The overexpression of Rab9 promotes tumor progression regulated by XBPI in breast cancer

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Background: Rab9 is a small GTPase that localizes to the trans-Golgi Network (TGN) and late endosomes and is involved in the recycling of mannose-6-phosphate receptors (MPRs).

Materials and methods: To determine new treatment strategies for breast cancer and to elucidate the mechanism underlying the phenomenon, we investigated the effects of Rab9 in the human breast cancer cell lines MCF7 and MDA-MB-231.

Results: We observed that knockdown of Rab9 inhibited the survival and proliferation of MCF7 and MDA-MB-231 cells, whereas Rab9 overexpression facilitated cell survival and proliferation by inducing or suppressing apoptosis. These results were further confirmed by the Bax/Bcl-2 ratio in affected MCF7 and MDA-MB-231 cells, which demonstrated whether the mitochondrial apoptotic pathway was triggered. Furthermore, the AKT/PI3K pathway is implicated in cell growth and survival and Rab9 changed the expression and phosphorylation of PI3K signaling pathway members. XBPI is a key regulator of Rab9 and further confirmed that Rab9 play important roles in breast cancer tumorigenesis.

Conclusion: These data suggest that Rab9 is a good candidate for a novel therapeutic strategy for the treatment of breast cancer.

Keywords: transcription factor, proliferation, MCF7, MDA-MB-231

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer death among women.¹,³ The transition from nonmetastatic to metastatic breast cancer is characterized by the translocation of the primary lesion towards lymphatic sites. Therefore, accurate evaluation of metastasis in axillary lymph nodes is a crucial factor affecting medical management, surgery, and prognosis.⁴,⁵ In clinical treatment, tumor metastasis accounts for 90% of breast cancer deaths.⁶ The small GTPase Rab9 was identified from a screen of Rab family proteins demonstrating localization similar to Arf-like3 GTPase of the tracheal system.⁷ Rab9 belongs to the Ras superfamily of small monomeric GTPases which are central regulators of intracellular trafficking events.⁸,⁹ Late endosomes carry Rab9 protein bound to its organelle membranes¹⁰–¹² by using different machineries for recruitment¹³ and locating to different domains while on the same endosome.¹⁰ Moreover, Rab9 has been proven to be a vital member for the retrograde transport of the M6P-R along the trans-Golgi Network (TGN) pathway.¹⁰,¹⁴ Rab9 is also involved in sorting pathways toward the endolysosomal compartments, which was characterized by the spatio-temporal dynamics of Rab9 shown in live cell imaging.⁹

In this study, we reported the correlation between Rab9 expressions in breast cancer tumorigenesis. To explore the physiological role of Rab9 in breast cancer, we manipulated Rab9 expression by RNAi technique in human breast cancer cell lines MCF-7 and MDA-MB-231 and studied its effect on cell proliferation, migration, invasion,
and apoptosis. Our results revealed the mechanism of Rab9’s involvement and demonstrated that Rab9 is a potential novel candidate for breast cancer therapy.

Materials and methods

Agents
DMEM was ordered from HyClone Company (Cat# SH30021, Logan, UT, USA). Antibiotics and CCK-8 were ordered from Beijing Solarbio Science & Technology Company (Beijing, China). Lipofectamine6000 was purchased from Thermo Fisher Scientific (Cat# 11668–027, Waltham, MA, USA). Ultrapure RNA extraction kit, HiFiScript cDNA Synthesis Kit, and fluorescence quantitative PCR kit UltraSYBR Mixture were all purchased from Beijing Kangwei Century Company (CwBio, Beijing, China). Primers were synthesized from Genewiz Company (Beijing, China). Matrigel was ordered from BD Company (Cat# 356234, Franklin Lakes, NJ, USA). ECL developer was ordered from PTG (Cat# B500024, Proteintech, Chicago, IL, USA).

