Int J Clin Exp Pathol 2018;11(1):224-231
www.ijcep.com /ISSN:1936-2625/IJCEP0066980

Original Article

Rab5a promotes the migration and invasion of hepatocellular carcinoma by up-regulating Cdc42

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Received October 11, 2017; Accepted November 17, 2017; Epub January 1, 2018; Published January 15, 2018

Abstract: There are many factors participating in the process of human hepatocellular carcinoma (HCC) occurrence and development. In this study, we found that Rab5a expression was higher in the HCC tissues and 3 cell lines than normal liver tissues. Overexpression of Rab5a promoted cell invasion and migration *in vitro* and *in vivo*. In contrary, inhibition of Rab5a suppressed cell invasion and migration. Mechanistic studies revealed that Rab5a was positively regulated cell division cycle 42 (Cdc42) expression by enhancing its promoter activity. These data suggested that Rab5a is highly expressed in HCC, and promote the invasion and migration by targeting Cdc42 expression.

Keywords: HCC, Rab5a, invasion, migration, Cdc42

Introduction

HCC is the most common tumor and the third primary cause of cancer-related deaths worldwide [1]. It is highly prevalent in Eastern and South-Eastern Asia, with incidence rates of 31.9/100,000 and 22.2/100,000 respectively [2]. Although there are many established therapeutic strategies including surgery, chemotherapy and radiotherapy, the 5-year overall survival has reached 30%-50%, the 5-year recurrent rate is still up to 70-85% due to recurrence and distant metastasis of HCC [3, 4]. To date, the precise mechanisms lead to the development and progression of HCC still unclear [5].

Rab5 belongs to Rab GTPases family, which contains three subtypes including Rab5a, Rab5b and Rab5c [6]. Rab5a is localized at human chromosome 3p24.3 and its molecular weight is 23.66 kDa [7]. Rab5a is abnormally expressed in a variety of tumors (such as breast cancer, lung cancer and ovarian cancer), which plays a vital role in the pathogenesis and distant metastasis [8-10]. However, there is rare report concerning the role Rab5a in HCC.

In this study, we examined the expression of Rab5a in HCC and determined the role of Rab5a in invasion and migration. The results showed that Rab5a may act as a tumor stimulator in HCC.

Materials and methods

Cell lines

All the cell lines (LO2, SMMC7721, HepG2, SK-Hep1) for this study were preserved in our lab, which were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China), supplemented with 10% fetal bovine serum (FBS, Cell-box), 100 units/mL penicillin and 100 μg/ml streptomycin. A humidified incubator with 5% CO_2 at 37°C was used for maintaining the cells.

RNA extraction and qPCR

Total RNAs were extracted with Trizol reagent (Invitrogen, Carlsbad). The RNA integrity was estimated by denatured agarose gel. The cDNA was acquired using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions (Takara). Quantitative real-time PCR (qPCR) was performed to confirm the expressions of Rab5a, Cdc42 and some other genes related to invasive function. All qPCR

