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Overexpression of RhoGDI, a novel predictor of distant metastasis, promotes cell proliferation and migration in hepatocellular carcinoma

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ABSTRACT

RhoGDI (Rho GDP-dissociation inhibitor alpha, or RhoGDIα) was identified as a regulator of Rho GTPases, but its role in cancer remains controversial. In this study, increased expression of RhoGDI was detected in hepatocellular carcinoma (HCC) cell lines and tissues with highly metastatic potential. RhoGDI overexpression correlated with postoperative distant metastasis. Enforced expression of RhoGDI in HCC cells significantly enhanced cell proliferation and migration. Conversely, knockdown of RhoGDI caused an inhibition of the aggressive phenotypes of HCC cells. Furthermore, RhoGDI up-regulated Rho, but not Rac, and enhanced PI3K/AKT and MAPK pathway activity. Our findings suggest that RhoGDI overexpression is a predictor of distant metastasis and plays an important role in the progression of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant disorders, becoming the third leading cause of cancer-related deaths globally [1]. Usually, it is common observed in certain areas of Asia and Africa, however, in recent years, an increasing incidence has been found in Western countries [2]. The prognosis of HCC after surgical or locoregional therapies is still in its poor position, resulting in ongoing researches [3]. With the increasing understanding of the tumor biology of HCC, more and more molecular markers with high sensitivity and specificity for HCC have been developed for early diagnosis and the development of future targeted HCC therapeutics.

Rho GDP-dissociation inhibitors (GDIs), the key regulators of Rho family GTPases, are typified by its ability to prevent nucleotide exchange and membrane association. Despite the initial negative roles attributed to RhoGDI, recent evidence has come to suggest that it may also act as a positive regulator necessary for the correct targeting and regulation of Rho activities by conferring cues for spatial restriction, guidance and availability to effectors [4,5]. The expression of RhoGDIs is altered in a variety of cancers, which could mediate several processes during tumorigenesis and cancer progression [6–9].

In our previous study, RhoGDI was identified as an up-regulated protein in metastatic colorectal cancer (CRC) by comparative proteomic analysis [10]. The further data suggested that RhoGDI may promote CRC progression by stimulating tumor cell growth and migration [11]. miR-151 was revealed as a crucial stimulus for HCC invasion and metastasis by directly targeting RhoGDI with small-samples assay [12]. Despite its critical cellular function, the clinical significance and role of RhoGDI in HCC progression is unclear.

In the present study, we detected RhoGDI expression in primary HCC using immunohistochemistry, and analyzed the relationship between its over-expression and clinicopathological features. Further, the potential predictive value of RhoGDI was evaluated in the post-resectional metastasis and survival of HCC patients. Gene transfection-mediated over-expression and RNA interference (RNAi)-mediated gene silencing were performed to investigate the effect of RhoGDI on the biological behavior of HCC cells and discussed the possible mechanisms involved in HCC progression.

2. Materials and methods

2.1. Tumor tissue sample

All cases of tumor tissue were provided by the Tumor Tissue Bank of Nanfang Hospital. A total of 163 patients were involved in the study. Fresh frozen tumor samples from 16 patients with

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HCC were selected for Western blot analysis. Formalin-fixed tumor tissues from other 147 patients including 126 tumor tissues and 106 adjacent non-tumors tissues were used for immunohistochemical analysis. The patients diagnosed of primary HCC had undergone elective surgery for HCC in Nanfang Hospital during 2001–2002. A comprehensive clinicopathological dataset including age, gender, size of primary tumor, tumor differentiation, serum alpha-fetoprotein (AFP), hepatitis B surface antigen (HBsAg), liver cirrhosis, local recurrence and distant metastasis were obtained from the Tumor Tissue Bank of Nanfang Hospital. Complete follow-up, ranging from 0 to 83 months, was available for all patients and the median follow-up was 42 months. At the time of censoring the data, there had been 89 (60.5%) deaths in the patient group. The pathological diagnosis was performed at the Department of Pathology of Nanfang Hospital, Southern Medical University. The study was approved by the Ethics Committee of Southern Medical University and all aspects of the study comply with the Declaration of Helsinki. Ethics Committee of the Southern Medical University specifically approved that not informed consent was required because data were going to be analyzed anonymously. All patient data were de-identified for analysis.

