Knockdown of RHOC by shRNA suppresses invasion and migration of cholangiocellular carcinoma cells via inhibition of MMP2, MMP3, MMP9 and epithelial-mesenchymal transition

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Abstract. Ras homolog family member C (RHOC) is important during the progression of several types of cancer, including prostate, breast and hepatocellular carcinoma. However, the function of RHOC in cholangiocellular carcinoma (CCC), a highly recurrent and metastatic carcinoma with poor prognosis, remains unclear. The aim of the present study was to investigate the involvement of RHOC in CCC tumor progression. RHOC expression levels were examined in CCC tissues and cells, and adjacent nontumorous bile duct tissues. The effects and molecular mechanisms of RHOC expression on cell migration and invasion were also investigated. The current study demonstrated that RHOC protein was frequently overexpressed in human CCC specimens and CCC cell lines. Downregulation of RHOC inhibited CCC cell invasion and migration partially via inhibition of matrix metalloproteinase 2, 3 and 9 expression. RHOC also modulated the expression of several epithelial-mesenchymal transition (EMT)-associated proteins, including E-cadherin, vimentin, Slug and Snail, to promote to EMT progression. The present results demonstrated that RHOC is important for the invasion and migration of CCC through simultaneous regulation of MMPs and EMT-associated protein, suggesting that RHOC is a potential molecular target for CCC treatment.

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Introduction

Cholangiocellular carcinoma (CCC) is a relatively rare malignant tumor of the bile duct epithelium. In Europe, approximately 10,000 new cases of CCC are diagnosed every year (1). CCC is classified into intrahepatic, perihilar and distal cholangiocarcinoma according to anatomical location. Currently, complete surgical resection is the only means for cure in patients with CCC at the early stage (2). However, most patients are initially diagnosed at the advanced stage, losing the opportunity of radical surgical resection (3). Even following surgery, the 5-year recurrence rate is in the range of 60-90% (4). The overall 5 year survival rate of patients with CCC is <5% and median survival time is ~12-30 months (5). Rapid invasion and metastatic capabilities of CCC contribute to the poor prognosis and resistance to the clinical therapeutic strategies (6). Thus, it is necessary to understand the precise mechanisms of this process to elucidate novel therapeutic modalities and improve the prognosis of patients with CCC.

Ras homolog family member C (RHOC) is a member of the ras superfamily of GTP-binding proteins, which act as molecular switches between active GTP-bound and inactive GDP-bound states (7). The family of RHO genes, which are important for cell proliferation and motility, have previously been implicated in tumorigenesis and metastatic progression (8). The RHO subfamily includes RHOA, RHOB and RHOC, which share 85% amino acid sequence identity (9). Despite this similarity, each protein has differing affinities for various downstream effectors and demonstrate different subcellular localization, suggesting that they have distinct functions in normal cellular activities and during tumor pathogenesis (10). Overexpression of RHOA has previously been reported to promote the invasiveness of tumor cells in several types of malignancy (11). By contrast, RHOB was

previously reported as a suppressor or negative modifier of cancer progression (12).

A previous study demonstrated RHOC to be correlated with the metastasis of various types of tumor (13). Other studies have demonstrated that RHOC expression is associated with aggressive phenotypes in a human cholangiocarcinoma cell line (14,15). However, the precise molecular mechanisms involved remain unclear. Thus, the current study aimed to investigate the pathological function of RHOC and the potential molecular mechanisms associated with cholangiocarcinoma.

Materials and methods

Patients and clinicopathological data. Clinical and pathological data were collected from 24 patients that underwent surgical resection of pathologically confirmed CCC between March 10, 2011 and May 15, 2014 at the First Affiliated Hospital of Henan Science and Technology University (Luoyang, China). Demographic data and pathological results were collected for each patient. The study was approved by Ethics Committee of the First Affiliated Hospital of Henan Science and Technology University in March 2011. All patients provided signed informed consent. Additionally, 24 samples of adjacent nontumorous bile duct tissues (NBD) were obtained as controls. All fresh samples were obtained from surgical resection and immediately preserved in liquid nitrogen. Clinicopathological staging was determined by the TNM classification of the 7th edition American Joint Committee on Cancer (16).

