postoperative OS and a shorter TTR compared those with high levels. Functional assays showed that loss of ARHGDIA promoted HCC cell migration and invasion *in vitro* and lung metastasis formation *in vivo*. We found that loss of ARHGDIA significantly induced Rac1 and RhoA activation which may contribute to invasion and metastasis of HCC. In conclusion, the present study has identified loss of ARHGDIA contributed to the processes of hepatic tumorigenesis, in particular invasion and metastasis which may provide a potential therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world, including China (1). It ranks as the second leading cause for cancer death worldwide. As far as great advances in the treatment of the disease is concerned, prognosis for HCC patients is not favorable due to the likelihood of intrahepatic and extrahepatic recurrence, which leads to a high mortality rate (2,3). Therefore, investigations into the molecular mechanisms involving in HCC metastasis have major importance to develop novel avenues for targeted therapies.

Cell motility plays an important role in tumor invasion and metastasis. Rho GTPases regulate actin polymerization, actomyosin contractility and microtubule dynamics controlling a wide range of cellular processes, including cell adhesion, and migration. They function as molecular switches in cell signaling, alternating between inactive GDP-bound states and active GTP-bound states. The active-GTP form of Rho is governed by a panel of inhibitors including ARHGDIs which block activation of Rho proteins by sequestering the GDP-bound Rho proteins in the cytosol (4). ARHGDIs includes three members, named ARHGDIA, ARHGDIB and ARHGDIG. ARHGDIA is ubiquitously expressed and interacts with several Rho GTPases, including RhoA, Rac1 and Cdc42 (5,6). As a regulator of Rho activity, ARHGDIA has attracted increasing attention. There are studies showing that ARHGDIA is aberrantly expressed in many tumors and plays an important role in the tumor process. However, the role of ARHGDIA in HCC remains to be unraveled. In this study, we found that ARHGDIA was frequently downregulated in HCC and significantly associated with prognosis of HCC patients. Loss of ARHGDIA promoted HCC cells invasion and metastasis *in vitro* and *in vivo*, which might be due to Rac1 and RhoA GTPase activation induced by silencing ARHGDIA.

Materials and methods

Patients and specimens. A total of 86 patients were enrolled in the present study. The patients did not receive any preoperative cancer treatment and their follow-up data were available. They were followed-up after surgical treatment until May 2011, with a median follow-up of 29 months (range 2-73. 2 months). During the follow-up, the patients were monitored every 2-3 months as described previously (7). CT scanning or MRI was performed when tumor recurrence was suspected. The recurrent tumors were treated as described

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previously (8). Clinical samples were collected from these patients after obtaining informed consent according to an established protocol approved by the Ethics Committee of Fudan University (Shanghai, China).

Immunohistochemical staining. Immunohistochemical staining was performed to detect the expression of ARHGDIA in HCC and matched non-cancer tissue. The primary antibody against ARHGDIA was obtained from Epitomics (1:50). Intensity of staining was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The extent of staining was based on the percentage of positive tumor cells: 0 (negative), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The final score of each sample was assessed by summarization of the results of the intensity and extent of staining. Therefore, each case was considered negative if the final score was 0-1 (-) or 2-3 (±) and positive if the final score was 4-5 (+) or 6-7 (++), respectively. These scores were determined independently by two senior pathologists.

Cell culture. Huh-7, SMMC-7721 and MHCC-97H cells were cultured in DMEM medium with 10% FBS, maintained at 37˚C in a humidified air atmosphere containing 5% carbon dioxide.

Construction of plasmids, lentivirus production and transduction. The coding sequence of human ARHGDIA was cloned into the expression vector pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA, USA). The siRNA against ARHGDIA were synthesized by Ribobio and inserted into the pLKO.1-TRC cloning vector (Invitrogen, Carlsbad, CA, USA). All constructs were verified by sequencing. A mixture of pCDH-ARHGDIA or pLKO.1-siARHGDIA cloning vector, and adjuvant vectors psPAX2 and pMDG2 were transfected into HEK293T cells using Lipofectamine 2000 reagent to generate lentiviruses. Huh-7, SMMC-7721 and MHCC-97H cells were infected with the recombinant lentivirus-transducing units plus 8 mg/ml polybrene (Sigma).

Cell proliferation assay. Cell proliferation was measured with the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Corp.); 5,000 cells were plated into each well of a 96-well plate, in which 10 μl CCK-8 was added to 90 μl of culture medium. The cells were subsequently incubated for 1 h at 37˚C and the attenuance was measured at 450 nm. Three independent experiments were performed.

Colony formation assay. The 500 cells were plated into 6-well culture-plates and cultured for 14 days to allow colony formation. Colonies were stained with 0.1% crystal violet (Amersco, Solon, OH, USA) in 50% methanol and 10% glacial acetic acid for counting.