2.2. Cell lines

Human liver immortal cell line L02 was obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HCC cell lines Huh-7 (RCB 1366) was obtained from the RIKEN cell bank (Ibaraki, Japan). Hep3B (HB-8064), HepG2 (HB-8065) and SK-Hep1 (HTB-52) were obtained from American Type Culture Collection (ATCC). MHCC-97L, MHCC-97H, HCC-LM3 and HCC-LM6, kindly provided by Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China) [13,14]. All the cells were maintained in RPMI1640 (Hyclone, Logan, UT) or Dulbecco's modified Eagle's medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Invitrogen, Paisley, UK) at a humidity of 5% CO_2 at 37 °C. All the cell lines were characterized by mycoplasma detection, DNA – Fingerprinting, isozyme detection and cell vitality detection. These cell lines were immediately expanded and frozen such that they could be restarted every 3–4 months from a frozen vial of the same batch of cells.

2.3. Western blot analysis

Sample preparation for immunoblotting was done as previously described [10,15,16]. The membrane was probed with anti-RhoGDI rabbit polyclonal antibody (1:1000; CST, Danvers, MA, USA), rabbit polyclonal antibodies to p-Akt (Ser473), p-Akt (Thr308), AKT, p44/42 MAPK (ERK1/2), p-p44/42 MAPK (ERK1/2) (1:1000; CST, Danvers, MA), rabbit monoclonal antibody to Rho, Rac/Cdc42 (1:500; Epitomics, California, USA). Protein expression was determined with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:10000; Zhongshan Jinqiao Biotech, China) and enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA). The immunoreactive bands were visualized and quantified on Kodak 2000 M camera system (Eastman Kodak, Rochester, NY, USA) according to the instructions of the manufacturer. The band intensity was measured by densitometry using the Quantity One software (Bio-Rad Lab, Hercules, CA). The protein levels were normalized with respect to that of β-actin or GAPDH (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). All experiments were repeated more than three times and representative results were shown.

2.4. Immunohistochemistry (IHC)

Immunohistochemistry was performed, as previously described [15]. The sections were incubated with primary antibodies against RhoGDI (1:50) for one hour at room temperature. The slides were reviewed by two or three pathologists blind to the study. To evaluate RhoGDI expression levels, immunostained slides were evaluated using a method described previously [15,17]. Scores representing the percentage of positive cells were as follows: 0% (absent), 1–5% (sporadic), 6–25% (local), 26–50% (occasional), 51–75% (majority) and 76–100% (large majority). Intensity of