Cells lines and cell culture. RBE and HCCC-9810 human cholangiocarcinoma cell lines were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China) and Wuhan Boster Biological Technology, Ltd. (Wuhan, China), respectively. QBC939 and SK-ChA-1 human cholangiocarcinoma cell lines were kindly provided by Dr. Chundong Yu (Xiamen University, Xiamen, China). All cell lines were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 units/ml) purchased from Hyclone; Thermo Fisher Scientific, Inc. (Logan, UT, USA) at 37˚C in 5% CO_2 atmosphere.

Lentivirus vector and cell transfection. Lentiviral-mediated RHOC short hairpin RNA (shRNA) and negative control shRNA were packaged and produced by Shanghai GenePharma Co., Ltd. (Shanghai, China). The RHOC shRNA target sequence (NM_175744, NCBI GenBank accession number) was cloned into the pGLV-3/H1/GFP + puro lentiviral vector (Shanghai GenePharma Co., Ltd.), which specifically expresses RHOC shRNA. The RHOC shRNA target sequence was as follows: 5'-GATCCCGCTATATTGCGG ACATTGAGTTCAAGAGACTCAATGTCCGCAATATAG TTTTTTGGAAA-3', as described by Wu *et al* (17). The shRNA nontarget sequence, 5'-TTCTCCGAACGTGTC ACGT-3' was also cloned into a pGLV-3/H1/GFP + puro lentiviral vector as a negative control. The recombinant lentivirus RHOC shRNA (Lv-shRHOC) and control shRNA (Lv-shCTRL) were packaged in 293T cells (Shanghai GenePharma Co., Ltd.) using a lentivector expression system. DNA sequencing results revealed that the shRNA interference sequence targeting the RHOC gene was successfully inserted into the recombinant lentivirus. For cell infection, 30-50% confluent RBE and HCCC-9810 negative control shRNA and RHOC shRNA cell lines were incubated with lentivirus for 72-96 h. Untransfected controls were used in preliminary shRNA experiments, and demonstrated no significant difference compared with the negative control shRNA group. Thus, negative control shRNA was used to represent normal controls in the subsequent experiments The expression of green fluorescent protein (GFP) was examined under an Eclipse Ti fluorescence microscope (Nikon Corporation, Tokyo, Japan), and the intensity of green fluorescence indicated the transduction efficiency. The CCC cells were transfected with high titres of Lv-shRHOC and Lv-shCTRL particles ($2x10^8$ TU/ml; MOI of 30 transfection concentration) according to the instructions of the lentivirus manufacturer. Stable knockdown of RHOC and negative control shRNA transfectants were obtained by continuous treatment with 2 μg/ml puromycin (Shanghai GenePharma Co., Ltd.) in transfected RBE and HCCC-9810 cell lines.

Western blotting assay. Proteins were extracted from the cells using radioimmunoprecipitation assay lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Lysates were centrifuged at 4˚C at 4,000 x g for 20 min, and the supernatants were collected. Protein concentration was quantified using a Bradford assay (Beyotime Institute of Biotechnology, Haimen, China). Lysates (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (Beyotime Institute of Biotechnology) electrophoresis and transferred onto nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked by 5% non-fat milk in Tris-buffered saline Tween 20 (TBST) then incubated with primary antibodies at 4-8˚C overnight. The primary antibodies used were monoclonal mouse anti-RHOC (1:300 dilution; cat. no. AT3636a; Abgent, Inc., San Diego, CA, USA), monoclonal mouse anti-MMP2, MMP3 and MMP9 (all 1:1,000 dilution; cat. nos. ab-86607, ab-17790 and ab-58803, respectively; Abcam, Cambridge, MA, USA), monoclonal mouse anti-MMP14 (1:2,000 dilution; cat. no. ab-78738; Abcam), polyclonal rabbit anti-E-cadherin, Vimentin, Snail and Slug (all 1:500 dilution; cat. nos. WL-01482, WL-01960, WL-01863 and WL-01508, respectively; Wanleibio Co., Ltd., Shenyang, China) and monoclonal mouse anti-β-actin (1:5,000 dilution; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were washed with TBST 3 times for 10 min then incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. sc-2004) and goat anti-mouse (cat. no. sc-2005) secondary antibodies (1:3,000 dilution; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein levels were determined by normalizing to β-actin. The proteins were visualized by enhanced chemiluminescence (Advansta, Inc., Menlo Park, CA, USA) and detected using Bio-Rad ChemiDoc MP imaging system (ImageLab 4.1 software; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were performed in triplicate.