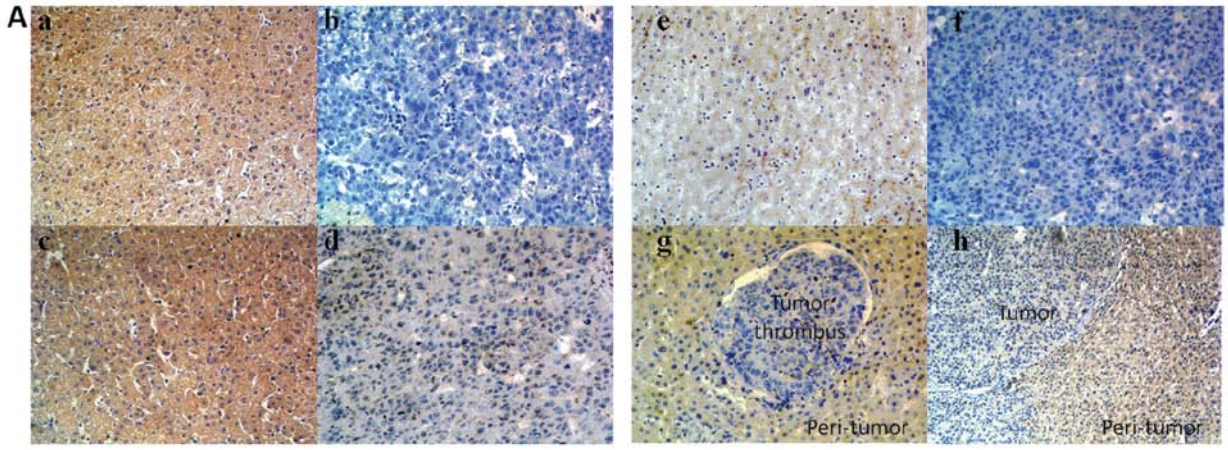
In vitro migration and invasion assays. For the migration assays, $2x10^4$ cells were added into the upper chamber of the insert with the non-coated membrane (Millipore, 8-mm pore size). For the invasion assays, each well insert was layered with 50 μl of a 1:4 mixture of Matrigel/Dulbecco's minimal essential medium (BD Bioscience). Cells ($1x10^5$) were added into the upper chamber of the insert. In both assays, cells were plated in medium without serum, and medium containing 10% FBS in the lower chamber served as chemoattractant. After several hours of incubation, the cells that did not migrate or invade through the pores were carefully wiped out with cotton swab. Cells on the lower surface of the membrane were fixed with methanol and stained with Giemsa and counted. Each experiment was performed in triplicates.

In vivo metastasis assays. For *in vivo* metastasis assays, SMMC-7721 cells infected with either the ARHGDIA-siRNAs or the vector were transplanted into nude mice (5-week-old BALB/c-nu/nu, 6 per group, $2x10^6$ cells for each mouse) through the tail vein. After 6 weeks, mice were sacrificed. The lungs were removed, fixed in formalin, and embedded in paraffin. Consecutive sections of the whole lung were subjected to hematoxylin and eosin staining. All of the metastatic foci in lung were calculated microscopically to evaluate the development of pulmonary metastasis. The lung metastases were calculated and evaluated independently by two pathologists.

Western blotting. Equal amounts of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis and transblotted onto nitrocellulose membrane (Bio-Rad). After blocking in 5% non-fat milk, the membranes were incubated with rabbit anti-ARHGDIA antibody (mAb; 1:1,000; Epitomics), rabbit anti-Rac1 antibody (mAb; 1:1,000; Epitomics), rabbit anti-RhoA antibody (mAb; 1:1,000; Epitomics), rabbit anti-Cdc42 antibody (mAb; 1:1,000; Epitomics) or rabbit anti-GAPDH mAb (1:5,000; Epitomics). The proteins were detected with enhanced chemiluminescence reagents (Pierce).

Immunoprecipitation of active Cdc42, RhoA, Rac1. The protocol used was based on the availability of a mouse monoclonal antibody directed against the active form of Cdc42, RhoA and Rac1 commercially through NewEast Biosciences (Malvern, PA, USA). Cells were lysed in 1 ml of ice-cold lysis buffer for 10 min. Aliquots of each cell lysate were added to two microcentrifuge tubes, one for analysis of the active and the other for the analysis of total potein content. Then 1 μl of anti-active Cdc42, RhoA or Rac1 monoclonal antibody was added, as well as 20 μl of Dynabead Protein G added, and samples were incubated overnight with rotation at 4˚C. Beads were pelleted by centrifugation for 1 min at 5,000 g, then washed three times with 0.5 ml of lysis buffer, resuspended in 20 μl of 2X reducing SDS-PAGE sample buffer, heated at 100˚C for 5 min, then separated on 12% polyacrylamide gels and processed for western blotting after transferring to PVDF membranes. Rabbit polyclonal antibody against total Cdc42, RhoA and Rac1 (mAb; 1:1,000; Epitomics) was used for western blotting.