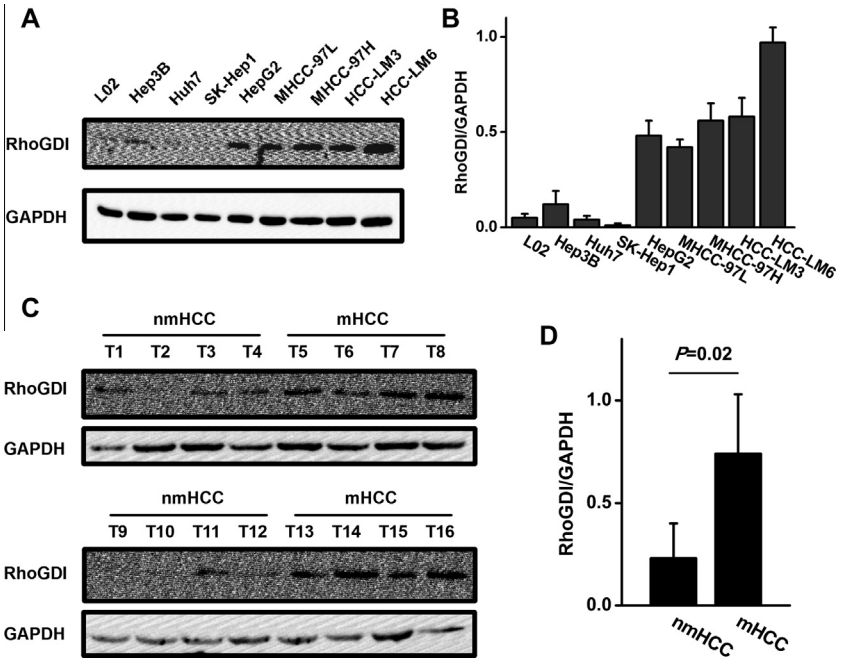

Fig. 1. Expression analysis of RhoGDI in HCC cells and tissues by Western blotting. (A) Expression of RhoGDI was detected in human liver immortal cell line L02 and HCC cell lines Hep3B, Huh7, SEK-Hep1, HepG2, MHCC-97L, MHCC-97H, HCC-L3 and HCC-L6 by Western blotting. (C) Expression of RhoGDI was detected in HCC tissues with or without metastasis. nmHCC, non-metastatic HCC. mHCC, metastatic HCC. (B and D) Immunosignals were quantified by densitometric scanning. RhoGDI expression in the individual cells (B) and tissues (D) was calculated as RhoGDI expression relative to GAPDH expression. Data are means ± SD from three independent experiments.

staining of cancer cells was scored as 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown), and 3 (strong staining, brown). An intensity score of ⩾2 with at least 50% of RhoGDI positive cells was considered as having high expression (or over-expression), and <50% of RhoGDI positive cells or <2 in intensity score was regarded as low expression. The discrepancies (<5%) were resolved by simultaneous re-evaluation.

2.5. Statistical analysis

All statistical analyses were carried out using the SPSS 12.0 statistical software package. Mann–Whitney U test was used to analyze the relationship between RhoGDI over-expression and clinicopathologic characteristics. Survival curves were plotted by the Kaplan–Meier method and compared by the log-rank test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. $P < 0.05$ in all cases was considered statistically significant.

3. Results

3.1. RhoGDI expression results analyzed by Western blot

The protein level of RhoGDI in HCC cell lines was detected by Western blot. As shown in Fig. 1, of the 16 total HCC tumorous tissues, an increase in LASP-1 expression was seen in HCC tissues of patients with metastasis compared with that without metastasis ($P = 0.02$). Compared with liver immortal cell line L02, LASP-1 expression was significantly increased in Hep3B, HepG2, MHCC-97L, MHCC-97H, HCC-LM3 and HCC-LM6 cells. Interestingly, a gradually increased trend of immunoreactivity was found in 4 cell lines (MHCC-97L, MHCC-97H, HCC-LM3 and HCC-LM6) derived from the same patient, along with the increase of metastatic potentials.

3.2. RhoGDI is over-expressed in HCC tissues

RhoGDI expression was detected in cytoplasm of liver cells. IHC was performed to localize RhoGDI expression in 58.5% (62 of 106) of adjacent non-tumorous tissues tested. As compared to these non-tumorous tissues, we observed RhoGDI protein expression in 68.3% (86 of 126) of all HCC samples ($P = 0.123$). According to reclassification as described above, RhoGDI was evaluated as high expression in 34.9% (44 of 126) of all HCC samples, compared with 2.8% (3 of 106) of adjacent non-tumorous tissues tested ($P < 0.001$; Fig. 2, Table 1).

Table 1
Correlation between the clinicopathological features and RhoGDI expression.