Table 1. Primer sequences used for PCR or constructions of various plasmids

Amplifier primers	Primer sequence
Rab5a-F	TGCTCTAGACGCCACCATGGCTAGTCGAGGCGCAAC
Rab5a-R	TGCTCTAGATTAGTTACTACAACACTGATTC
Rab5a-F	ATACACTCTCATCCTACGGG
Rab5a-R	GAACTTCCAGAATTCAAGGG
β actin-F	CCTTCTACAATGAGCTGCGT
β actin-R	CCTGGATAGCAACGTACATG
si-Rab5a-F (661)	GCCAAUUUCAUGAAUUUCATT
si-Rab5a-R (661)	UGAAAUUCAUGAAAUUGGCTT
si-Rab5a-F (817)	CAGCCAUAGUUGUAUAUGATT
si-Rab5a-R (817)	UCAUAUACAACUAUGGCUGTT
si-Rab5a-F (980)	GUCCUAUGCAGAUGACAAUTT
si-Rab5a-R (980)	AUUGUCAUCUGCAUAGGACTT
si-NC-F	UUCCUUUUCCGUAUUCGCGUU
si-NC-R	CGCGAAUACGGAAAAGGAAUG
Cdc42-F	TGACAGATTACGACCGCTGA
Cdc42-R	TCTCAGGCACCCACTTTTCT
FOXC2-F	TCCTGGTATCTCAACCACAG
FOXC2-R	CGAGGGTCGAGTTCTCAAT
TP53-F	TGGCCATCTACAAGCAGTCA
TP53-R	GGTACAGTCAGAGCCAACCT
VEGF-F	TCCCGGTATAAGTCCTGGAG
VEGF-R	ACAAATGCTTTCTCCGCTCT
MMP2-F	GTGGATGATGCCTTTGCTC
MMP2-R	CAGGAGTCCGTCCTTACC
Cdc42-F	TGACAGATTACGACCGCTGA
Cdc42-R	TCTCAGGCACCCACTTTTCT
RhoA-F	CTTCACCAGCACCCTCTACA
RhoA-R	GCTTACACACCACCACGTAC
TIMP1-F	TACTTCCACAGGTCCCACAA
TIMP1-R	AGCTAAGCTCAGGCTGTTCC
WNT5A-F	TCAGGACCACATGCAGTACA
WNT5A-R	GCAGTTCCACCTTCGATGTC
Twist 1-F	CATCCTCACACCTCTGCATT
Twist 1-R	TTCCTTTCAGTGGCTGATTG
MMP9-F	GAGTTCCCGGAGTGAGTTGA
MMP9-R	AAAGGTGAGAAGAGAGGGCC
NDRG1-F	GAAGGAAGCAAGCATCTCCG
NDRG1-R	CACCCGTTTGAACCAGGATG
KLF4-F	TCTCCCACATGAAGCGACTT
KLF4-R	CGTTGAACTCCTCGGTCTCT
IDO1-F	TGCAAGAACGGGACACTTTG
IDO1-R	CCCTTCATACACCAGACCGT
ZEB1-F	GGCCTGAAATCCTCTCGAAT
ZEB1-R	GTCTTCATCCTCTTCCCTTGTC
Nanog-F	ACCCAGCTGTGTGTACTCAA
Nanog-R	GGAAGAGTAAAGGCTGGGGT
Sox2-F	CGGAAAACCAAGACGCTCAT
Sox2-R	TTCATGTGCGCGTAACTGTC

samples were executed by using UltraSYBR mixture (Cwbio, China) and conducted using the CFX Connet TM real-time PCR system (Bio-Rad). The quantification analysis was calculated by the $2^{-\Delta\Delta CT}$. All experiments were performed in triplicate and repeated at least 3 times.

Immunohistochemistry

The tissue sections of hepatocellular carcinoma patients were obtained from pathology Department of Chongqing Medical University. The human subject protocol was approved by the Clinical Research Ethics Committee of Chongqing Medical University. Written consent was obtained from each patient. The tissue of transplanted tumors and human liver cancer tissue were paraformaldehyde fixed and paraffin embedded. Then the slides were deparaffinized with dimethylbenzene, followed by gradient alcohol dehydration and analyzed for Rab5a (Bioword, 1:100 dilution) and Cdc42 (Bioword, 1:100 dilution) expression. Visualized with 3,3-diaminobenzidine substrate (ZSGB-Bio, DAB) and counterstained with hematoxylin of the slides. The PBS staining was used as the negative staining control. Mounted and visualized under an inverted microscope.

Western blot analysis

Cells were lysed with RIPA Lysis Buffer (Beyotime, China) including 1 mmol/L PMSF, and protein concentration was measured using the BCA Assay Kit (Beyotime). Protein samples were separated by 12% SDS-PAGE and transferred to a PVDF membrane. Blots in PVDF membranes were incubated with primary antibody, followed by anti-rabbit HRP secondary antibody (Proteintech, 1:4000) or anti-mouse HRP secondary antibody (Bioword, 1:3,000) for 2:30 h at room temperature, and the signals were detected with an ECL Detection Reagent (Millipore, Billerica, MA).