Statistical analysis. Statistical analysis was performed with SPSS 15.0 (SPSS Inc, Chicago, IL, USA) and values are expressed as the mean ± standard deviation. The differences between groups were analyzed using Student's t-test (only two groups), or one-way analysis of variance (more than two groups were compared). $P<0.05$ was considered statistically significant.



B

ARHGDIA Staining	Tumor (N=40)	Adjacent Liver Tissue (N=40)	*P Value*
Negative	26	10	P<0.001
Positive	14	30	

Figure 1. Immunohistochemical staining of ARHGDIA in HCC, adjacent non-cancer tissue. Scoring criteria and grouping are described in Materials and methods. ARHGDIA is downregulated in HCC tissue. (A-a) Non-cancer tissue matched with HCC-1, score 7; (b) HCC-1, score 0; (c) Non-cancer tissue matched with HCC-2, score 7; (d) HCC-2, score 2; (e) Non-cancer tissue matched with HCC-3, score 2; (f) HCC-3, score 0; (g) Tumor thrombus and peri-tumor tissue; (h) HCC and peri-tumor tissue. (B) Statistical analysis of ARHGDIA expression according to the scoring and group.

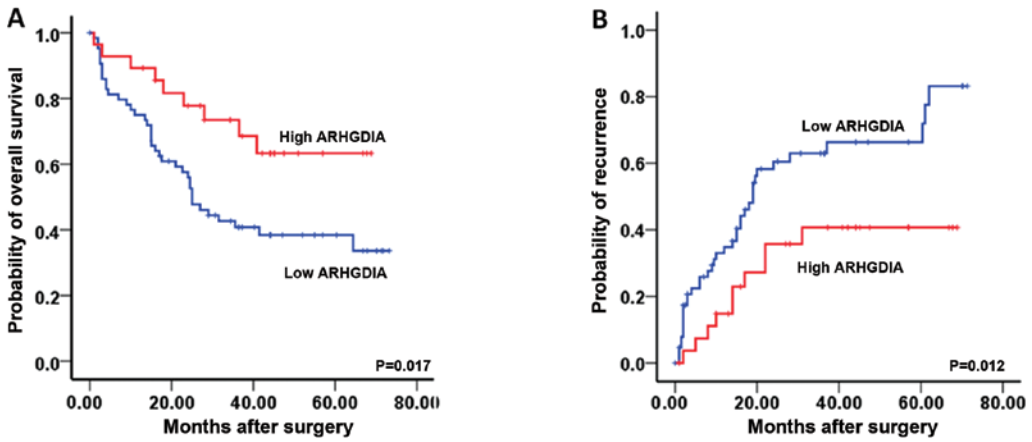


Figure 2. The association of ARHGDIA with prognosis of HCC patients assessed by Kaplan-Meier analyses. The patients with low level of ARHGDIA had a worse postoperative overall survival (OS) (A) and a shorter tumor recurrence time (TTR) (B).

Results

ARHGDIA is frequently downregulated in HCC and associated with tumor invasion and metastasis. The protein levels of ARHGDIA in 86 cases of HCC patient samples and corresponding non-cancer liver tissues (40 cases) were measured by immunohistochemical staining. Strong staining of ARHGDIA was observed in adjacent non-cancer liver tissue, but weaker in more than half (65%) of the HCC tissues (Fig. 1A). The expression level is significantly downregulated in HCC compared with non-cancer liver tissues (P<0.001) (Fig. 1B). At various regions of tumor within the same slide, it appeared that ARHGDIA expression remarkably decreased at invasive cancer *in situ* such as tumor embolus. Next, we analysed the relationships between ARHGDIA and clinical pathological features of HCC. Significant correlations were observed between ARHGDIA and vascular invasion (tumor invasion in blood vessel or bile duct) (P=0.0216). Low level of ARHGDIA expression was observated in 79.07% of vascular invasion group (Table I). The result indicated that ARHGDIA might correlated with HCC metastasis. Then, the ARHGDIA level was analyzed in a panel of human HCC cell lines with different metastatic potential. The level of ARHGDIA in the high-metastatic HCC cell lines (MHCC-97H) was much lower than that in the less-metastatic HCC cell lines (Huh-7, SMMC-7721) (Fig. 3C), indicating that the downregulation of ARHGDIA was related to the metastatic ability of HCC.

The association of ARHGDIA with prognosis of HCC patients. In the Kaplan-Meier analyses, the expression level

Table I. Expression of ARHGDIA detected by IHC and the clinicopathologic features of HCC patients (n=86).

