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ORIGINAL ARTICLE

Rab23 Promotes Hepatocellular Carcinoma Cell Migration Via Rac1/TGF-β Signaling

Li Zhang[1] · Bingqiang Zhang[1] · Wenxian You[1] · Pan Li[1] · Youlin Kuang[2]

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Abstract

Rab23 is a member of Ras-related small GTPase family, which plays a critical role in the progression of wide range of tumors. However, its biological function in hepatocellular carcinoma still remains unclear. Here, we investigated the effects of Rab23 on proliferation and migration in hepatocellular carcinoma cell and its potential mechanisms. We found over-expression of Rab23 promoted the Hep3B hepatocellular carcinoma cell migration, which could be reversed by Rab23 silencing. Rab23 induced Rac1 activation and followed progression of epithelial-mesenchymal transition (EMT) along with upregulation of N-cadherin, snail as well as vimentin and downregulation of E-cadherin via upregulating Transforming Growth Factor-β (TGF-β). Silencing Rac1 significantly attenuated Rab23-induced HepG2 migration and TGF-β. Moreover, knockdown of TGF-β effectively attenuated Rab23-induced EMT. Taken together, we demonstrated a mechanistic cascade of Rab23 enhangcing Rac1 activation and subsequent TGF-β expression, leading to hepatocellular carcinoma cell migration.

Keywords Hepatocellular carcinoma cell · Rab23 · Rac1 · TGF-β · Migration

Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed malignancies and also the third leading cause of cancer-related death [1]. Patients with HCC in late stage usually are accompanied with a poor prognosis, mainly due to the occurrence of local and remote metastasis [2]. In present, the mechanisms underlying HCC metastasis are still not totally understood. Therefore, exploring the potential mechanisms by which HCC metastasizes is quite important for probably promoting the prognosis of this disease.

Rab23, a member of Rab GTPase family, has been reported to play a potent role in the process of endocytic recycling and vesicle transportation [3]. It was first demonstrated in open brain mouse mutants as a cell autonomous negative regulator of the mouse Shh signaling pathway [4]. Recently, Rab23 was shown to be highly expressed in several tumor types and be closely related to the tumor development, such as promoting cell migration and invasion of human bladder cancer, astrocytoma, and pancreatic duct adenocarcinoma cells [5–7]. In gastric cancers, Rab23 was identified as an invasion mediator gene in diffuse-type gastric cancer by using integrative genomics and overexpression of Rab23 could enhance gastric cancer cells invasion [8]. In addition, Rab23 was also discovered in some thyroid malignant cohorts, such as follicular thyroid carcinoma, papillary thyroid carcinoma and follicular variant of papillary thyroid carcinoma [9]. In hepatocellular carcinoma tissue and cells, Rab23 has been convinced to be overexpressed and is associated with tumor size [10], but whether and how it influences HCC progression is unknown.

Rac1, a member of Rac subfamily belonging to human Rho GTPase family, transduces signals from tyrosine-kinase, G protein-coupled receptors (GPCRs), and integrins, and controlles a number of essential cellular functions including motility, adhesion, and proliferation [11]. Aberrant Rac1 activation has been found in several cancers and promotes cancer cell motility, invasion, and metastasis [12]. TGF-β has been detected in a variety of human tumors, including HCC. The crucial role of TGF-β signaling in carcinoma progression is highlighted by the fact that is overexpressed in the tumor tissue and that overexpression correlates with poor prognosis

✉ Youlin Kuang
kyl361@163.com

[1] Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

[2] Department of Urology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

[13]. In this study, we demonstrated Rab23 increased TGF-β expression through Rac1 activation to induce hepatocellular carcinoma cell migration.