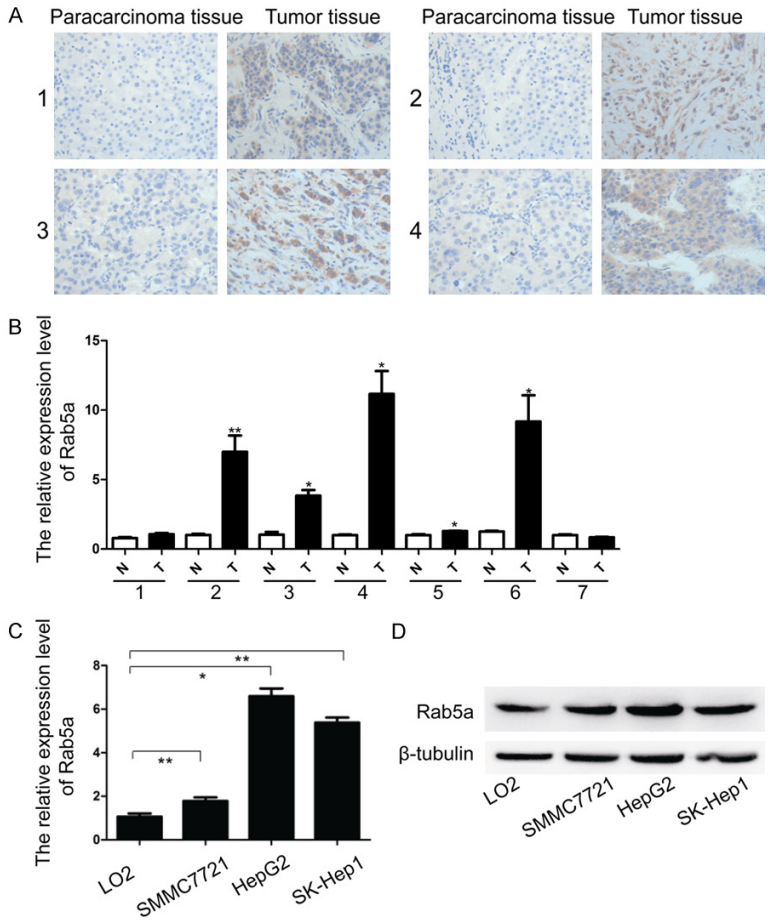

Figure 1. The expressions of Rab5a in HCC tissues and HCC cell lines. A, B. The expressions of Rab5a in HCC tissues by IHC and qRT-PCR, paracarcinoma tissue and N were used as control; C, D. Rab5a mRNA and protein expressions in LO2 and HCC cell lines by qRT-PCR and Western blot (SMMC7721, SK-Hep1 and HepG2). β-tubulin was used as the endogenous control. *P < 0.05, **P < 0.01.

Plasmids, small interfering RNA (siRNA) and transfection

The CDS section of Rab5a was amplified by PCR with primers (**Table 1**). The PCR product was purified and linked into the *Xho*I sites of pcDNA3.1 (-) expression vector. Fragment of si-Rab5a and negative control (si-NC) were purchased from TsingKe (**Table 1**). Transfections were performed with a Lipofectamine^{2000} kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Wound healing assays

Transfected cells were plated in 6-well plates and incubated 24 h. When cells grew in full monolayer on cover slips in six well plates and then created the wound with using a 200 μl pipette tip. PBS washed the cells gently and cells were replaced incubated in DMEM with 2% FBS. Then take photos at 0 h, 24 h and 48 h after wounding.

Transwell assays

After 24 h transfection, 1 × 10^5 cells suspending in 200 μl of serum-free DMEM were seeded to the 8 μm transwell migration chambers (Costar). 600 μl of DMEM media containing 10% FBS was added to the bottom chamber. After cultured 24 h at 37°C incubator, the membrane was cleaning out with PBS. Migrated cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 10 min. Then washed with distilled water and took photograph, counting the cells with using the inverted microscope.

Luciferase reporter assay

SK-Hep1 cells were cultured in a 24-well plate and co-transfected with 250 ng pcDNA3.1-Rab5a or pcDNA3.1 vector, 150 ng pGL3-Basic-Cdc42 or pGL3-Basic, and 25 ng pRL-TK. Cells were collected in 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega).