Variables	ARHGDIA expression		P-value
	Low (n=57)	High (n=29)	
Gender			
Female	6	4	0.655
Male	51	25	
Age (years)			
≤51	23	13	0.691
>51	34	16	
Preoperative			
AFP (ng/ml)			
≤20	15	11	0.268
>20	42	18	
HBsAg			
Negative	6	2	0.479
Positive	51	27	
Liver cirrhosis			
No	5	2	0.764
Yes	52	27	
ALT (U/l)			
≤75	48	21	0.194
>75	9	8	
Tumor size (cm)			
≤5	18	13	0.226
>5	39	16	
Tumor number			
Single	43	26	0.118
Multiple	14	3	
Tumor encapsulation			
None	34	14	0.315
Complete	23	15	
Vascular invasion			
No	23	20	0.012
Yes	34	9	
TNM stage			
I	18	16	0.063
II	17	8	
III	22	5	
Tumor differentiation			
I-II	44	13	0.321
III-IV	25	4	

of ARHGDIA was significantly associated with OS and TTR. The patients with low level of ARHGDIA exhibited a decreased postoperative OS and a shorter TTR compared those with high level (Fig. 2). The 1-, 3- and 5-year OS rates of the patients with low level were 74.8, 42.2 and 36.6%, respectively, which were significantly lower than those with high level group (86.9, 65.4 and 51.5%, respectively; P=0.017). The 1-, 3- and 5-year cumulative recurrence rates of low level group were 37.8, 63.9 and 75.6%, respectively, which were significantly higher than those of the high level group (20.5, 47.2 and 60.4%, respectively; P=0.012).

ARHGDIA has no effect on HCC cell proliferation or the colony formation ability. To explore the functions of ARHGDIA in HCC, specific siRNAs against ARHGDIA were exploited to knockdown expression in SMMC-7721, and Huh-7 cell lines. As shown in Fig. 3D, siRNA significantly reduced the expression of ARHGDIA protein. We also constructed a lentivirus vector expressing ARHGDIA and established the stable cell line MHCC-97H, which has low basal levels of ARHGDIA (Fig. 3E). In cell proliferation assays, knocking down ARHGDIA showed no obvious impact on the proliferation of SMMC-7721 and Huh-7 cells (Fig. 3A). Similarly, overexpression of ARHGDIA did not affect MHCC-97H cell growth either (Fig. 3B). Next, the colony formation assays were performed to observe the effects of ARHGDIA on the anchoring growth ability of HCC cells. No obvious effects were observed on the colony formation ability of HCC cells after infection with ARHGDIA-siRNAs or lenti-ARHGDIA (Fig. 4).

Loss of ARHGDIA promotes HCC cell invasion and metastasis in vitro and in vivo. Given that expression of ARHGDIA is highly associated with the metastatic property of HCC, we wondered whether ARHGDIA could play an important role in HCC cell invasion and metastasis. Transwell assays without Matrigel demonstrated that downregulation of ARHGDIA could significantly promote migration of Huh-7 and SMMC-7721 cells when compared with vector groups (Fig. 5A). Transwell assays with Matrigel showed that the invasive capacities were dramatically enhanced in these two stable cell lines when compared with the control cells (Fig. 5B). However, the migration and invasion of MHCC-97H cells decreased when ARHGDIA was upregulated (Fig. 5). These results indicated that loss of ARHGDIA could significantly enhance HCC cell migration and invasion *in vitro*. To further explore the role of ARHGDIA in tumor metastasis *in vivo*, SMMC-7721 cells infected with si-ARHGDIA were transplanted into nude mice through the tail vein. Interestingly, the number of the metastatic nodules in the lung were dramatically increased in si-ARHGDIA groups compared with vector control (P=0.0377) (Fig. 6). Taken together, these observations suggested that ARHGDIA is a negative metastatic regulator for HCC.

Loss of ARHGDIA significantly increases the activities of Rac1 and RhoA GTPases in HCC cells. Regulation of the cytosol-membrane cycling of the Rho GTPase by ARHGDIs has a major role in controlling Rho GTPase activity and function. Given the important role of ARHGDIA in HCC cell migration and invasion, we conducted immunoprecipitation assays to determine the status of Rho GTPases in HCC cells. The mouse monoclonal antibody directed against