Materials and Methods

Cells Culture

HCC cell line Hep3B obtained from the Chinese Academy of Sciences (Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 mg/mL penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection

For siRNA transfection, HCC cells were plated in six well plates and grew without antibiotics to 70–90% confluence, and then transfected with the Rab23-targeting siRNA (si-Rab23) (sc-95,163), Rac1-targeting siRNA (si-Rac1) (sc-36,351), TGF-β-targeting siRNA (si-TGF-β) (sc-40,222) as well as negative control siRNA (c-siRNA) (sc-37,007) (Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. For Rab23 overexpression, the recombinant plasmid pcDNA3.1-Rab23 (p-Rab23) encoding sequence of human Rab23 was transfected into HCC cells using Lipofectamine 2000, and cell samples were collected at 24 h after transfection for further analysis.

Rac1 GTPase Activity Assay

The activities of Rac1 were measured by using an Active Rac1 Pull-Down and Detection Kit (Pierce, Rockford, IL, USA) according to the manufacture's instruction. Briefly, Rac1-GTP-bound proteins were separated by 12% SDS-PAGE and then transferred to PVDF membrane (Millipore). The membrane was incubated in the anti-Rac1 antibody and detected with the Bio Imaging System.

Western Blot

Cell lysates were prepared. Briefly, cells after being washed with ice-cold PBS were harvested and then solubilized in RIPA buffer with PMSF on ice for 30 min. Subsequently, supernatants were collected after centrifugation at 16000G for 20 min at 4 °C. Total cellular proteins (50 μg) were subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Amersham, USA). Specific polyclonal antibodies against Rab23, TGF-β, snail, vimentin, N-cadherin and E-cadherin (Cell Signaling Technology, MA, USA) were used to detect indicated proteins. The appropriate horseradish peroxidase (HRP) conjugated IgG was used as secondary antibody. Antibodies on membrane were visualized by enhanced chemiluminescence (Pierce Biotechnology, IL, USA). Western blot for GAPDH (Santa Cruz, CA, USA) was used as an internal sample.

Cell Proliferation Assay

The cellular proliferation of transfected cells was measured by Cell Counting Kit-8 (dojindo, Japan) assay. Briefly, 10 μl CCK-8 resolution was added to each well of 100 μl medium. Absorbance was measured at 450 nm on automatic ELISA reader (TRITURUS). All determinations were carried out in triplicate and repeated three times.

Cell Migration Assay

The ability of cells in vitro migration assays were performed in Transwell chambers (Corning, NY, USA) following the manufacturer's instructions. Briefly, 5 × 105 cells were cultured in serum-free medium on 8 μm porous polycarbonate membranes in the upper chambers. The lower chambers were added with medium containing 10% FBS. After incubation for 24 h, the cells that migrated to the underside of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa (Sigma, MO, USA). Cells were calculated in 10 randomly selected fields under 200× magnification and expressed as the average number of cells/field of view. These data were represented as the average of the three independent experiments.

Statistical Analysis

Statistical analyses were performed using SPSS version 13.0. Values were expressed as the mean ± standard deviation. Statistically significant differences between groups in each assay were determined by ANOVA, or student's t test. $p < 0.05$ was considered as statistically significant.

Results

Overexpression of Rab23 Enhanced Hepatocellular Carcinoma Cells Growth and Migration

To explore the effects of Rab23 on hepatocellular carcinoma cells, we performed Rab23 overexpression by transfecting recombinant plasmid p-Rab23 into Hep3B cell (Fig. 1a) and observed the cell growth and migration. Rab23 overexpression significantly promoted Hep3B cells growth at 24 h and 48 h after transfection ($P < 0.05$, Fig. 1b). Also, it enhanced the migration ability of the cells with increasing migrated cells ($P < 0.05$, Fig. 1c).