Recombinant lentiviral overexpression vectors

The Rab5a gene fragment was ligated into pCDH-CMV-MCS-EF1-copGFP Vector. Sequencing was performed in 293T cells (Human embryonic kidney stem cells) after packaging, virus solution was collected and virus titer detected. Then the virus was infected with the target SK-Hep1 cells. Recombinant lentiviral overexpression vectors in cells can be selected for GFP positive cells by FACS.

Tumor migration assay

Male nude mice (8 weeks old) were purchased from the Laboratory Animal Services Cen-

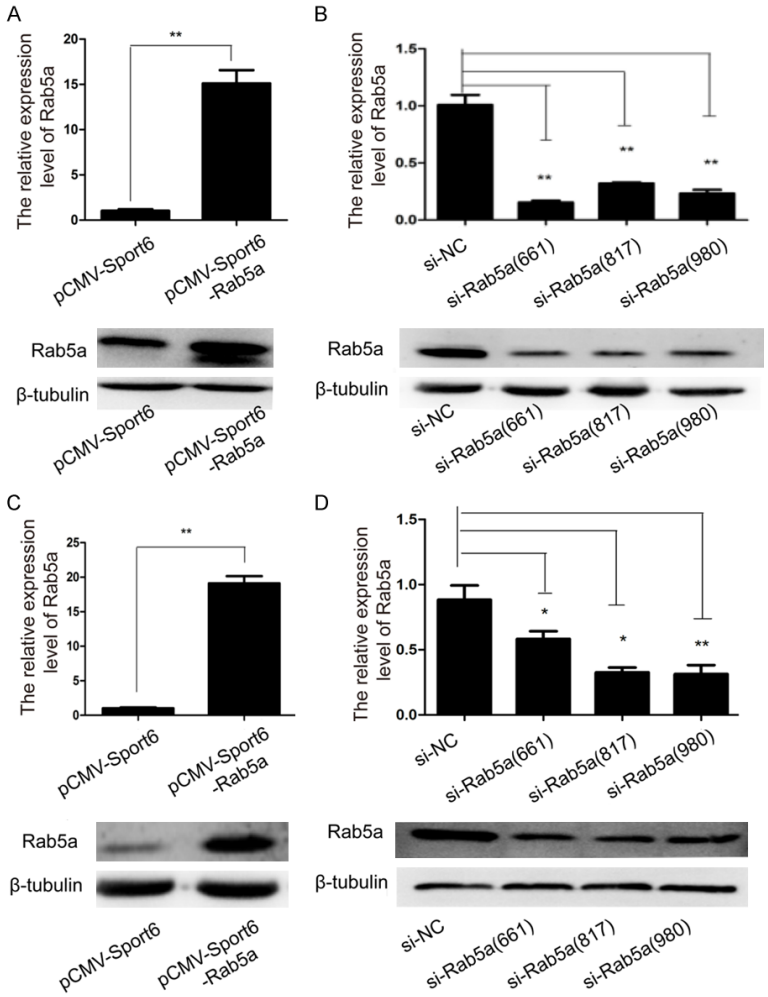

Figure 2. The efficiencies of over-expression and siRNA of Rab5a. A, B. The efficiencies of PCMV-Sport6-Rab5a and siRNA by qRT-PCR and WB in SK-Hep1 cells; C, D. The efficiencies of PCMV-Sport6-Rab5a and siRNA by qRT-PCR and WB in SMMC7721 cells. *P < 0.05, **P < 0.01.

ter of CQMU. Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of Chongqing Medical University. pCDH-CMV-copGFP and pCDH-CMV-copGFP-Rab5a with a total of 0.8 × 10^7 cells, respectively, were injected into the liver of nude mice, and sacrificed them at 30 days.

Statistical analysis

All results were expressed as the means ± sd of at least three independent experiments. Statistical analysis was performed by X^2 analysis and Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Up-regulation of Rab5a in HCC tissue and HCC cell lines

Firstly, we examined Rab5a expression level by both IHC and qRT-PCR in human HCC tissues and precancerous tissues. Compared with the paracarcinoma tissues, the expression of Rab5a was significantly up-expressed in human HCC tissues (**Figure 1A**, **1B**). Equally, qRT-PCR and western blotting were done in HCC cell lines to detect the expression of Rab5a. The results showed that Rab5a mRNA and protein levels were markedly up-regulated in 3 HCC cell lines (SMMC7721, SK-Hep1 and HepG2) compared with the hepatic immortal cell line L02 (**Figure 1C**, **1D**).