Primary antibodies, including anti-Rab9 (Cat# ab179815, 1:1,000), Bim (Cat# ab32158, 1:1,000), Bcl-2 (Cat# ab32124, 1:1,000), Active-Caspase3 (Cat# ab32042, 1:1,000), Bax (Cat# ab182733, 1:1,000), AKT (Cat# ab8805, 1:1,000), p-AKT (Cat# ab38449, 1:1,000), Cyclin D1 (Cat# ab134175, 1:1,000), p-P70/S6K (Cat# ab32158, 1:1,000), and P70/S6K (Cat# ab184551, 1:1,000), were rabbit anti-human, and were purchased from Abcam (Cambridge, UK). The primary antibody, anti-GAPDH, was purchased from PTG (Cat# 10494–1-AP, 1:5,000). HRP sheep anti-rabbit/mouse secondary antibodies (1:5,000) were ordered from PTG.

Cell culture
Human breast cancer cell lines MCF7 and MDA-MB-231 were ordered from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL Penicillin, and 0.1 mg/ml streptomycin at 37°C with 5% CO₂. Cells were trypsinized during their logarithmic phase and re-plated to fresh 6-well plates for transfection.

Transfection
The plasmid containing the pcDNA3.1-Rab9 or siRNA-Rab9 interference sequence was confirmed by enzyme cutting and sequencing. 5 µL of lipofectamine 6,000 liposomes was dissolved into 150 µL serum-free non-antibiotic medium. The 2.5 µg plasmids were dissolved in 150 µL serum-free antibiotic-free medium, and the control group used an empty vector of pcDNA3.1. The liposome and plasmid mixture were mixed within 30 minutes. Then, 500 µL of the liposome and plasmid mixture was added into each cell and incubated for 24 hours.

Fluorescence quantitative PCR
Total RNA was extracted with the Ultrapure RNA extraction kit and cDNA was synthesized with the HiFiScript cDNA Synthesis Kit. The expression of Rab9 was detected using fluorescence quantitative PCR. The primers are listed below:

Rab9:
Forward: 5′-GGACAACGCGACTATCCTT-3′
Reverse: 5′-TGAGCTAGGCTTGGGCTTTC-3′.
The expression volume was calculated by the 2⁻ΔΔCt method from three independent experiments. Experiments were performed in triplicate.

Western blot
Transfected cells were incubated on 6-well plates to 90%–95% confluent. Forty-eight hours after transfection, cells were collected and lysed with RIPA buffer. Twenty micrograms of protein samples were heated at 95°C for 5 minutes to denature and then loaded onto the 10% SDS-PAGE gel. After transfer, the PVDF membrane was blocked with 5% fat-free milk for 1 hour and probed with primary and secondary antibodies in blocking solution. After washing, ECL substrate was added to the membrane for immunoblot analysis. QUANTITY ONE software scanned the grayscale value, taking GAPDH as the internal reference control, and calculated the relative expression amount of each protein by protein/internal reference. Experiments were performed in triplicate.

CCK8 cell viability and proliferation assays
Normal cultured MCF7 and MDA-MB-231 cells were trypsinized and counted to make the suspension, seeding ~1,000 cells to each well of a 96-well plate. Every 24 hours, the proliferation was determined by adding 10 µL of CCK8 reagent and incubating for 90 minutes. Then, the OD value at 450 nm was measured on a microplate reader. The proliferation curves were drawn with the OD values. Experiments were performed in triplicate.

Cell migration and invasion assays
For the invasion assay, the chilled Matrigel was diluted with serum-free DMEM at a ratio of 1:6, and 100 µL was applied to the transwell chamber before 4 hours of incubation. The migration experiment procedure was similar to the invasion experiment, but the cubicle wasn’t required to be processed with Matrigel.
After transfection for 24 hours, 100 µL cell suspensions were transferred to the chamber, and 600 µL DMEM containing 10% FBS were added to the outside of the chamber. Following 48 hours of incubation, cells were fixed with 4% paraformaldehyde for 15 minutes and stained with 0.1% crystal violet for 5 minutes. Then, the filters were cut off and mounted on slides. The images were analyzed, and the number of invading cells was counted under the microscope. Experiments were performed in triplicate.

