Rab11-FIP2 promotes colorectal cancer migration and invasion by regulating PI3K/AKT/MMP7 signaling pathway

Chang-long Xua, Jian-zhang Wang a, Xuan-ping Xia a, Chen-wei Pan b, Xiao-xiao Shaoa, Sheng-long Xiaa, Shou-xing Yanga, Bo Zheng a, b

a Department of Gastroenterology, The Second Affiliated Hospital & Yuying Children’s Hospital of Wenzhou Medical University, Wenzhou, China
b Department of Infectious Disease, The Second Affiliated Hospital & Yuying Children’s Hospital of Wenzhou Medical University, Wenzhou, China

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A B S T R A C T
Rab11-family interacting proteins (Rab11-FIPs) belong to an evolutionarily conserved protein family and act as effector molecules for the Rab11 family of small GTPases. Recent evidence suggests that Rab11-FIPs have important roles in tumor progression and metastasis. However, the contribution of Rab11-FIPs to colorectal carcinoma (CRC) remains elusive. Our study focuses on elucidating the role of Rab11-FIP2 in the migration and invasion of colorectal cancer cells. We firstly found upregulation of Rab11-FIP2 in CRC tissues compared with peritumor tissues by oncomine data-mining analysis, western blot analysis and immunohistochemistry (IHC) analysis, respectively. Then, we demonstrated that knockdown of Rab11-FIP2 via siRNAs transfection resulted in a decrease in migration and invasion of CRC cells, while over-expression of Rab11-FIP2 via lentiviral infection increased migration and invasion of CRC cells. In addition, we verified that Rab11-FIP2 promoted migration and invasion of CRC cells through upregulating MMP7 expression. Finally, using several kinase inhibitors, our results showed that Rab11-FIP2 regulated MMP7 expression through activating PI3K/Akt signaling. Our data suggested a potential role of Rab11-FIP2 in tumor progression and provided novel insights into the mechanism of how Rab11-FIP2 positively regulated cell migration and invasion in CRC cells.

1. Introduction
Colorectal cancer (CRC) is one of the most commonly diagnosed malignances and has highly mortality worldwide [1]. Recent progress in diagnosis and treatment has successfully improved the survival state of the CRC patients in the early stage, but the prognosis of malignant CRC patients that undergo metastasis in the advanced stage is still poor [2,3]. Metastasis process of cancer is complicated and involves individual cancer cells detaching from the primary tumor, migrating to the blood or lymph, resisting to anoikis and colonizing distant organs [4,5]. Therefore, further investigation into the metastasis mechanism of CRC is urgently needed.

The Rab11-family of small GTPases (Rab11a, Rab11b and Rab25) play critical roles in membrane identity, vesicle budding, uncoating, cytokinesis, motility and fusion through the recruitment of specialized effector proteins [6–8]. Rab11-family interacting proteins (Rab11-FIPs) are newly identified family which comprises of at least six mammalian genes, Rip11, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, RCP, and Rab11-FIP4 [7,9,10]. Each of these members possesses a predicted alpha-helical coiled-coil domain, a Rab-binding domain (RBD), a hydrophobic Rab11-binding patch near the carboxy-terminus, and either a C2 or EF-hand domain in the amino-terminal region [10–12]. A growing body of research showed that Rab11-FIPs played important roles in tumor progression and metastasis. For example, Jin M et al. [13] demonstrated that Rab11-FIP1 regulated plasma membrane recycling of HeLa cells with regional specificity within the Rab11A-containing recycling system. Jing J et al. [14] reported that Rab11-FIP3 regulated breast cancer cell motility by modulating the actin cytoskeleton. In hepatocellular carcinoma (HCC), hypoxia up-regulated Rab11-FIP4 through HIF-1α to promote tumor metastasis [15]. Rab11-FIP2, which also contains a conserved carboxyl-terminal Rab11-binding domain, is one of the Rab11-FIPs members [9,11]. Rab11-FIP2 was firstly identified as a Rab11A-binding protein in a yeast two hybrid
screen [9], and subsequently proved to interact with Myosin Vb, participating in Rab11-mediated recycling pathways [16]. Naslavsky N et al. [17] reported that Rab11-FIP2 and Eps15 homology domain (EHD) 1 played a coordinated role in mediating early endocytic recycling. Ducharme NA et al. [18] figured out that phosphorylation of Rab11-FIP2 on serine 227 by MARK2 was necessary for the timely establishment of polarity in Madin–Darby canine kidney cells. Recently, Gidon A et al. [19] proved that Rab11-FIP2, together with myosin Vb and Rab11A, constituted to a membrane platform and regulated two late recycling steps of laminin from the endosomal recycling compartment (ERC) to the plasma membrane in melanoma cells. However, to date, the potential role of Rab11-FIP2 in CRC progression remains unknown.