Table I. Clinicopathological information of cholangiocellular carcinoma cases studied and RHOC expression in cholangiocellular carcinoma tissue.

Case no.	Gender	Age years	Type	Stage[b]	Differentiation	RHOC expression (Fold change[a])
1	Male	65	Intrahepatic	T2aNxMx	NA	1.81
2	Female	45	Extrahepatic	T3N1M0	NA	1.93
3	Male	47	Intrahepatic	T1N0M0	NA	1.02
4	Male	34	Extrahepatic	T3N2M0	NA	6.21
5	Male	29	Intrahepatic	T4N2M0	NA	9.45
6	Female	67	Extrahepatic	T3N0M0	Well	0.67
7	Male	71	Extrahepatic	NA	Poor	3.15
8	Female	58	Intrahepatic	T2aNxMx	Poor	5.17
9	Male	49	Extrahepatic	T2bN0M0	Well	1.10
10	Male	44	Intrahepatic	T3N1M0	Moderately	4.23
11	Male	73	Extrahepatic	T1N0M0	Well	0.77
12	Female	54	Extrahepatic	T2aN2M0	Moderately	4.89
13	Male	65	Extrahepatic	T1N1M0	Moderately	3.78
14	Male	41	Intrahepatic	TxN1M0	Poor	2.56
15	Male	38	Intrahepatic	T1N1M0	Moderately	2.66
16	Female	44	Extrahepatic	NA	Poor	5.66
17	Male	57	Extrahepatic	T2bN0M0	Well	0.57
18	Female	55	Extrahepatic	NA	Moderately	3.40
19	Male	63	Extrahepatic	T2bN2M0	Moderately	7.34
20	Female	68	Intrahepatic	T3N1M1	Moderately	2.91
21	Male	36	Extrahepatic	T2bN1M0	Moderately	3.35
22	Female	47	Intrahepatic	T4N2M0	Poor	6.23
23	Male	36	Extrahepatic	T2aN1M0	Well	1.88
24	Female	42	Extrahepatic	T1N0M0	Moderately	1.67

[a]Fold change indicates ratios of the relative RHOC levels in tumor tissues compared with NBD specimens. A >2-fold increase was considered as RHOC upregulation. The result of paired t-test demonstrated that RHOC protein was overexpressed in tumor tissues compared with NBD (t=7.199, P<0.001). [b]Stages were classified according to the American Joint Committee on Cancer TNM system. RHOC, ras homolog family member C; NBD, nontumorous bile duct; NA, not available.

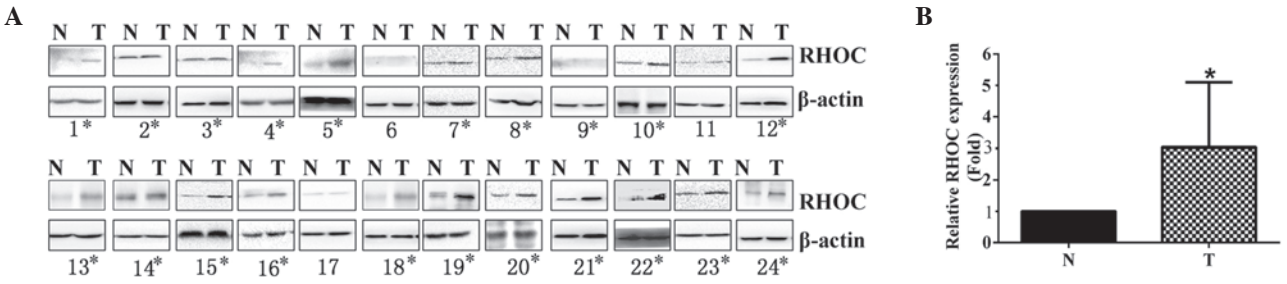

Figure 1. Overexpressed RHOC in human CCC tissues. (A) Expression of RHOC protein in human CCC specimens and adjacent N tissues. *P<0.05 indicates significant overexpression of RHOC in CCC specimens. Western blot analysis was repeated in triplicate. (B) The protein levels of RHOC were significantly higher in CCC specimens compared with N specimens. Values are presented as the mean ± standard deviation. *P=0.000 vs. N. RHOC, ras homolog family member C; CCC, cholangiocellular carcinoma; N, nontumorous bile duct; T, tumor.