**Cell apoptosis analysis with flow cytometer**

After transfection and incubation in serum-free medium, cells were harvested and washed with precooled PBS. Five microliters of Annexin V-FITC was added and incubated at 25°C, followed by the addition of 10 µL PI for an additional 2 minutes of double staining. The number of apoptotic cells was determined using a flow cytometer (BD FACS Canto II, BD Biosciences, San Jose, CA, USA). Statistical analysis was performed using Flowjo software. Experiments were performed in triplicate.

**Bi-luciferase reporter assay**

According to the promo and GeneCard database, the promoter of Rab9 has the ability and transcription factor XBP1. In the binding region, the binding sequence is AGTCAT, suggesting that the expression of Rab9 may be regulated by the transcription factor XBP1. A synthetic dual luciferase report carrier pGl3.0-Basic-Rab9-promoter and a XBP1 expression vector pcDNA3.1-XBP1 were used. Three nights prior to the experiments, 293 t-cells were counted. The second day, cell density reached 80% transfection. During the experiments, the dual luciferase report carrier and pcDNA3.1-XBP1 expression vector were transfected into 293 t-cells. The experiment was divided into three groups: the pGl3.0-Basic group, the ppGl3.0-Basic-Rab9-promoter group (Rab9-promoter), and the pGl3.0-Basic-Rab9-promoter and pcDNA3.1-XBP1 sublet (Rab9-promoter+XBP1). After 48 hours, cells were collected, and fluorescence values were detected using the Dual Luciferase Reporter Gene Assay Kit (Beyotime, China) and the TECAN multifunctional enzyme marker instrument.

**Statistical analyses**

The results were denoted as a plus or minus SD, indicated by the figure legend. The data represented three separate experiments. SPSS 18.0 software was used for statistical analysis of data. The Student’s t-test was used to determine the meaning of all pairs of interest comparisons. P<0.05 was statistically significant.

**Ethical statement**

Ethical approval for this investigation was obtained from the Research Ethics Committee, Shandong Cancer Hospital Affiliated to Shandong University.

**Results**

**Rab9 shows abnormal high expression levels in breast cancer tissues**

The expression of Rab9 in breast cancer tissues was significantly higher than that in adjacent cancer tissues. Tissue sections were analyzed immunohistochemically with an EliVision™ (IHC two-step assay kit) plus kit. The breast cancer tissue sections showed much more yellow staining, which was indicative of Rab9 expression (Figure 1A).

Experimental results from 30 cases of cancer and 30 cases of para-cancer tissues showed that breast cancer tissue expressed a significantly higher expression rate (24/30) than that of adjacent tissue (8/30) (Figure 1B).

We next detected the mRNA levels and protein expression of Rab9 in different breast cell lines, including MCF10A, MCF7, MDA-MB-435, MDA-MB-231, and MDA-MB-415. Our results showed that the mRNA and protein expression levels of Rab9 were significantly higher in MCF7 and MDA-MB-231 cells (Figure 1C and D). Therefore, we chose these two cell lines to perform our experiments.

**Patients with low expression of Rab9 in breast cancer have a better prognosis**

The GEPIA database was used to analyze the prognostic value of Rab9 in breast cancer. Through online data analysis, we found that the overall prognosis of patients with low expression of Rab9 in breast cancer was significantly better than that of patients with high expression of Rab9. The correlation between Rab9 expression and OS was assessed using Kaplan–Meier survival analysis. Patients with high Rab9 expression showed worse OS than those with lower Rab9 expression (Figure 1E, P<0.01). These results showed that Rab9 was upregulated in breast cancer tissues compared with adjacent tissues. This suggests the possible involvement of Rab9 in the tumorigenesis of breast cancer and that Rab9 can be a good candidate for breast cancer treatment.