Characteristics	RhoGDI expression		
	Negative (%)	Positive (%)	*P* value
Group			
Non-tumor	103 (97.2)	3 (2.8)	<0.001*
Tumor	82 (65.1)	44 (34.9)	
Gender			
Male	76 (67.9)	36 (32.1)	0.064
Female	6 (42.9)	8 (57.1)	
Age			
<50	51 (72.9)	19 (27.1)	0.041*
⩾50	31 (55.4)	25 (44.6)	
Tumor size (cm in diameter)			
⩽5	27 (62.8)	16 (37.2)	0.698
>5	55 (66.3)	28 (33.7)	
Tumor differentiation			
Low	14 (82.4)	3 (17.6)	0.742
Middle	27 (57.4)	20 (42.6)	
High	41 (66.1)	21 (33.9)	
Liver cirrhosis			
No	38 (67.9)	18 (32.1)	0.559
Yes	44 (62.9)	26 (37.1)	
HBsAg status			
Negative	7 (43.8)	9 (56.2)	0.055
Positive	75 (68.2)	35 (31.8)	
Serum AFP			
Negative	20 (58.8)	14 (41.2)	0.371
Positive	62 (67.4)	30 (32.6)	
Local recurrence			
No	23 (29.5)	55 (70.5)	0.487
Yes	17 (35.4)	31 (64.6)	
Distant metastasis			
No	74 (71.8)	29 (28.2)	0.001*
Yes	8 (34.8)	15 (65.2)	

* Statistically significant ($P < 0.05$).

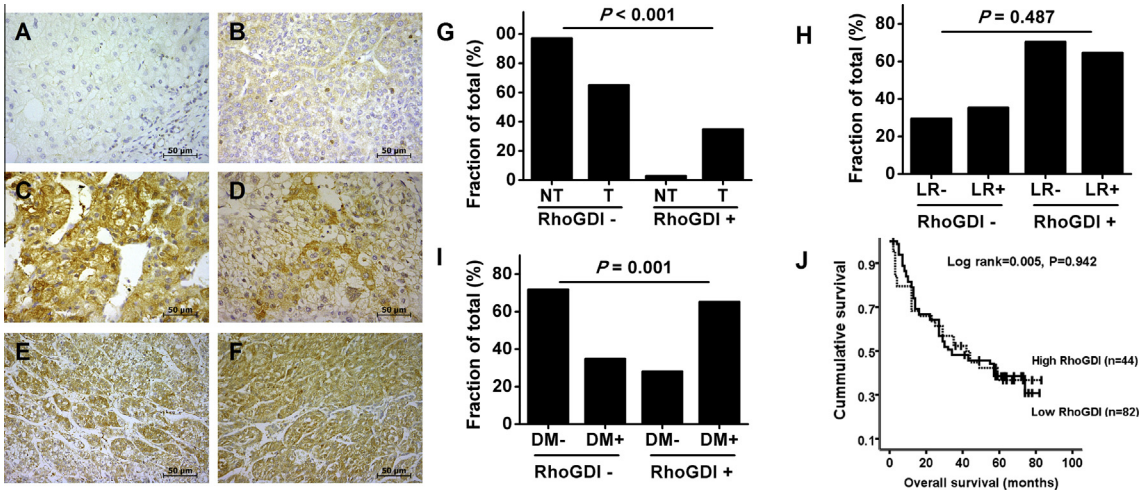

Fig. 2. Expression analysis of RhoGDI in HCC by immunohistochemistry. (A) Negative expression of RhoGDI in adjacent non-tumorous tissue. (B) Positive expression of RhoGDI in adjacent non-tumorous tissue. (C–F) High expression (or overexpression) of RhoGDI in HCC tissues. Scale bars were showed in the lower right corner of each picture. (G) Graphical illustration of statistical RhoGDI distribution. RhoGDI is significantly higher in HCC (T) than adjacent non-tumorous tissue (NT). (H) Distribution of high RhoGDI (RhoGDI+) and low RhoGDI (RhoGDI−) expression in HCC with or without local recurrence (LR− or LR+). (I) High expression of RhoGDI is more frequently in HCC with distant metastasis (DM+) than in HCC without distant metastasis (DM−). (J) Kaplan–Meier survival analysis of overall survival in patients with HCC according to RhoGDI overexpression. The log-rank test was used to calculate *P* values.