Over-expression and siRNA efficiency in SK-Hep1 and SMMC7721 cells

Due to the highly expression of Rab5a, we suppose that Rab5a maybe play a crucial role in the occurrence and development in HCC. Therefore, we constructed a plasmid expressing Rab5a (pCMV-Sport6-Rab5a) and synthesize interference fragments (si-Rab5a). Over-expression and siRNA efficiencies were measured by qRT-PCR and Western blot in SK-Hep1 and SMMC7721 cells. The results showed that pCMV-Sport6-Rab5a increased approximately 15 times in SK-Hep1 cells (**Figure 2A**) and 20 times in SMMC7721 cells (**Figure 2C**). In contrast, si-Rab5a resulted in an obvious inhibition of Rab5a in SK-Hep1 cells (**Figure 2B**) and SMMC7721 cells (**Figure 2D**), specially si-Rab5a (661). Then, we chose si-Rab5a (661) to undergo the following experiments.

Up-regulation of Rab5a promoted the invasion and migration in HCC cells

To clarify whether Rab5a could affect the invasion and migration in HCC cells, transwell assay

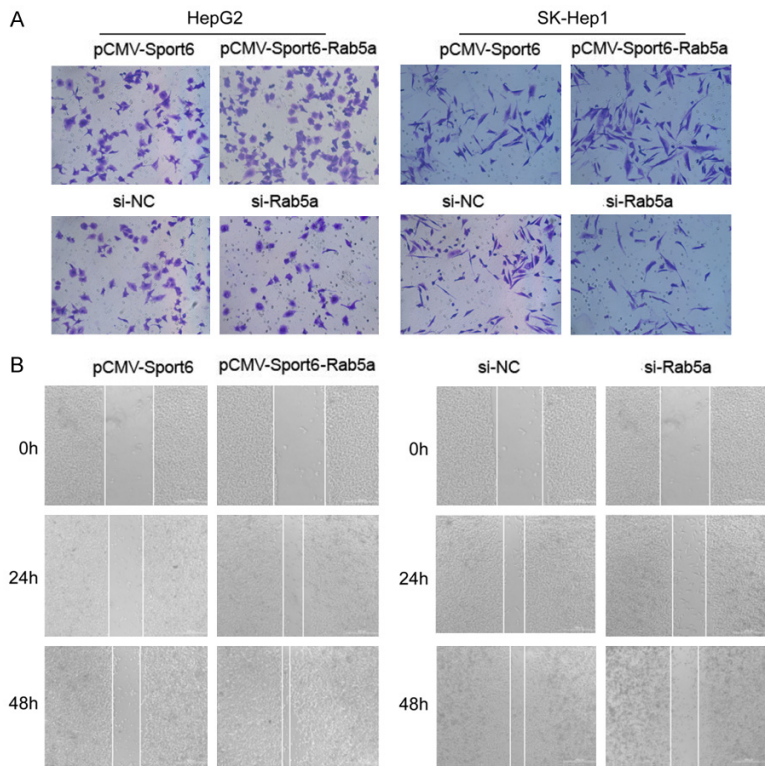

Figure 3. Rab5a promoted invasion and migration in HCC cells. A. Cell migration was detected by transwell assay in HepG2 and SK-Hep1 cells; B. Wound healing assay in SK-Hep1 cells transfected over-expressing Rab5a, si-Rab5a and their controls.

and wound healing assay were performed. pCMV-Sport6-Rab5a (pCMV-Sport6 as control) and si-Rab5a (Si-NC as control) were transfected in HepG2 and SK-Hep1 cells respectively. We found that Rab5a overexpression promoted the invasion activity while Rab5a silencing repressed the invasion in HepG2 and SK-Hep1 cells (**Figure 3A**). On the other hand, wound healing assay showed that pCMV-Sport6-Rab5a boosted the migration while Rab5a silencing depressed the migration of SK-Hep1 cells (**Figure 3B**). These findings indicated that Rab5a promoted the invasion and migration of HCC cells.