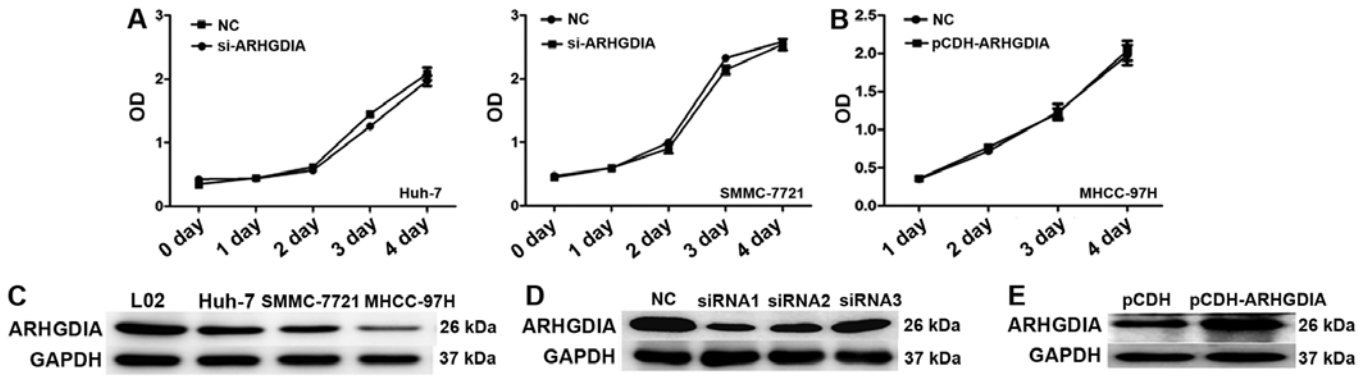


Figure 3. The effects of ARHGDIA downregulation or upregulation on the *in vitro* proliferation of HCC cells. (A) The proliferation assay of SMMC-7721 and Huh-7 cells infected with ARHGDIA-siRNAs or negative control. (B) The proliferation assay of MHCC-97H infected with lenti-ARHGDIA or lenti-mock control. No obvious effects on HCC cell growth *in vitro* were observed after up- or downregulation of ARHGDIA. (C) The expression level of ARHGDIA in normal human liver cells (LO2) and HCC cell lines. (D) Western blotting of ARHGDIA protein levels in Huh-7 cells infected with ARHGDIA-siRNAs or negative control-siRNA. (E) MHCC-97H cells transfected with lenti-ARHGDIA or lenti-mock control.