3.3. Correlation between RhoGDI over-expression and the clinicopathological features

To evaluate the clinical relevance of RhoGDI over-expression, RhoGDI protein was compared to clinicopathological and biological parameters. As shown in Table 1, there was no significant correlation between expression of RhoGDI protein and gender, tumor size, tumor differentiation, hepatitis B surface antigen (HBsAg), liver cirrhosis except age of HCC patients.

3.4. RhoGDI over-expression is closely related to postsurgical metastasis of patients with HCC

To investigate the predict value of RhoGDI over-expression, we performed a long-term follow-up study to understand local recurrence, distant relapse and overall survival of patients with HCC. Importantly, RhoGDI over-expression were associated with a significantly higher incidence of distant relapse ($P = 0.001$; Table 1), while no significant association with local recurrence was found ($P = 0.487$; Table 1). Further, no significant trend toward poorer survival for patients whose primary tumors showed high expression of RhoGDI, compared with those patients whose primary tumors showed low expression of RhoGDI (Log rank = 0.005, $P = 0.942$; Fig. 2J).

3.5. RhoGDI promotes the aggressive phenotype of HCC cells in vitro

We performed gene transfection and RNA interference to investigate the role of RhoGDI in biological behavior of HCC cells in vitro. Human RhoGDI cDNA was successfully transfected into HepG2 cells to establish a RhoGDI-overexpressing cell line. Inversely, siRNA transfection was employed to knock-down RhoGDI expression in HepG2 and MHCC-97H cells. The transfection efficiency was confirmed by Western blot analysis (Fig. 3A). The two most effective siRNA was used to down-regulate endogenous RhoGDI expression. A significantly slower proliferation rate was observed in RhoGDI siRNA-transfected HepG2 and MHCC-97H cells when compared with control cells (Fig. 3B). Cell migration analysis demonstrated that depletion of RhoGDI in cancer cells strongly reduced cell migration (Fig. 3C), suggesting that RhoGDI was