Rab5a up-regulated Cdc42 expression by enhancing its promoter activity

We measured a large number of genes related with invasion and migration biologic functions by qRT-PCR in SK-Hep1 cells, such as FOXC2, TP53, VEGF, MMp2, Cdc42, etc. As a result, the expressions of TP53, Cdc42, RhoA, Twist1 and KLF4 were all increased in pCMV-Sport6-Rab5a group (**Figure 4A**) and decreased in si-Rab5a (**Figure 4B**). Then, Western blotting was used to further confirm. However, only three genes (TP53, Cdc42 and RhoA) protein level corresponded with the mRNA level (**Figure 4C**). We finally chose Cdc42 to undergo the following experiments.

To investigate the potential mechanism of Cdc42 up-regulated by Rab5a, we constructed pGL3-Basic-Cdc42 vector which contains the Cdc42 promoter (-1002 bp~+20 bp) for luciferase reporter assay. As a result, Cdc42 promoter activity was higher in the pcDNA3.1-Rab5a and pGL3-Basic-Cdc42 co-transfected group compared to pcDNA3.1 and pGL3-Basic-Cdc42 co-transfected group (**Figure 4D**). These data suggested that Rab5a could up-regulate Cdc42 expression by increasing its promoter activity.

Rab5a influenced tumor migration in vivo

To further definite the effects of Rab5a on tumorigenesis *in vivo*, nude mice were subcutaneously injected with SK-Hep1 cells stably transfected with the lentiviral vector expressing Rab5a (pCDH-CMV-copGFP-Rab5a) (**Figure 5A**). 30 days after infection, nude mice were sacrificed and the migration potential were analyzed (**Figure 5B**). Tumor cell migration in liver was detected by IHC staining assay. As a result, Cdc42-staining increased in the pCDH-CMV-copGFP-Rab5a group compared with control (**Figure 5C**). Therefore, Rab5a promoted development in HCC *in vivo* by regulating Cdc42 expression.

Discussion

The occurrence and development of HCC is a complex process based on multiple genetic changes. The invasion and metastasis of HCC is a more complicated procedure, which involved the mutation and activation of oncogenes, deletion of anti-oncogenes, and disor-