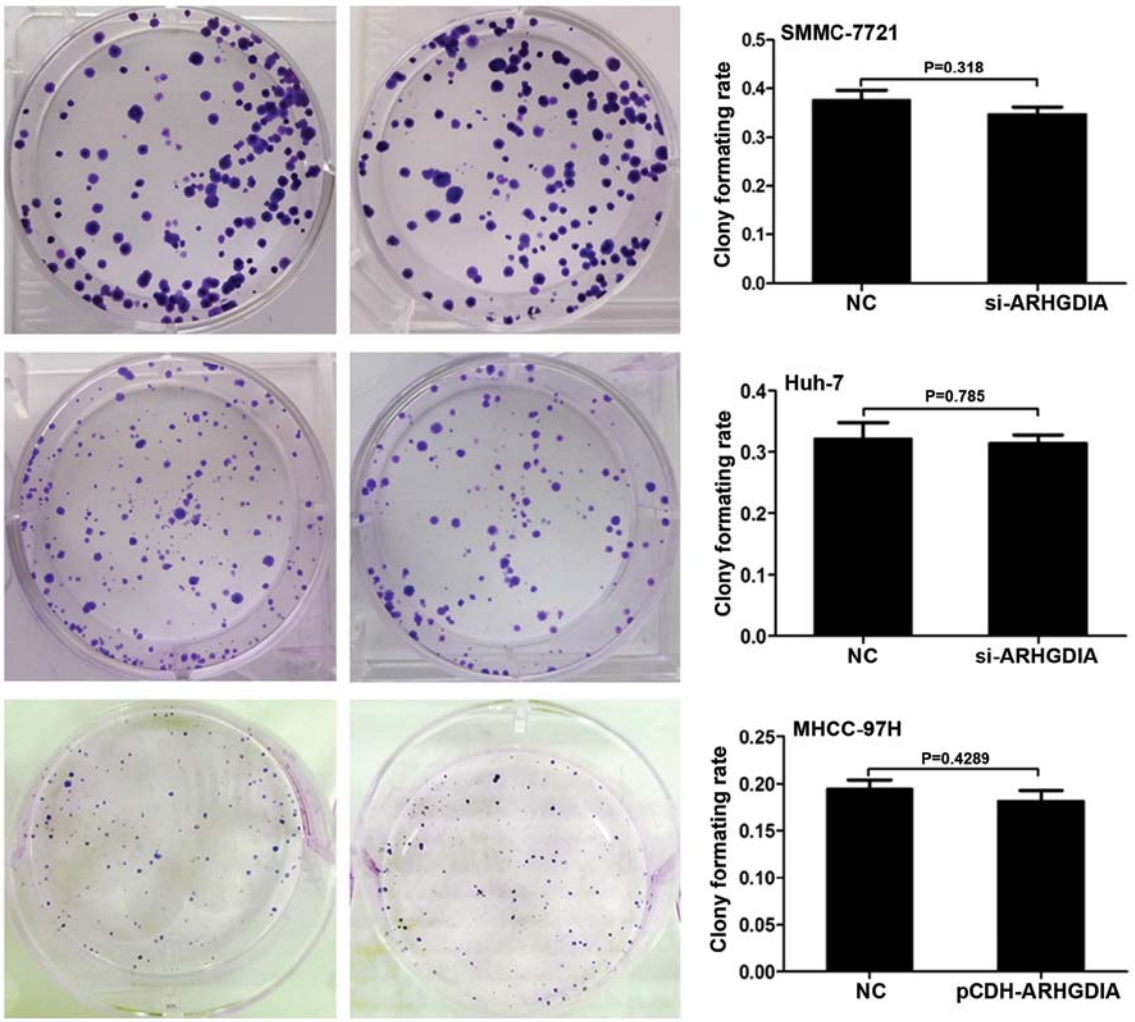


Figure 4. The effects of ARHGDIA up- or downregulation on the colony formation assay of HCC cells. SMMC-7721, Huh-7 cells infected with ARHGDIA-siRNAs or negative control and MHCC-97H infected with lenti-ARHGDIA or lenti-mock control. No obvious effects were observed on the colony formation ability of HCC cells after up- or downregulation of ARHGDIA.

the active form of Cdc42, RhoA and Rac1 were used in the immunoprecipitation assays. The results indicated that loss of ARHGDIA significantly induced RhoA and Rac1 activation in SMMC-7721 cells (Fig. 7A), particularly the activity of RHOA increased nearly 4-fold compared to the control. The Cdc42 activity was also slightly increased, but did not

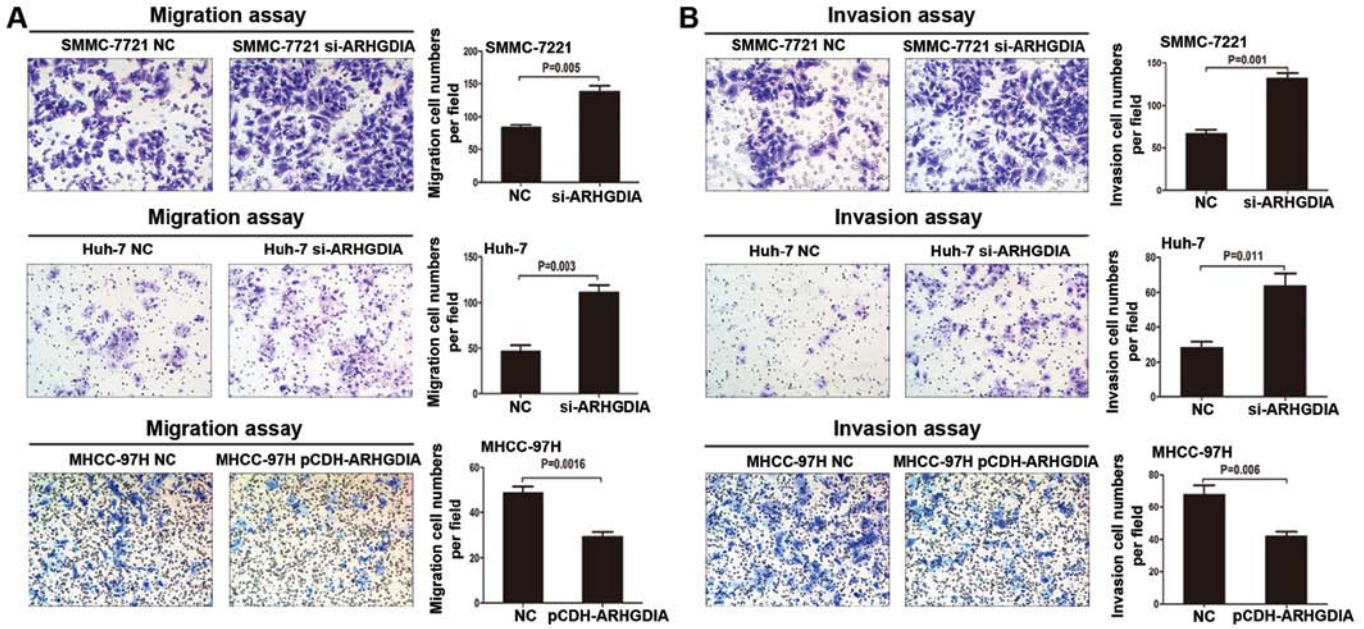


Figure 5. Loss of ARHGDIA promotes HCC cell migration and invasion *in vitro*. (A) Transwell migration assays of Huh-7 and SMMC-7721 cells infected with ARHGDIA-siRNAs or negative control, and of MHCC-97H transfected with lenti-ARHGDIA or lenti-mock control. (B) Transwell Matrigel invasion assay of Huh-7 and SMMC-7721 cells infected with ARHGDIA-siRNAs or negative control, and of MHCC-97H transfected with lenti-ARHGDIA or lenti-mock control.