In this study, we firstly found that the expression of Rab11-FIP2 was upregulated in CRC and significantly correlated with poor prognosis of CRC patients. Moreover, we demonstrated that Rab11-FIP2 dramatically contributed to migration and invasion of CRC cells through activating PI3K/Akt signaling and increasing MMP7 expression. Overall, our results provide critical roles for Rab11-FIP2 in CRC progression.

2. Methods and materials

2.1. Ethics statement

Tissue specimens (20 tissue pairs and 82 tumor tissues) from CRC patients were obtained between 2010 and 2015, and histologically confirmed by a pathologist at The Second Affiliated Hospital of Wenzhou University (Wenzhou, China). Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of The Second Affiliated Hospital of Wenzhou University.

2.2. Immunohistochemical staining

Formalin fixed paraffin-embedded (FFPE) specimens of CRC cancer and peritumor tissues were obtained from The Second Affiliated Hospital of Wenzhou University (Wenzhou, China). Specimens were deparaffinized, rehydrated, and blocked with 2% normal goat serum (Gibco). Immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s protocol. Incubations with primary antibodies against Rab11-FIP2 (1:100; Santa Cruz, USA) were conducted overnight at 4 °C in a humidified chamber. Substrate interactions were visualized by incubation in DAB (Vector Laboratories). Negative controls were incubated in pre-immune serum. Immunostaining scores were performed according to previously reported method [20].

2.3. Cell lines and cell culture

The human colorectal carcinoma cell lines LoVo and HCT116 were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 10 units/mL penicillin and 10 mg/mL streptomycin (1% penicillin/streptomycin, Thermo Scientific HyClone) and incubated in a humidified atmosphere at 37 °C.

2.4. Construction of Rab11-FIP2 overexpression lentivirus

The Rab11-FIP2 ORF sequence (NM_014904) was PCR amplified using specific primers and cloned into the lentiviral expression vector pWPXL (Addgene) to develop a pWPXL-Rab11FIP2 recombinant plasmid. Virus packaging was performed in HEK 293T cells, which were co-transfected with pWPXL-Rab11FIP2, psPAX2 (the packaging plasmid) and pMD2.G (the envelope plasmid) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Viruses were harvested after 40 h transfection, and viral titers were determined. HCT116 was infected with recombinant lentivirus-transducing units in the presence of polybrene (Sigma).

2.5. Small interfering RNA transfection

Small interfering RNA (siRNA) duplexes targeted Rab11-FIP2 (siRNA#1: forward 5'-GUGCGGUAUCACUUCAUA-3'; siRNA#2: forward 5'-GGUGCGGAUCACUUCAUA-3'; siRNA#3: forward 5'-GCAUAUCACUCCUAUAUA-3'), MMP7 (siRNA#1: forward 5'-GGCAUCAGAGAAACUAAUG-3'; siRNA#2: forward 5'-GCACUGAGUUCUCACUCA-3') and negative control (NC) siRNA duplex (forward: 5'-UUUCGAGAACGUGACAGUTT-3') were chemically synthesized by Biomics Biotechnologies Co. Ltd (Shanghai, China). Transfection was performed using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions.

2.6. Quantitative real-time polymerase chain reaction assay

Total RNA was extracted from human colorectal carcinoma cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using PrimeScript™ RT Reagent Kit (Takara, Dalian, China) according to manufacturer’s instructions. Then, quantitative real-time PCR was subsequently performed with SYBR Premix Ex Taq (Takara, Dalian, China) using an ABI 7500 instrument (Applied Biosystems Inc). The PCR conditions were as follows: 95 °C for 15 s followed by 38 cycles of 95 °C for 5 s and 60 °C for 45 s. The sequences of primers used were as follows:

- Rab11-FIP2: Forward Primer 5'-TGTCCGCGACCAAGCCCAAGAAG-3', Reverse Primer 5'-CTCTCTTTIAAATGTCACACCTT-3', Reverse Primer 5'-TGTTGTTCCACCCATTTTCTC-3', MMP1: Forward Primer 5'-GATACCCGTTGACCTGAAGA-3', Reverse Primer 5'-CCCTCTCAGAAGGCTATAGC-3'; MMP3: Forward Primer 5'-CGTGCTCCCTGTTCAAG-3', Reverse Primer 5'-CGCCAAAGTGGCTGTTCTT-3'; MMP7: Forward Primer 5'-GAGTGCTACAGTGAGGAAA-3', Reverse Primer 5'-CTATGACGGGGAGTTAAACAT-3'; MMP9: Forward Primer 5'-GGAGGCAGAGATGGCATC-3', Reverse Primer 5'-TTCGCTACGTCGAAATGGGC-3'; MMP10: Forward Primer 5'-TGCTGTGGCTATCTGGTGT-3', Reverse Primer 5'-TCA- CATTCTTTCAGGGTGTAG-3'; MMP13: Forward Primer 5'-ACTGAGGCGTCGGAATAAG-3', Reverse Primer 5'-GAACCCCGCATTCGGCTT-3'; β-actin: Forward Primer 5'-TTGTTACAAGGAGCTCCTTCC-3', Reverse Primer 5'-ATGCTAT- CACTCCTTCCGTTG-3'. Data were normalized to β-actin, and mRNA abundance was calculated using the 2-ΔΔCT method.

2.7. Western blotting

CRC cells and tissues were harvested and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris–HCl at pH 7.4, and protease inhibitors). Cell debris was removed via centrifugation at 14,000 × g for 15 min at 4 °C. For western blotting analysis, a total of 60–80 μg protein was separated using SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked in 5% fat-free milk at room temperature for 2 h. Specific primary antibodies against Rab11-FIP2 (1:800, Santa Cruz, USA), MMP7 (1:1000), MMP9 (1:1000), MMP13 (1:1000) and β-actin (1:1500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific primary antibodies against phospho-PI3K (p-PI3K, 1:1500), total-PI3K (T-PI3K, 1:1500), phospho-
Akt (p-Akt, 1:1000), and total-P13K (T-Akt, 1:1000) were purchased from Cell Signaling Technology (CST, Beverly). The specific primary antibodies were incubated at 4 °C overnight and secondary anti-rabbit antibody (1:5000, Bioworld, USA) was incubated for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence (Amersham; Buckinghamshire, UK).

2.11. Statistical analysis

Data were analyzed with GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software, San Diego, CA, USA). Average values were expressed as mean ± SD. Statistical significance between different groups was determined by repeated-measures ANOVA test. A p value < 0.05 was accepted as statistically significant.

3. Results

3.1. Rab11-FIP2 was significantly increased in CRC tissue

To investigate the role of Rab11-FIP2 in CRC, we firstly analyzed the expression level of Rab11-FIP2 through oncomine data-mining analysis. In Hong Colorectal Statistics, we found that level of Rab11-FIP2 mRNA was significantly increased from colon tissue to CRC tissue (Fig. 1A). Subsequently, Rab11-FIP2 expression by western blot analysis was performed to further confirmed. Seen from Fig. 1B, the results validated that Rab11-FIP2 was significantly increased in CRC tissues compared with peritumor colon tissues. Next, IHC analysis of Rab11-FIP2 expression was done using tissue microarrays which contained 20 paired CRC tissue samples. The representative clinical data were summarized in Supplemental Table 1. The immunohistochemical results (Fig. 1C–D) showed that the staining density of Rab11-FIP2 in CRC group was obviously stronger than that in matched peritumor nontumor colon tissues.

3.2. Correlation of Rab11-FIP2 expression with prognosis of CRC patients

Next, we investigated whether Rab11-FIP2 expression was associated with overall survival of CRC patients. Tissue microarray analysis of CRC tissues from 82 postsurgical patients was carried out. The representative clinical data were summarized in Supplemental Table 1. As shown in Fig. 1E, Kaplan–Meier overall survival (OS) analyses showed that patients with high Rab11-FIP2 expression had much shorter OS times.

3.3. Rab11-FIP2 knockdown inhibited migration and invasion of LoVo cells in vitro

In order to further explore the role of Rab11-FIP2 in CRC cells, LoVo cells were transfected with siRNA control (LoVo siNC) or siRNAs against Rab11-FIP2 (LoVo siRab11FIP2#1, LoVo siR-ab11FIP2#2 and LoVo siRab11FIP2#3), respectively. The knockdown efficiency of Rab11-FIP2 was verified by qRT-PCR analysis and western blot analysis. And the results (Fig. 2A–B) showed that Rab11-FIP2 was significantly knocked down by infection with siRNAs against Rab11-FIP2. Then LoVo siRab11FIP2#2 and LoVo siR-ab11FIP2#3 were used to further investigate the role of Rab11-FIP2 in CRC cells. The result from CCK8 assay (Fig. 2C) showed that Rab11-FIP2 knockdown had no effect on proliferation of LoVo cells. In addition, Rab11-FIP2 knockdown also had no effect on colony formation of LoVo cells detected by clonogenic assay (Fig. 2D). Moreover, LoVo cells were transfected with siRNAs against Rab11-FIP2 (siRab11FIP2#2 and LoVo siRab11FIP2#3), and transwell assays were conducted. The results (Fig. 2E–F) showed that the migration and invasion cells were significantly decreased after Rab11-FIP2 knockdown.