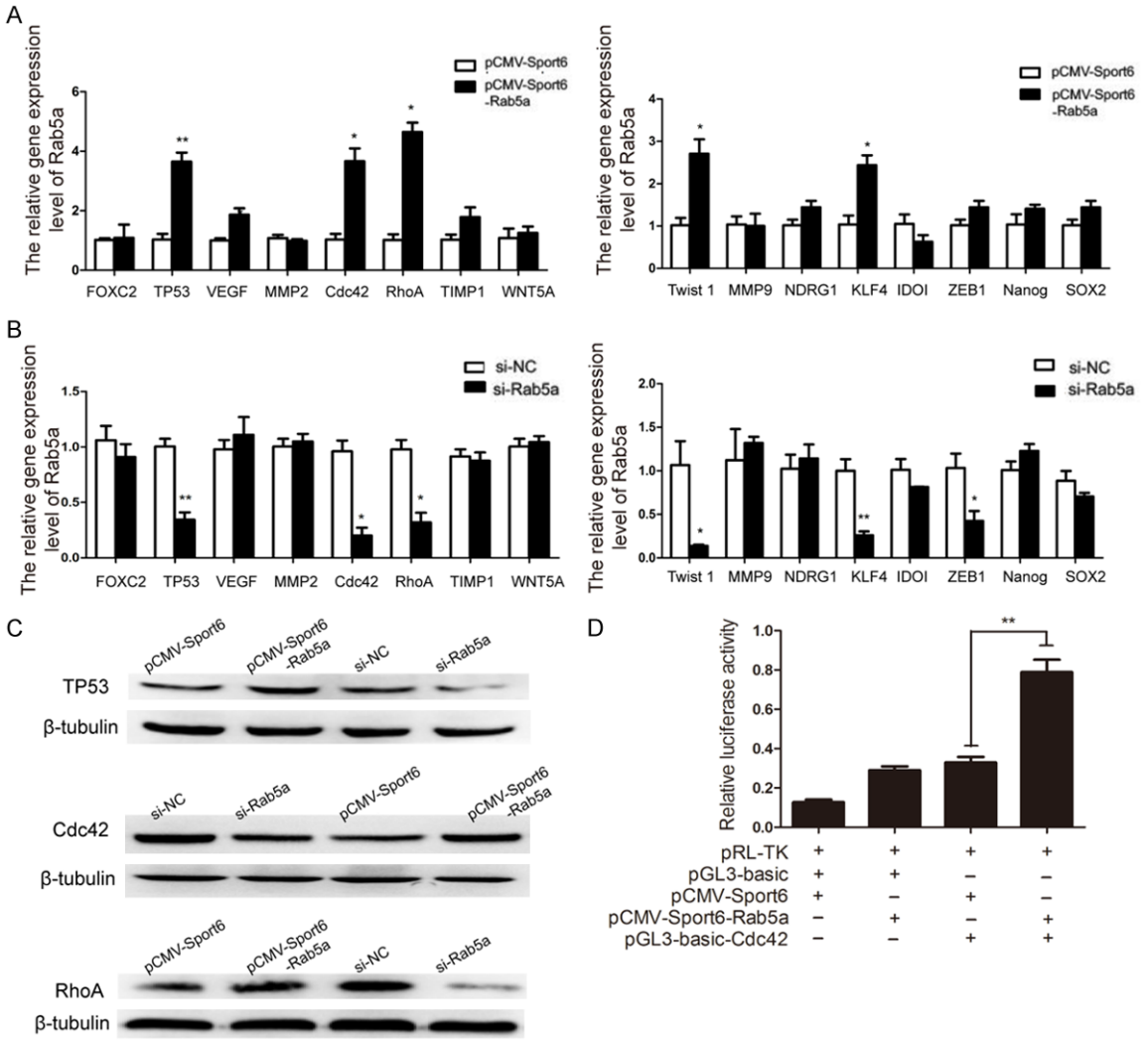

Figure 4. Cdc42 was a target gene of Rab5a. A, B. Relative genes expressions were detected in SK-Hep1 cells cotransfected with PCMV-Sport6-Rab5a and siRNA by qRT-PCR. *P < 0.05, **P < 0.01; C. TP53, Cdc42 and RhoA protein expressions were detected in SK-Hep1 cells transfected with PCMV-Sport6-Rab5a and siRNA by WB; D. Luciferase reporter assays in SK-Hep1 cells, which co-transfected with plasmids as indicated.

der of apoptosis regulation mechanism. With the development of molecular biology, more and more genes closely related to HCC metastasis were found to provide a new method for the treatment of individuals.

As a member of Rab family, Rab5a regulates the formation and transport in early vesicles, adjusts the fusion of endocytosis and early endosomes, and participates in the process of receptor internalization, intracellular transport of substances, signal transduction and cytoskeletal remodeling. In a word, Rab5a is a signal regulator in protein transmembrance pathways [7]. In recent years, it is reported that aberrant expression of Rab5a contributes to tumor progression and distant metastasis [8]. Yang et al [9] have found that Rab5a is closely related to breast cancer axillary lymph node metastasis. Zhao [10] have showed that Rab5a can transfer tumor cells from G1 phase to S phase via regulating APPL1-related epidermal growth factor (EGF) signaling pathway in ovarian cancer. Yu [11] revealed that increased expression of Rab5a was significantly correlated with poor prognosis in colorectal cancer patients. Lu [12] explained vacuolin-1 activated Rab5a to block autophagosome-lysosome fusion in cancer cells. Fukui et al [13] found that Rab5a enhance the EGF signaling and Rab5a upregulation predict poor prognosis in HCC patients.