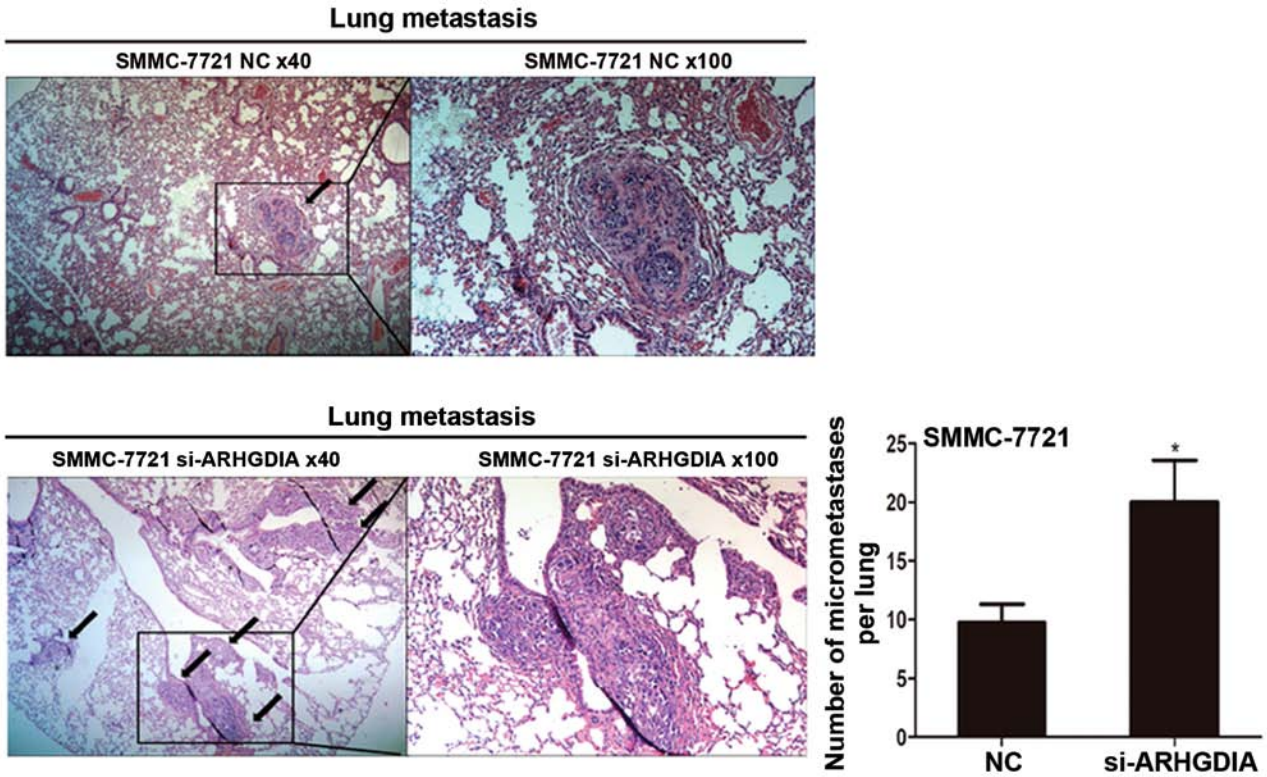


Figure 6. Loss of ARHGDIA promotes HCC metastasis *in vivo*. Representative H&E-stained sections of the lung tissues isolated from mice injected with SMMC-7721 vector or SMMC-7721 ARHGDIA-siRNA cells through the tail vein; arrowhead points to the tumor focus formed in the lung. The numbers of metastases in the lungs were counted.

reach statistical significance (Fig. 7B). Numerous studies have confirmed that activation of signaling of Rho GTPases plays an important role in cancer progression and metastasis (9). Therefore, the activation of Rho GTPase proteins induced by silencing ARHGDIA might contribute to tumor invasion and metastasis of HCC.