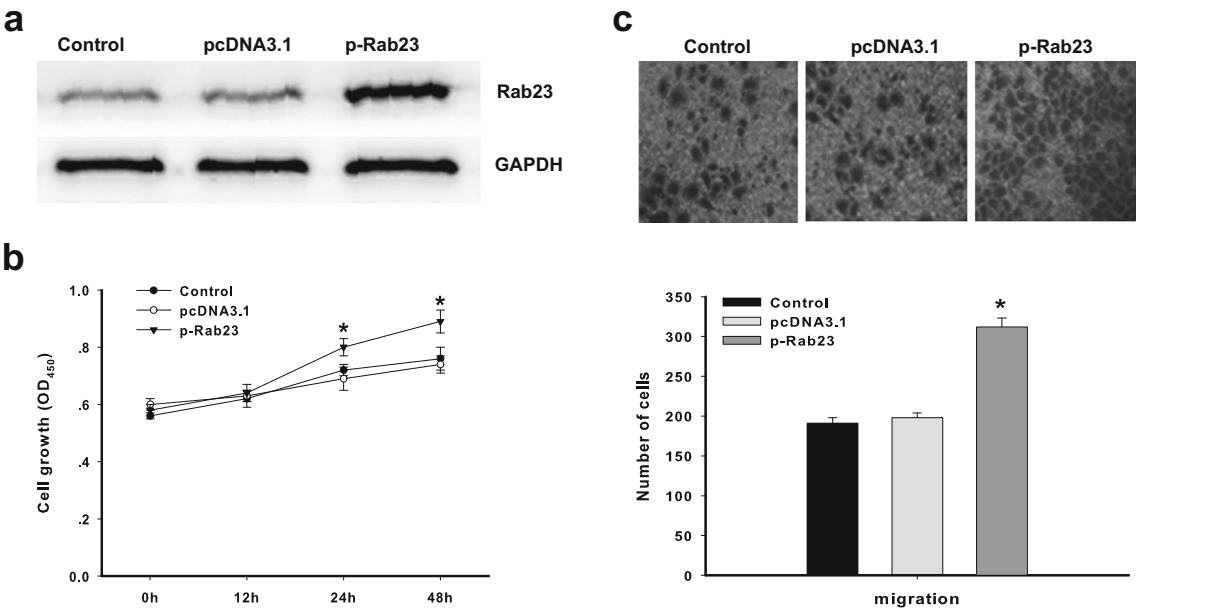

Fig. 1 Rab23 overexpression promoted hepatocellular carcinoma cells growth and migration. Hep3B cells were transfected with recombinant plasmid pcDNA3.1-Rab23 (p-Rab23), empty vector as control. **a** Western blot was used to detect Rab23 expression. **b** Cells growth capacity was tested by CCK-8 assay, showing cells transfected with recombinant plasmid had higher growth capacity compared with control groups at 24 and 48 h after transfection, data presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*$P < 0.05$). **c** Cells migration was analyzed by transwell chambers, data presented as the mean ± SD of 3 independent experiments (*$P < 0.05$)

Rab23 Silencing Suppressed the Progression of Hepatocellular Carcinoma Cells

Hep3B cells were transfected with Rab23-specific siRNA. Western blot showed Rab23 expression was reduced at protein levels (Fig. 2a). Silencing Rab23 suppressed Hep3B growth and migration (Fig. 2b,c).

Rac1 Activation Was Critical for Rab23-Induced Hepatocellular Carcinoma Cells Migration

To observe the role of Rac1 in Rab23-induced hepatocellular carcinoma cells migration, we measured the activity of Rac1. Upregulation of Rab23 by p-Rab23 efficiently increased the active Rac1 levels, which was decreased when Hep3B cells were treated with si-Rab (Fig. 3a). When Rac1 was silenced by Rac1-specific siRNA, the Hep3B migration improved by enhanced expression of Rab23 was significantly attenuated (Fig. 3b).

Rab23 Promoted Hepatocellular Carcinoma Cell EMT Via TGF-β Signaling

Upregulation of Rab23 expression enhanced TGF-β expression and Hep3B EMT with increased N-cadherin, snail, vimentin and decreased E-cadherin (Fig. 4a). When TGF-β was silenced by si-TGF-β, Hep3B EMT factors induced by overexpressed Rab23 were reversed with increase of E-cadherin and decrease of N-cadherin, snail as well as vimentin (Fig. 4a). To further confirm whether TGF-β was regulated by Rac1, we used Rac1-specific siRNA. We found silencing Rac1 attenuated the enhanced TGF-β by Rab23 (Fig. 4b).