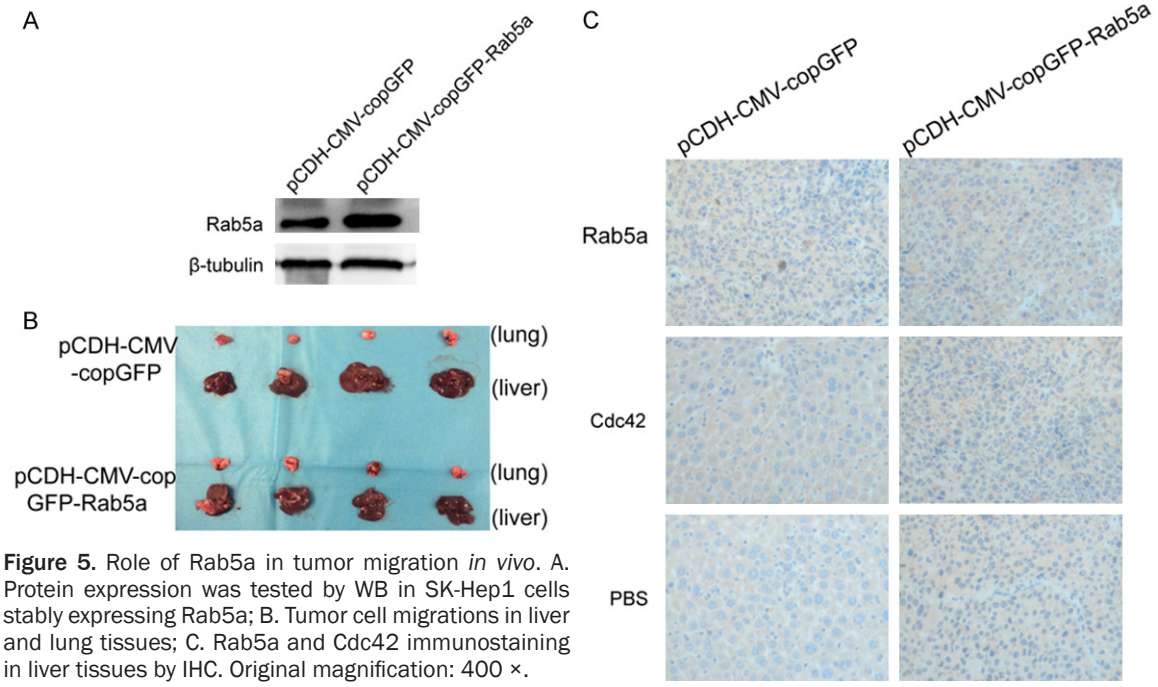

Figure 5. Role of Rab5a in tumor migration *in vivo*. A. Protein expression was tested by WB in SK-Hep1 cells stably expressing Rab5a; B. Tumor cell migrations in liver and lung tissues; C. Rab5a and Cdc42 immunostaining in liver tissues by IHC. Original magnification: 400 ×.

In our study, we examined the expression of Rab5a in HCC tissues and cell lines.

The results showed that the expression of Rab5a mRNA and protein were upregulated in HCC tissues as well as that of HCC cell lines, which can be used as a molecular marker in HCC. In addition, it is well known that metastasis is severely responsible for HCC reoccurrence. We found that overexpression of Rab5a promoted cell invasion and migration *in vitro* and *in vivo* by accelerating Cdc42 expression via enhancing its promoter activity. Cell division cycle 42 (Cdc42) is a member of the Rho subfamily, which is known to regulate the dynamic organization of the cytoskeleton and membrane trafficking for physiologic processes such as tumor cell proliferation, motility, polarity, cytokinesis and growth [14]. A large number of studies found that Cdc42 was overexpressed in tumors (breast cancer, lung cancer, testicular cancer, bladder cancer and so on), and it plays an important role in cell proliferation, transformation, invasion and metastasis [15-18]. More interesting, Cdc42 is a member of Rho subfamily belongs to Ras super family GTPase as well as Rab5a, which may plays a similar role in the occurrence and development of HCC. The next step is to elucidate the interaction mechanism of Rab5a and Cdc42, to promote the development of clinical treatment in HCC.

Based on all the above results, Rab5a presents an oncogenic function and plays a significant role in HCC development.

Acknowledgements

This work were supported by the Scientist Culture Plan of Chongqing Medical University (162014) and the Young Scientist Culture Plan of Chongqing (cstc2014kjrc-qnrc10007).

Disclosure of conflict of interest

None.

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