In vitro invasion assay. For the invasion assay, $3x10^4$ cells were added to the cell culture inserts with microporous membrane and Matrigel coating (BD Biosciences, Franklin Lakes, NJ, USA). Medium containing 10% FBS was added to the bottom chamber. The cells were then incubated for 48 h at 37˚C and the upper chamber was removed. The cells on the bottom surface of the upper chambers fixed in 95% ethanol (Sangon Biotech Co., Ltd., Shanghai, China) for 15 min and stained with 0.1 mg/ml crystal violet solution and the number of cells was counted under a Eclipse Ti microscope (magnification, x200). Individual experiments had triplicate inserts and five randomly selected fields were counted per insert.

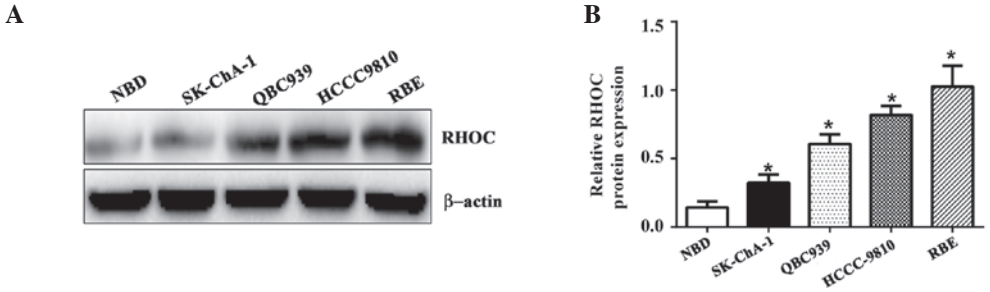

Figure 2. RHOC is overexpressed in human CCC cell lines. (A) Expression of RHOC protein in human CCC cell lines (SK-ChA-1, QBC939, HCCC9810 and RBE) and adjacent NBD samples. (B) Compared with NBD epithelium, RHOC expression was significantly increased in the 4 CCC cell lines. *P<0.01 vs. NBD. Values are presented as the mean ± standard deviation. RHOC, ras homolog family member C; CCC, cholangiocellular carcinoma; NBD, nontumorous bile duct.