3.4. Overexpression of Rab11-FIP2 promoted migration and invasion of HCT116 cells in vitro

HCT116 cells were transfected with lentivirus that stably expressed Rab11-FIP2 (HCT116-Rab11FIP2) and control vector (HCT116-Vector), respectively. Then, qRT-PCR analysis and western blot analysis were used to determine the overexpression efficiency, respectively. As shown in Fig. 3A–B, both mRNA and protein levels of Rab11-FIP2 in HCT116-Rab11FIP2 cells were significantly increased by more than 4-fold compared with HCT116-Vector cells. The results of CCK8 assay and clonogenic assay showed that overexpression of Rab11-FIP2 had no effect on proliferation and colony formation of HCT116 cells (Fig. 3C–D). Migration and invasion
capacities of HCT116 cell were also detected. Transwell assay showed that overexpression of Rab11-FIP2 promoted migration of HCT116 cells in vitro (Fig. 3E). Likewise, transwell invasion assay showed that overexpression of Rab11-FIP2 significantly promoted invasion of HCT116 cells (Fig. 3F).

3.5. Rab11-FIP2 promoted migration and invasion of CRC through regulating MMP7 expression

MMPs play important roles in progression and metastasis of CRC through extracellular matrix (ECM) turnover, migration and invasion [21–23]. In our study, we analyzed the mRNA levels of MMPs (including MMP1, MMP2, MMP3, MMP7, MMP9, MMP10, and MMP13) in CRC cells transfected with siRNAs against Rab11-FIP2 or Rab11-FIP2 lentivirus, using qPCR analysis. As shown in Fig. 4A, mRNA level of MMP7 was significantly decreased in Rab11-FIP2 knockdown group (siRab11FIP2#2 and siRab11FIP2#3), compared with control group in LoVo cells. On the contrary, the mRNA level of MMP7 was significantly increased in Rab11-FIP2 overexpression group, compared with control group in HCT116 cells (Fig. 4B). Then, western blot analysis was used to verify these results. As shown in Fig. 4C–D, the knockdown of Rab11-FIP2 significantly down-regulated MMP7 expression, while the overexpression of Rab11-FIP2 significantly up-regulated MMP7 expression. However, dysregulation of Rab11-FIP2 didn’t affect levels of MMP9 and MMP13.

Next, we tested whether MMP7 was involved in the migration and invasion of CRC cells in vitro. HCT116-Rab11FIP2 cells were further transfected with siRNA against MMP7 (siMMP7) for evaluating the effects of MMP7 on migration and invasion. The expression levels of MMP7 were assessed by qRT-PCR and western blot analysis (Fig. 4E–F). As shown in Fig. 4G–H, knockdown of MMP7 significantly decreased migration and invasion of in HCT116-Rab11FIP2 cells. These results suggested that MMP7
functioned downstream of Rab11-FIP2 and was necessary for CRC progression.

3.6. Rab11-FIP2 regulated MMP7 expression via PI3K/Akt pathway in CRC

To elucidate which signaling pathway mediates the regulatory effect of Rab11-FIP2 on MMP7 expression, HCT116-Rab11FIP2 cells were preincubated with inhibitors of Erk, Jak, JNK, p38 kinase, PI3K, or Akt for 24 h. Our results showed that blockade of PI3K/Akt pathway with PI3K inhibitor (LY294002) or Akt inhibitor (Wortmannin) significantly inhibited MMP7 expression in HCT116-Rab11FIP2 cells (Fig. 4I). In addition, levels of p-PI3K and p-Akt were significantly decreased in Rab11-FIP2 knockdown LoVo cells (siRab11FIP2#2 and siRab11FIP2#3), whereas it was increased in HCT116-Rab11FIP2 cells, compared with their relative control cells.