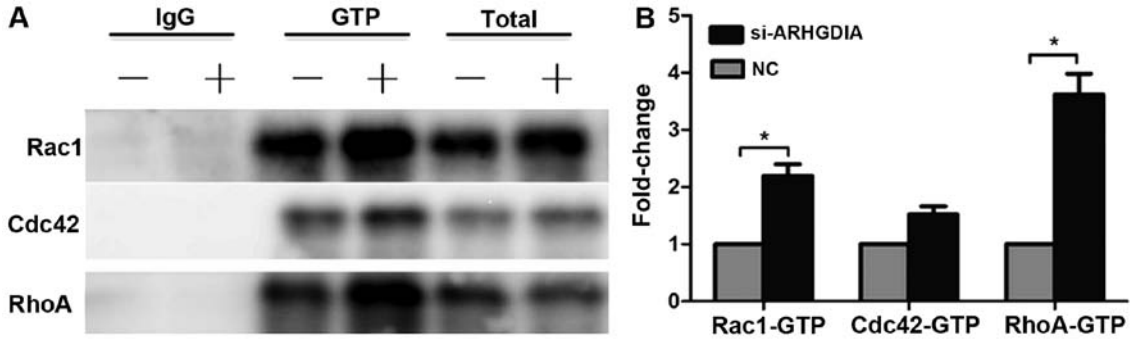


Figure 7. Loss of ARHGDIA increases the activity of Rho GTPases. (A) Rho GTPase activity of SMMC-7721 cells transfected with siRNA or vector was analyzed by pull-down assay. Lysates were incubated with an anti-active Rho GTPases monoclonal antibody. The precipitated active Rho GTPases were immunoblotted with an anti-Rho GTPase rabbit polyclonal antibody, the protocol is described in Materials and methods. Total protein levels of Rho GTPases present in whole cell lysates are shown in the right panel to demonstrate amounts of protein in samples. (B) The fold change of active-form of Rho GTPases/total Rho GTPases, gray value of vector group was set as 1.

Discussion

Changes in ARHGDIA expression levels have been associated with many cancers (10). Previous studies indicated that the changes vary depending on the tumor type. For instance, ARHGDIA expression is upregulated in colorectal and ovarian cancers, and high expression levels correlate with increased invasion and resistance to chemotherapy (11-13). By contrast, ARHGDIA expression is reduced in brain cancers, and inversely correlate with the degree of malignancy (14). In breast cancers, Jiang *et al* found a significant reduction of ARHGDIA expression in tumor versus normal breast (15). Furthermore, the reduction of ARHGDIA had a significant, poor prognostic correlation when tumors were stratified by node status or by recurrence and disease-specific death. Therefore, the effects of the ARHGDIA on cancers are complex and context-dependent. In the present study, we first clarified the role of ARHGDIA in HCC to ensure that ARHGDIA is indeed a tumor suppressor gene involved in HCC invasion and metastasis. We found the following evidence: a) ARHGDIA was frequently downregulated in HCC compared with non-cancer liver tissues. Within the same IHC slide, ARHGDIA expression remarkably decreased at invasive cancer *in situ* such as tumor embolus. b) ARHGDIA expression level was significantly associated with vascular invasion. HCC with vascular invasion had a lower ARHGDIA expression than those without vascular invasion. c) The level of ARHGDIA in the high-metastatic HCC cell lines was lower than that in the low-metastatic cell lines. d) In the Kaplan-Meier analyses, the expression level of ARHGDIA was significantly associated with OS and TTR. The patients with low level of ARHGDIA exhibited a decreased postoperative OS and a shorter TTR. e) The functional assay indicated that *in vitro* and *in vivo* phenotypes of ARHGDIA correlated well with the patterns of its expression and prognosis in HCC, as well as clinical profiles. Loss of ARHGDIA could promote HCC cell migration and invasion *in vitro* and increase lung metastasis *in vivo*. Therefore, the evidence above proves that ARHGDIA is a tumor suppressor and plays an important role in HCC progression especially in invasion and metastasis.

The changes in ARHGDIA expression are manifested through their actions on multiple RHO GTPases, and the levels and activity vary significantly in the different cell types and cancers. A single Rho family member can have opposite effects in different tumor types (16,17), possibly leading to the biological diversity of ARHGDIA. Many experiments have reported that loss of ARHGDIA might reduce inhibition on endogenous Rho family GTPases exerting a negative regulator of Rho-family GTPase activity. Turner *et al* found that the amount of RhoGTP increased significantly in the HEL cells transfected with ARHGDIA siRNA (18). In ARHGDIA-knockout mice, renal abnormality is associated with increased Rac1 (but not RhoA) (19), while the abnormal basal permeability of the pulmonary vascular endothelium correlates with the increasing activity of RhoA (20). On the contrary, overexpression of ARHGDIA significantly inhibits the activities of RhoA, Rac1, Cdc42 and reduces the positioning of these active proteins in membranes of myocardial cells (21). In HCC, we confirmed that loss of ARHGDIA significantly induced Rac1, RhoA activation in SMMC-7721 cells. Numerous studies indicate that deregulated signaling of Rho GTPases plays an important role in HCC progression and metastasis (9). RhoA pathway associates with venous invasion, cell differentiation and poor prognosis (22), correlating with tumor progression and metastasis (23-25). Rac1 GTPase is crucial for actin cytoskeleton reorganization at the cell cortex and is involved in processes of HCC migration and invasion (26-29). Therefore, activating Rho GTPase initiated by silencing ARHGDIA in HCC cells may at least in part mediate the effect of tumor invasion and metastasis.

In conclusion, the present study identified ARHGDIA as a suppressor of HCC invasion and metastasis by the RhoGTP pathway. The above findings may contribute to better understanding of the processes of hepatic tumorigenesis, especially invasion and metastasis thus providing a potential therapeutic target in HCC.

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