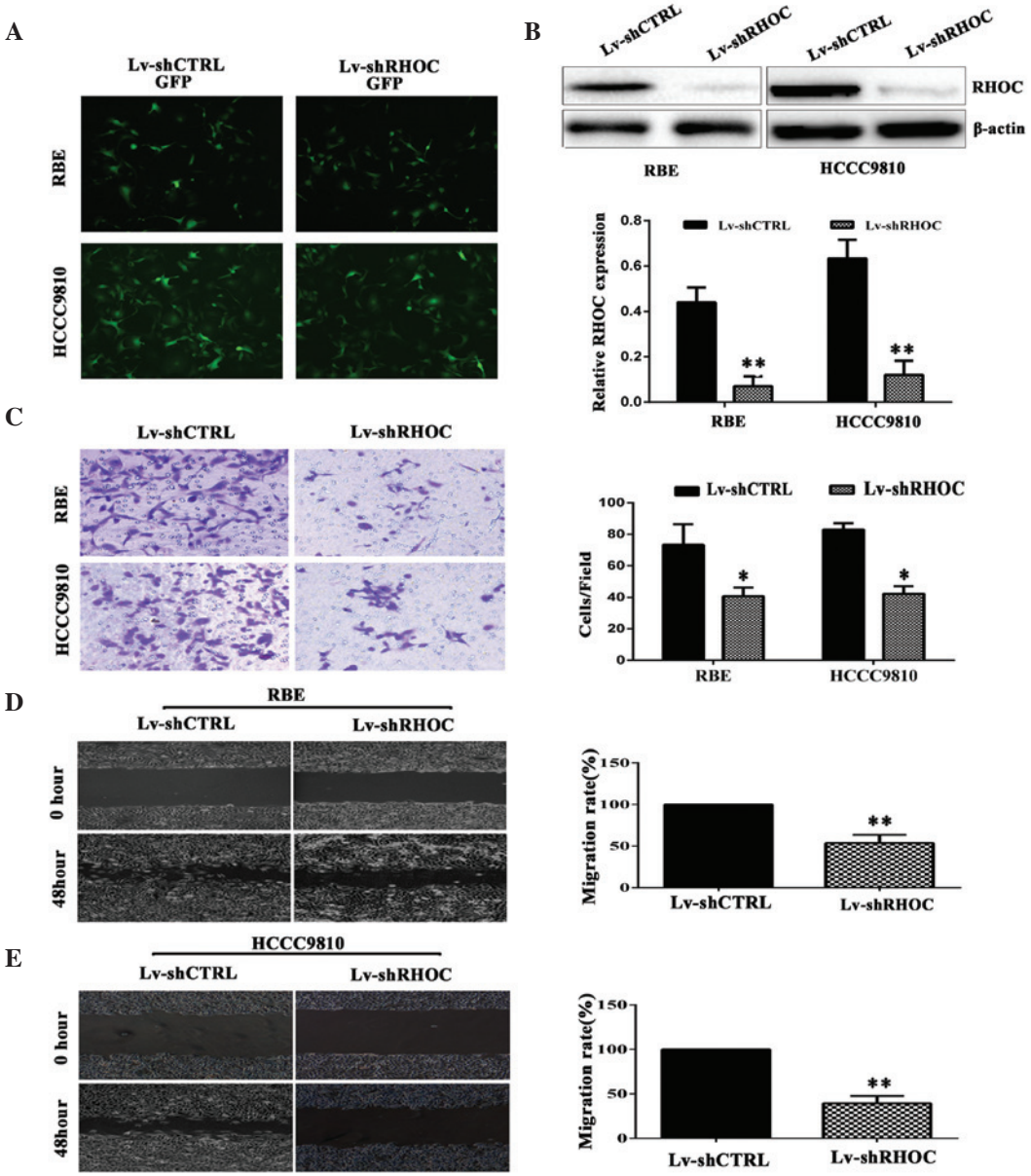

Figure 3. Effect of RHOC knockdown on RBE and HCCC-9810 cell invasion and migration. (A) Lentiviral transduction efficiency. Fluorescence microscopy (magnification, x100) demonstrated >95% RBE and HCCC-9810 cells were effectively transfected with Lv-shRHOC and Lv-shCTRL vector 4 days after transfection at a multiplicity of infection of 30. (B) Significantly decreased RHOC protein expression following lentiviral shRNA stable transfection. (C) Cell invasion capacity of transfected RBE and HCCC-9810 cells by a Transwell assay and quantification of the number of invaded RBE and HCCC-9810 cells in each group. Scratch assays showed that RHOC shRNA knockdown (D) RBE and (E) HCCC9810 cells had reduced motility compared with control cells. Representative images are presented and quantification of mean migration rates of RBE and HCCC-9810 cells in each group. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. Lv-shCTRL. RHOC, Ras homolog family member C; Lv, lentivirus; Lv-shRHOC, RHOC small hairpin RNA interference group; Lv-shCTRL, nontarget shRNA interference group; GFP, green fluorescent protein.

Scratch assay. For wound-healing assays, RBE and HCCC9810 cells were plated at $2x10^6$ cells/dish density in 60 mm-diameter dishes. Wounds were created in the confluent cells using a 200 μl pipette tip after cells had reached confluency. The cells were then rinsed with medium to remove free-floating cells and debris. Medium with 1% FBS was added and the culture plates were incubated at 37˚C for 48 h. Different stages of wound healing were observed along the scrape line and representative scrape lines were imaged with an Eclipse Ti microscope. Wound closure was measured using AxioVision software version 4.7 (Zeiss GmbH Jena, Germany). Quantification was performed by measuring the uncovered areas compared with the controls. Each experiment was repeated in triplicate.

Statistical analysis. All data were analyzed for statistical significance using SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation. The protein expression levels were measured using densitometry with ImageLab 4.1 software. Two-tailed Student's t-test was used for comparisons of two independent groups, and analysis of variance followed by Dunnett's test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

RHOC is highly expressed in human CCC tissues and cell lines. To evaluate the expression of RHOC in CCC, western blotting was performed to assess the levels of RHOC protein in 24 tumor and 24 adjacent NBD tissues. As presented in Fig. 1 and Table I, the levels of RHOC protein were significantly upregulated in 21 CCC specimens, including 12 extrahepatic CCC and 9 intrahepatic CCC samples, compared with NBD specimens (P=0.000). Additionally, RHOC expression was significantly increased in CCC cell lines, including HCCC9810, QBC939, RBE and SK-ChA-1, compared with NBD epithelium (Fig. 2; P=0.006). Thus, the overexpression of RHOC in CCC specimens and CCC cell lines suggests that RHOC may be important for the tumorigenesis of CCC.