Fig. 2. Rab11-FIP2 knockdown inhibited migration and invasion of LoVo cells in vitro. LoVo cells were transfected with siRNA control (LoVo siNC) or siRNAs against Rab11-FIP2 (LoVo siRab11FIP2#1, LoVo siRab11FIP2#2 and LoVo siRab11FIP2#3), respectively. (A) qRT-PCR analysis verified that mRNA level of Rab11-FIP2 was knocked down by infection with siRNAs against Rab11-FIP2. (B) Western blot analysis validated that protein level of Rab11-FIP2 was knocked down by infection with siRNAs against Rab11-FIP2. (C) CCK8 assay showed that Rab11-FIP2 knockdown had no effect on proliferation of LoVo cells. (D) Clonogenic assay showed that Rab11-FIP2 knockdown had no effect on colony formation of LoVo cells. (E) Transwell assay showed that Rab11-FIP2 knockdown significantly inhibited migration of LoVo cells. (F) Transwell invasion assay showed that Rab11-FIP2 knockdown significantly inhibited invasion of LoVo cells. *P < 0.05; **p < 0.01.
These findings demonstrated that PI3K/Akt pathway might play a critical regulatory role in MMP7 expression.

4. Discussion

The basis of tumor treatment is to understand the molecular mechanisms for tumorigenesis and cancer metastasis. The activation of oncogene is one of the important factors that result in the development of CRC. In this study, we revealed that Rab11-FIP2 was upregulated in CRC and enhanced the migration and invasion of colorectal cancer cells via PI3K/Akt/MMP7 axes. Our findings uncover a novel mechanism by which Rab11-FIPs regulates tumor progression and invasion.

Matrix metalloproteinases (MMPs) constitute more than 20 related zinc-dependent endopeptidases capable of degrading and shedding several extracellular matrix (ECM) components, such as growth factors, receptors, and cell–cell adhesion molecules [24,25]. MMP7 (also called matrilysin) is an important member of the MMPs and exhibits proteolytic activity against components of the ECM. Previously studies showed that MMP7 was frequently overexpressed in human CRC tissues, especially in the invasive fronts and was associated with poor prognosis of CRC patients [26–29]. For example, Li M et al. [27] reported that MMP7 inhibited cancer-cell apoptosis, decreasing cell adhesion, and inducing angiogenesis, resulting in promoting the development and progression of CRC cells. Lin BR et al. [30] demonstrated that MMP7 played a critical role in connective tissue growth factor (CTGF) mediated CRC invasion and metastasis. In our study, the results also indicated that MMP7 could promote the migration and invasion of CRC cell lines (HCT116 and LoVo) in vitro. Moreover, we firstly found that MMP7 functioned as downstream target of Rab11-FIP2 in CRC cells. However, overexpression or knockdown of Rab11-FIP2 had no effect on the expression of other MMPs (including MMP1, MMP2, MMP3, MMP9, MMP10, and MMP13).

Multiple previous researches have showed that PI3K/Akt pathway was closely associated with MMP7 expression. Zhang J et al. [31] reported that miR99a modulates MMP7 expression via PI3K/Akt pathway to regulate invasiveness of Kaposi’s sarcoma. Ye Y et al. [32] found that an Akt inhibitor could significantly inhibit EGF–induced activation of MMP7 in gastric cancer. In addition, EGF/EGFR signaling of larynx carcinoma could activate downstream PI3K/Akt to induce FoxO1 nuclear exclusion, which activates MMP7 to promote tumor metastasis [33]. Similarly, by using both an inhibitor for EGFR and an inhibitor for Akt, Liu G et al. [34] found the blockage of either EGFR or Akt could significantly inhibit the EGF-induced activation of MMP7 in gastric cancer. Our current studies identified that the PI3K/Akt pathway was involved in the regulatory effect of Rab11-FIP2 on MMP7 expression in CRC cells. Our results clearly demonstrated that the PI3K inhibitor LY294002 and Akt inhibitor Wortmannin could significant inhibit the expression of MMP7.
MMP7 in HCT116 cells, which increased by Rab11-FIP2. Meanwhile, a significant positive correlation of PI3K/Akt pathway activation
and Rab11-FIP2 expression was observed in both HCT116 cells and LoVo cells. These findings demonstrate that PI3K/Akt pathway may play a critical role in the regulatory effect of Rab11-FIP2 on MMP7 expression. However, the detail mechanism of how Rab11-FIP2 activated PI3K/Akt pathway is still unclear and need further investigating.

In conclusion, our findings unravel a novel mechanism that Rab11-FIP2 activates PI3K/Akt pathway, which in turn promotes expression of MMP7 and leads to facilitated migration and invasion of CRC cells. These data suggest that Rab11-FIP2 may be a novel candidate gene involved in CRC progression.

5. Conflict of interest

No potential conflicts of interest were disclosed.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbrc.2016.01.031.

References