Discussion

Rab23 has been reported to be overexpressed in several human cancers. Previous study discovered Rab23 was overexpressed in HCC using immunohistochemistry and that was correlated with tumor size [10]. However, whether and how it influences HCC progression is not clear. In some other tumor types, Rab23 has been showed multiple functions in cancer development dependent on the context of the tumor. It was found that ectopic expression of Rab23 inhibited the growth and proliferation as well as inducing cell apoptosis in breast cancer cells [14], while promoting invasion and migration of esophageal squamous cell carcinoma and squamous cell carcinoma cells [15, 16]. In several cancer types, Rab23 was found to be regulated by some kinds of miRNA, such as miR-802 in gastric cancer [17], miR-665 in osteosarcoma [18]. To figure out that, we overexpressed Rab23 in HCC cells using recombinant plasmid pcDNA3.1-Rab23 and observed that upregulation of Rab23 promoted the HCC cells growth and migration, while silencing Rab23 by Rab23 siRNA inhibited tumor progression. Moreover, enhanced expression of Rab23 promoted the occurrence of EMT with downregulated E-cadherin expression and upregulated N-cadherin. These results demonstrated the importance of Rab23 in the

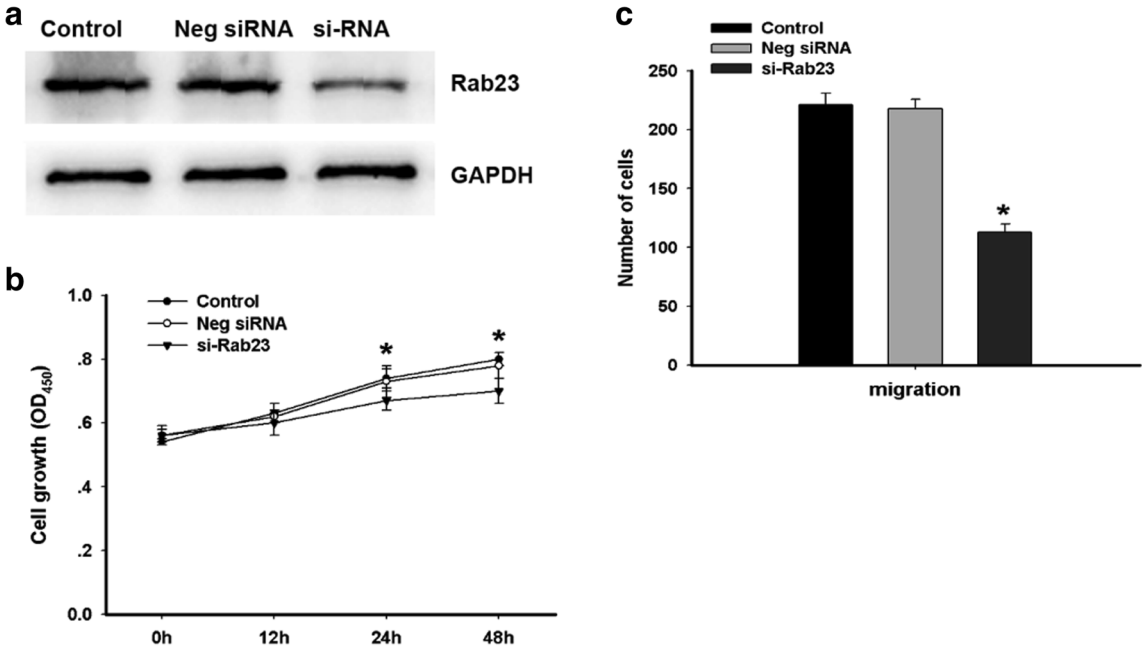

Fig. 2 Rab23 silencing suppressed the progression of hepatocellular carcinoma cells. Hep3B cells were transfected with negative siRNA (neg siRNA) or Rab23-specific siRNA (si-Rab23). Non-transfected cells were used as control. **a** Western blot was used to detect Rab23 exptession. **b** The cells' proliferation activity was tested by CCK-8 assay, data presented as the mean ± SD of 3 independent experiments, each performed in triplicate ($*P < 0.05$). **c** Cells migration was observed by transwell chambers, data presented as the mean ± SD of 3 independent experiments ($*P < 0.05$)