RHOC protein expression is inhibited by lentiviral-mediated shRNA interference in RBE and HCCC-9810 cell lines. Analysis of western blotting results demonstrated that the relative protein expression levels of RHOC were 1-3-fold higher in RBE and HCCC-9810 cells compared with QBC939 and SK-ChA-1 cell lines (Fig. 2). Thus, the RBE and HCCC-9810 cell lines were selected for use in knockdown experiments. The cells were transfected with Lv-shRHOC and Lv-shCTRL lentiviral vectors. The transfection efficiency of >90% was determined by detecting the expression of GFP 96 h after infection (Fig. 3A). Western blotting indicated RHOC was significantly downregulated in RBE and HCCC-9810 cells transfected with Lv-shRHOC vector compared with Lv-shCTRL. In comparison with Lv-shCTRL, the protein levels of RHOC were decreased by 83.73±9.09% and 81.2±8.53% in Lv-shRHOC-transfected RBE and HCCC-9810 cells, respectively (P<0.003, Fig. 3B).

RHOC silencing impairs CCC cell invasion and migration in vitro. The results of the Matrigel invasion assay indicated that inhibition of RHOC reduced the invasive ability of RBE and HCCC-9810 cells compared with Lv-shCTRL-transfected cells (P<0.016; Fig. 3C). The effects of RHOC on the migration of CCC cell lines were assessed by scratch assay. The results demonstrated that compared with Lv-shCTRL-transfected cells, RHOC knockdown significantly reduced cell migration in RBE and HCCC-9810 cells (P<0.003; Fig. 3D and E). Collectively, these findings provided evidence that elevated RHOC expression levels were involved in promoting the migratory and invasive phenotype of CCC cells.

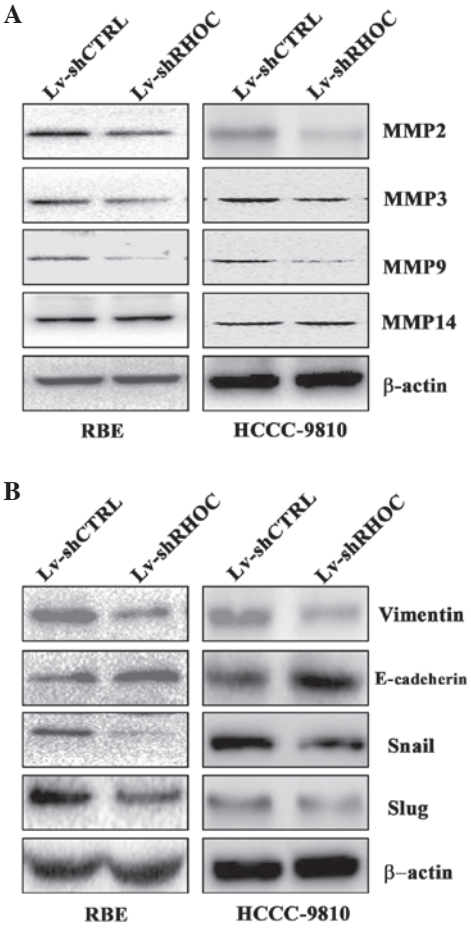

Figure 4. Expression levels of MMPs and epithelial mesenchymal transition-associated genes in RBE and HCCC-9810 cells following knockdown of RHOC. (A) The relative protein levels of MMP2 and 9 were decreased, and the expression levels of MMP3 and 9 were unchanged in Lv-shRHOC transfected RBE and HCCC-9810 cells compared with control. (B) Protein expression levels of vimentin, Snail and Slug were reduced, and E-cadherin increased in RBE and HCCC-9810 cells compared with control. MMP, matrix metalloproteinase; RHOC, ras homolog family member C; Lv, lentivirus; Lv-shRHOC, RHOC small hairpin RNA interference group; Lv-shCTRL, nontarget shRNA interference group.

Effects of RHOC knockdown on the expression of tumor invasion-associated molecules. As the invasive ability of tumor cells is often correlated with the production of secretory proteases, the effect of RHOC knockdown on the expression of tumor invasion-associated molecules was determined. As demonstrated in Fig. 4A, western blotting analysis indicated that downregulation of RHOC reduced the protein expression level of MMP2, 3 and 9, whereas, no change was observed in the expression level of MMP14 (Fig. 4A).