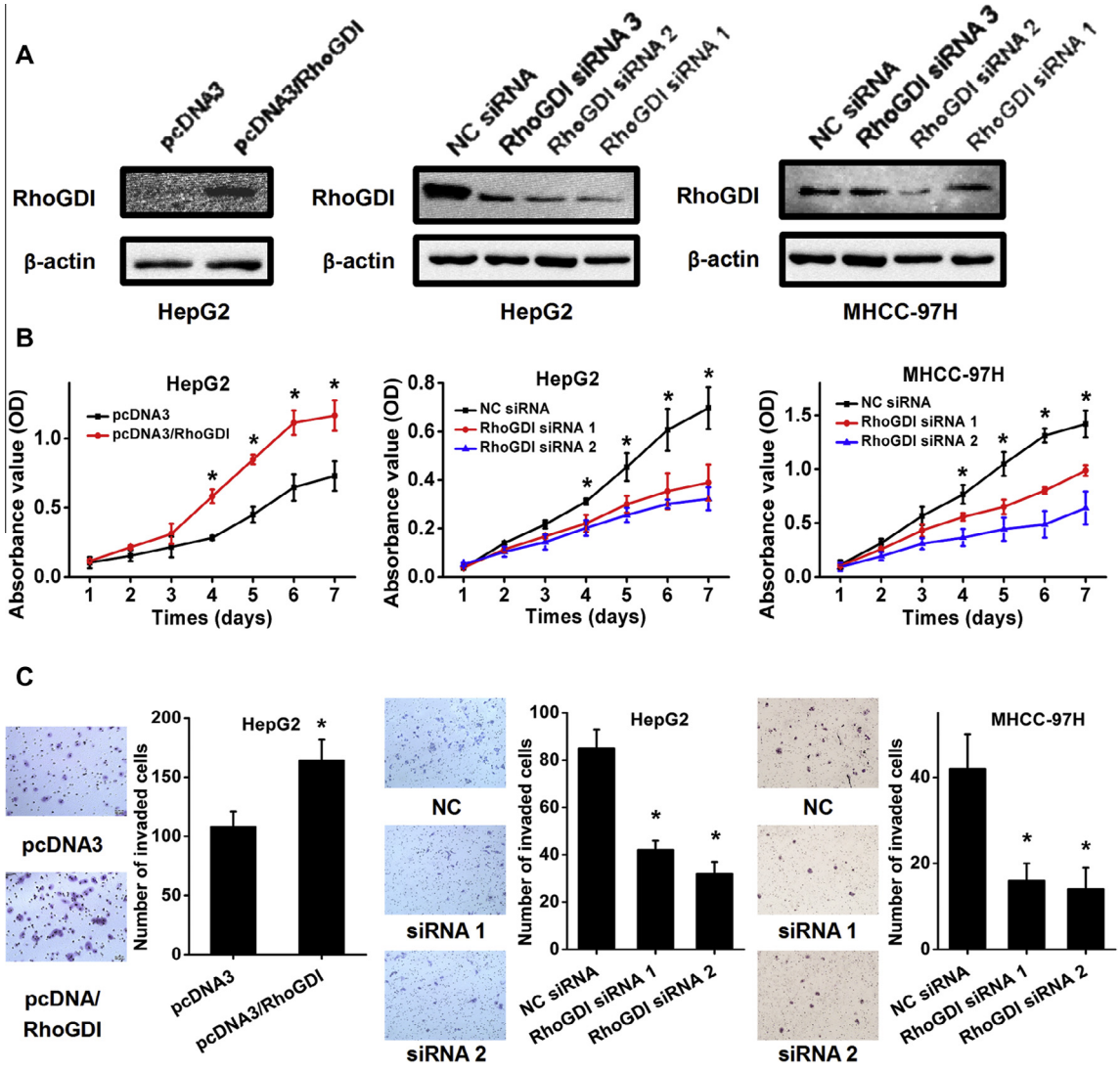

Fig. 3. RhoGDI promoted aggressive phenotypes of HCC cells. (A) Transfection efficiency of HepG2 and MHCC-97H cells. RhoGDI cDNA efficiently up-regulated RhoGDI protein expression compared to empty vector transfected HepG2 cells (Left panel). Three RhoGDI specific siRNA, especially siRNA 1 and 2, significantly down-regulated RhoGDI protein expression compared to negative control (NC) siRNA transfected HepG2 (middle panel) and MHCC-97H (right panel). (B) The ability of in vitro cell proliferation was detected by CCK-8 assay. (C) Migration potential was measured in transwell cell culture chamber. Cells were counted under high power field of microscopy. (B and C) Left panel, HepG2 cells transfected with RhoGDI cDNA. Middle panel, HepG2 cells transfected with RhoGDI siRNA 1 and 2. Right panel, MHCC-97L cells transfected with RhoGDI siRNA 1 and 2. Data represent mean ± SD of three different experiments.

necessary for cell migration. On the contrary, RhoGDI over-expression increased the proliferative ability of HepG2 cells. Migration analysis indicated that exogenous expression of RhoGDI in cancer cells cased a significant increase in cell motility.

3.6. RhoGDI regulated Rho expression, activated PI3K/Akt and MAPK pathways

To better understand the mechanisms that facilitate the aggressive behavior mediated RhoGDI, the expression levels of signal pathway. As shown in Fig. 4, RhoGDI may regulate expression of Rho, but not Rac/Cdc42, which are the members of Rho family small GTPases. RhoGDI overexpression significantly promoted the phosphorylation of p44/42 MAPK (ERK1/2) and AKT at Thr308 in HepG2 cells. Inversely, RhoGDI knockdown inhibited the phosphorylation of p44/42 MAPK (ERK1/2) and AKT at Thr308 in HepG2 and MHCC-97H cells.