regulation of HCC cells progression. Further, we observed that TGF-β was involved in Rab23-mediated EMT. Our findings revealed that Rab23 overexpression significantly increased the expression of TGF-β in HCC cells. It has been showed that TGF-β involves numerous cellular functions, such as cell growth, development and apoptosis, both in adult and in embryonic stages [19]. In hepatocellular carcinoma, TGF-β has been exhibited both tumour suppressing and promoting effects [20, 21]. In addition, TGF-β signalling is also a master mediation of initiating and maintaining cell EMT, the process directing cancer cells towards invasion [22]. In HCC cells, suppression of TGF-β has been showed to increase E-cadherin and thereby lower invasion and migration potential [23]. In this study, we found that TGF-β silencing significantly reversed EMT with increase of E-cadherin and decrease of N-cadherin, snail as well as vimentin. These

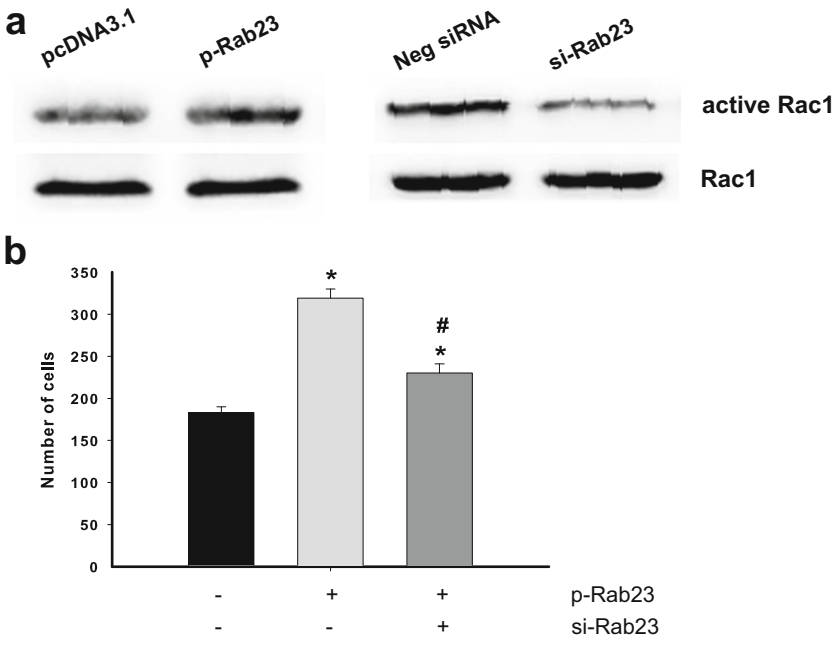

Fig. 3 Rac1 activity was required for Rab23-induced hepatocellular carcinoma cells migration. **a** Hep3B cells were transfected with Rab23 expression vector or Rab23-specific siRNA for 48 h. Rac1 activity was analyzed by GTPase activity assay. **b** Hep3B cells were transfected with Rab23 recombinant vector for 48 h, and then transfected with Rac1-specific siRNA (si-Rac1) or not for another 24 h. Then, the cells migration was observed by transwell chambers, data presented as the mean ± SD of 3 independent experiments ($*^{\#}P < 0.05$)