Effects of RHOC knockdown on the expression of epithelial mesenchymal transition (EMT)-associated genes in CCC cell lines. To further investigate the mechanism by which RHOC regulates cell invasion and migration, the protein expression levels of EMT-associated genes in CCC cells was analyzed. It was demonstrated that RHOC knockdown decreased the expression of the mesenchymal marker, vimentin, and the EMT central regulators, Snail and Slug, compared with Lv-shCTRL-transfected cells. In contrast to Lv-shCTRL-transfected cells, the expression of epithelial marker E-cadherin was upregulated in CCC cell lines following RHOC knockdown (Fig. 4B). Thus, the results of the current study demonstrated that knockdown of RHOC altered the expression levels of MMP2, 3 and 9, and EMT-associated genes, therefore, suppressing the invasive and migratory phenotype of CCC cells.

Discussion

CCC is a highly metastatic disease characterized by invasive growth along the lymphangion or perineurium, or direct invasion into the liver (18). Thus, intensive studies for potential candidate molecules involved in the metastatic process are urgently required to provide effective treatments for patients with advanced CCC.

RHOC has been shown to promote cancer metastasis in a variety of tumor types, including breast cancer (19), non-small cell lung carcinoma (20), colon carcinoma (21), malignant melanoma (22), hepatocellular carcinoma (23), head and neck cancer (24) and prostate cancer (25). However, the function of RHOC in human CCC remains unclear. The current study demonstrated that the expression levels of RHOC were significantly increased in CCC tissues and cell lines compared with normal biliary epithelium, suggesting that RHOC may be involved in the progression of CCC. Additionally, the present study specifically and efficiently inhibited RHOC gene expression in CCC cell lines by lentiviral-mediated shRNA interference, and the results demonstrated that the invasion and migration capacities of transfected RBE and HCCC9810 cell lines were significantly inhibited by RHOC knockdown *in vitro*. These results collectively suggested that RHOC may be important in CCC progression.

To date, the molecular mechanisms by which RHOC promote tumor development and metastasis are not fully understood (26). Previous studies have demonstrated that RHOC promote cancer progression by regulating the expression of MMP genes (14,23,27). It is well established that MMPs induce cancer cell invasion and metastatic spread by degrading the extracellular matrix and other barriers (28). In hepatocellular carcinoma, Liao *et al* (29) reported that hepatocellular carcinoma cell invasion and migration are modulated by the genes RHOC, MMP-2 and MMP-9 (29). Wang *et al* (14) also demonstrated that RHOC promotes the malignant progression of CCC cells via regulation of MMP expression levels (14). The present study demonstrated that RHOC promotes CCC cell invasion partially via inducing the expression of MMP2, 3 and 9, but not that of MMP14, which is consistent with previous observations in other types of tumor (27,30,31). Further studies are required to determine the precise molecular mechanisms of the invasion process in CCC.

Emerging evidence has established that EMT is an important event during carcinoma progression (32). EMT promotes carcinoma progression by increasing migratory and invasive properties, and cancer stem cell-like phenotype of cells, which may be prerequisites for cancer cell metastasis (32). Thus, understanding the regulatory mechanisms of EMT may provide greater insight into the signaling programs that control CCC metastasis. EMT is characterized by decreased expression of epithelial proteins, such as E-cadherin, and increased expression of mesenchymal proteins, such as vimentin (32). Additionally, Snail and Slug are crucial for the transcriptional regulation of EMT (33,34). Reportedly, either upregulation or increased activity of RHOC promotes the invasive potential of cancer cells, which is closely associated with EMT (35). Bellovin *et al* (21) reported that RHOC expression and activation are induced by EMT and RHOC promotes post-EMT cell migration. In ovarian carcinoma, a study by Gou *et al* (36) indicated that ectopic RHOC expression enhanced migration, invasion and altered the expression of EMT markers. Similarly, the current study in CCC demonstrated that RHOC knockdown markedly altered the expression of EMT-associated genes. These results consistently suggested that RHOC has an important function during the malignant progression of CCC by regulating MMPs and EMT.

In summary, the results of the present study indicate that the expression of RHOC is significantly increased in CCC cell lines and clinical samples. Furthermore, knockdown of RHOC expression significantly inhibited CCC cell migration and invasion *in vitro*, and regulated the expression of MMPs and EMT-associated genes. Thus, strategies interfering with RHOC expression may provide a novel and promising alternative approach for the treatment of aggressive CCC.

Acknowledgements

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