4. Discussion

Rho family GTPases are essential in a variety of cellular functions, which were initially linked to changes in the filamentous actin system involving the formation of stress fibres, membrane ruffles/lamellipodia and filopodia respectively [18]. Now, they play roles in many aspects of cellular regulation including morphology and migration, gene transcription, cell cycle progression [19]. Rho GDIs could prevent nucleotide exchange and membrane association by extracting Rho family GTPases from membranes and solubilizing them in the cytosol. Moreover, they interact only with prenylated Rho proteins both in vitro and in vivo [20,21]. One important member of human Rho GDIs has been identified as RhoGDI (or GDIa/GDI1) [22,23].

Despite their critical cellular function, the role of RhoGDI in various kinds of cancers remains controversial. It is reported that the expression levels of RhoGDI have been up- or down-regulated in certain cancers [10–12,24–27] and in other pathological conditions [28,29]. A study based on proteomic analysis showed that RhoGDI was upregulated in oral squamous cell carcinoma [26]. Our previous study showed that RhoGDI was up-regulated in CRC [11], suggesting its important role in CRC genesis. However, a recent research showed that deregulation of RhoGDI is frequently down-regulated in HCC [12]. In the study based on cell lines and a larger number of clinical samples, however, a significantly high incidence rate of RhoGDI overexpression was found in HCC cells and samples compared with control groups tested.

Recent evidence has come to suggest that it may also act as a key regulator necessary for the progression of HCC. Our previous study has identified RhoGDI as an up-regulated protein in metastatic colorectal carcinoma [10]. The further data suggested that RhoGDI may promote tumor progression by stimulating tumor cell growth and migration [11]. Similarly, a comparative proteomic analysis revealed that RhoGDI was upregulated in oral squamous cell carcinoma and validated as an independent prognostic indictor for overall survival [26]. Recently, a proteomics-based study searched and identified RhoGDI as a metastasis-related protein in colon and prostate cancer [24]. In other research about prostate cancer, however, RhoGDI was identified as a tumor suppressor and inhibited the growth in vitro and tumorigenic ability of prostate tumor xenografts in vivo [25]. To sum up, the role of RhoGDI in cancer progression remains controversial. Recently, Ding et al. showed that RhoGDI functions as a metastasis suppressor and can block miR-151-induced HCC cell migration and invasion, suggesting its role in HCC progression [12]. Unfortunately, the research was involved in a small-samples analysis with incomplete clinicopathological parameters. To address this question, we analyzed RhoGDI protein expression in 147 clinicopathologically characterized HCC cases by immunohistochemistry. The statistical evaluation showed RhoGDI overexpression was closely associated with distant relapse of patients with HCC, suggesting its potential role in HCC metastasis. Further, our unvariate and multivariate survival analysis still cannot support RhoGDI overexpression as an independent predictor of HCC patients.

Until now, no functional evidence of RhoGDI has been documented. Our previous data suggested that RhoGDI may promote CRC progression by stimulating tumor cell growth and migration [11]. A recent research revealed that miR-151 is a crucial stimulus for HCC invasion and metastasis by directly targeting RhoGDI [12]. In the present study, RhoGDI overexpression promoted cell proliferation and migration in vitro. RhoGDI knockdown inhibited aggressive phenotype of HCC cells, suggesting its role in tumor