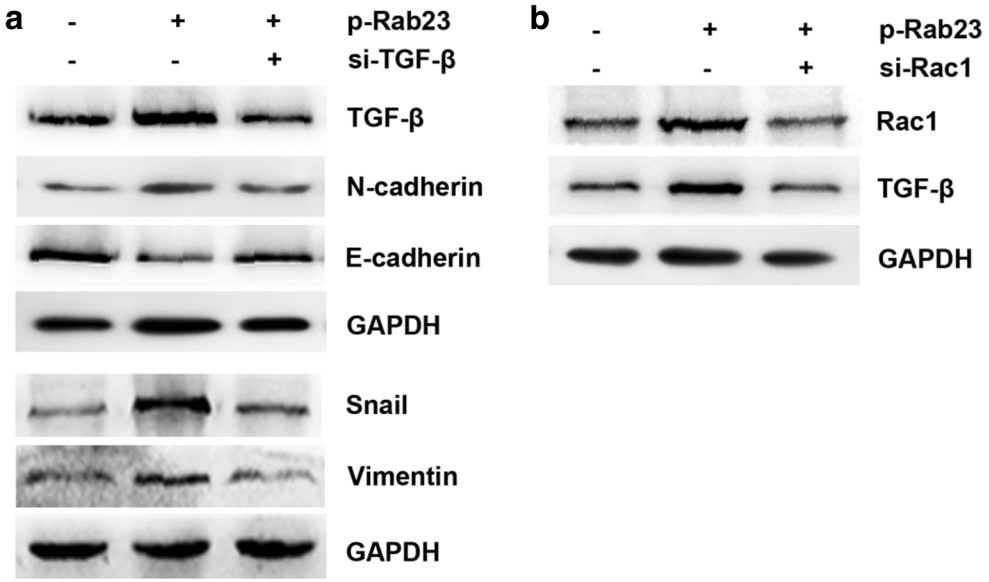

Fig. 4 Rab23 promoted hepatocellular carcinoma cell EMT via TGF-β signaling. **a** Hep3B cells were transfected with Rab23 recombinant vector for 48 h, and then transfected with TGF-β-specific siRNA (si- TGF-β) or not for another 24 h. The expression of TGF-β, E-cadherin, N-cadherin, snail and vimentin was detected by Western blot. **b** Hep3B cells were transfected with Rab23 recombinant vector for 48 h, and then transfected with Rac1-specific siRNA (si-Rac1) or not for another 24 h. Western blot was used to detect the Rac1 and TGF-β expression

data suggested that upregulation of TGF-β might represent a novel mechanism by which Rab23 facilitated hepatocellular carcinoma cells EMT and migration.

Rac1, as a member of the Rac subfamily of the Rho family GTPases, has been found that aberrant activation of which in several cancers promotes cancer cell motility, invasion, and metastasis [12]. In this study, we found Rac1 activation was critical for Rab23-induced hepatocellular carcinoma cells migration. Upregulation of Rab23 efficiently increased the active Rac1 levels, while silencing Rab23 decreased Rac1 activation. When Rac1 was silenced, the Hep3B cell migration improved by enhanced expression of Rab23 was significantly attenuated. To understand whether Rac1 was involved in Rab23 mediated TGF-β expression, we further found silencing Rac1 by Rac1-specific siRNA strongly attenuated Rab23-induced TGF-β expression. These findings demonstrated that Rab23 promoted hepatocellular carcinoma cells migration could via increase of TGF-β expression by activating Rac1. Activated Rac1 can also exert its function via other effectors such as p21-activated kinase 1 (PAK1), or via Rac1-dependent NADPH oxidases which generate reactive oxygen species (ROS) [13]. In squamous cell carcinoma cells, Rab23 promotes migration and invasion by regulating Integrin β1/Tiam1/Rac1 pathway [16], and NF-κB in bladder cancer [6]. All of these suggest that the full regulation mechanisms of Rab23 in cancer progression is intricate. We also speculate Rab23 promotes proliferation and metastasis of hepatocellular carcinoma maybe in multitude mechanisms, and Rab23 might be a rational molecular therapeutic target for hepatocellular carcinoma. More studies should be done to explore the regulation of Rab23.

Taken together, we demonstrated a mechanistic cascade of Rab23 up-regulating Rac1 activation and subsequent TGF-β expression, leading to hepatocellular carcinoma migration. Rab23 could be taken as a critical determinant of cancer cellular behavior and serve as a promising therapeutic target for hepatocellular carcinoma.

Compliance with Ethical Standards

Conflict of Interest Statement There is no potential conflicts of interest in this paper.

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