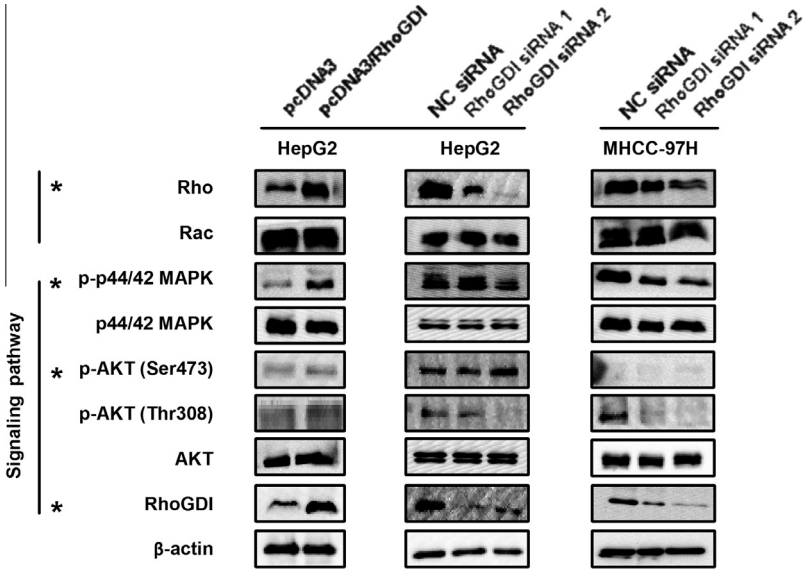

Fig. 4. RhoGDI up-regulated Rho expression and activated MAPK and PI3K/AKT pathway. Western blot analysis was used to detect expression of Rho and Rac, the phosphorylated level of p44/42 MAPK (ERK1/2) and AKT. Left panel, HepG2 cells transfected with RhoGDI cDNA. Middle panel, HepG2 cells transfected with RhoGDI siRNA 1 and 2. Right panel, MHCC-97L cells transfected with RhoGDI siRNA 1 and 2. The asterisk (∗) indicates $P < .05$. The representative results were shown from three independent experiments.

progression. To combine in vitro study with the result of immunohistochemistry, we proposed RhoGDI overexpression as a novel biomarker to predict postoperative distant metastasis of patients with HCC.

Despite its critical cellular function little is known about molecular mechanism of RhoGDI in HCC progression. As key regulators of Rho family GTPases, here, we showed RhoGDI significantly upregulated Rho, but not Rac/Cdc42, which are the members of Rho family small GTPases. A large number of researches have demonstrated that Rho family GTPases play important roles in a variety of cellular functions, such as cell migration and reorganization of the filamentous actin system [18,19]. Further studies revealed that PI3K/AKT and MAPK signal transduction cascades were activated by RhoGDI because of increased phosphorylation of ERK and AKT. This helps to provide evidence for diverse molecular mechanism by which RhoGDI promote cell growth and proliferation.

5. Conclusion

Our study evaluated clinical significance of RhoGDI and its predictive value for distant metastasis in a large number of HCC clinical tissue specimens at protein level by immunohistochemical analysis. Further in vitro study was performed to achieve a better understanding of its functions in HCC progression. The most valuable finding of this study is that distant metastasis of our study cohort was significantly more frequently in high RhoGDI expression cases that in low RhoGDI expression cases. It indicates that RhoGDI overexpression is a new predictor for postoperative distant metastasis of HCC patients. Therefore, its clinical value lies in that closer monitoring and more aggressive treatment should be indicated for the HCC patients with RhoGDI overexpression. This may be a useful way to reduce mortality and prolong the survival time as much as possible. Modulation of the tumor aggressive effect through inhibiting PI3K/AKT or MAPK activation mediated by RhoGDI overexpression might be used as a potential target for HCC prevention and therapy.

Statement of author contributions

Conceived and designed the experiments: L.Z. Performed the experiments: H.W., H.Y.A., B.W., Q.L. Analyzed the data: L.Z. Contributed reagents/materials/analysis tools: W.D.L., X.J.J., S.Z.C. Wrote the paper: L.Z.

Competing interests

The authors have declared that no competing interests exist.

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