**Rab9 plays important roles in the proliferation of breast cancer cells**

The cells were sorted in five different groups based on treatment type: Blank cells (Con, blank control group), siRNA control group (siNC, interference of negative control group), pcDNA3.1 empty vector group (O-NC, express the negative control group),
Figure 1 Rab9 is overexpressed and predicts poor prognosis in breast cancer. (A) Immunohistochemical detection of breast cancer tissue microarray. (B) Rab9 expression in breast cancer tissues (24/30) was significantly higher than that of adjacent cancer tissues (8/30). A total of 30 cases of cancer and 30 cases of adjacent tissues were tested. (C) Rab9 mRNA levels in different breast cancer cell lines. (D) Rab9 protein expression in different breast cancer cell lines. (E) GEPIA was used to analyze the prognosis of Rab9, and the prognosis of patients with high expression of Rab9 was poor.

Notes: *P<0.05; **P<0.01; ***P<0.001.

pcDNA3.1-Rab9 expression group (O-Rab), and siRNA-Rab9 (siRab9, Rab9 interference group). In the siNC and O-NC groups, the mRNA and protein levels of Rab9 were similar to that of the blank control group (Figure 2A–E). The siRNA-Rab9 significantly decreased the mRNA and protein levels of Rab9, while the transfection of pcDNA3.1-Rab9 resulted in robust overexpression of Rab9 in both MCF7 and MDA-MB-231 cells (Figure 2A–E). These results indicated successful knockdown and overexpression of Rab9 in both cell lines.

We next detected the effect of overexpression and knockdown of Rab9 on the proliferation of breast cancer cells. CCK8 assays were performed to evaluate the effect of the overexpression and reduction of Rab9 in cell proliferation. The OD curves showed that overexpression of Rab9 significantly increased the viability at 72 hours. On the contrary, knockdown of Rab9 significantly decreased the viability of both MCF7 and MDA-MB-231 cells (Figure 2F and G). The results of the CCK8 proliferation experiment showed that Rab9 is a key factor in the proliferation of MCF7 and MDA-MB-231 cells.

Rab9 affects the migration and invasion of breast cancer cells

Trans-well assays were performed to investigate the effect of Rab9 on the cell migration and invasion in breast cancer in vitro. The migration and invasion cells in siNC and O-NC groups showed no difference from the blank control group (Figure 3). Crystal violet stained cells on the siRNA-Rab9 transfected group were much lower than the siNC groups and in the O-Rab9 groups. The numbers of migrating cells and invading cells were significantly increased in both MCF7 and MDA-MB-231 cells (Figure 3). These results showed that Rab9 facilitates the migration and invasion of breast cancer cells.

Knockdown of Rab9 induces apoptosis of breast cancer cells

The apoptosis of MCF7 and MDA-MB-231 cells were analyzed by cell flow-cytometry. Cells were transfected with siRNA of Rab9 (siRab9) or control siRNA (siNC) for 48 hours...
and then double stained by Annexin V and PI. Annexin V-positive and PI-negative staining identified apoptotic cells. Our results showed that the transfection of siRab9 significantly increased the rate of apoptosis of both MCF7 and MDA-MB-231 cells compared to those transfected with siNC or cells that were non-transfected cells, respectively (Figure 4A–C).

In order to investigate the mechanism of apoptosis induction by siRNA of Rab9 in breast cancer cells, we performed Western blot analysis to determine the role of siRab9 on the regulation of the expression of apoptosis-related gene expression. We detected the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax in siRab9 transfected MCF7 and MDA-MB-231 cells by Western blot. Compared to the control and siNC groups, siRab9 transfection suppressed Bcl-2 expression, but increased Bax expression in MCF7 cells (Figure 4D and E). In MDA-MB-231 cells, there was a significant increase in Bax expression, but only a mild decrease in Bcl-2 expression (Figure 4D and F). There are three Bim isoforms, which can only neutralize certain members of the pro-survival Bcl-2 sub-family; all induce apoptosis. The transfection of siRab9 significantly increased the expression of Bim in both MCF7 and MDA-MB-231 cells (Figure 4D–F). These results indicated that siRab9 increased apoptosis through involvement with Bcl-2 family members. The Bax/Bcl-2 ratio and the expression of Bim in both breast cancer cells was increased which suggests that the apoptosis triggered by Rab9 knockdown may occur through the mitochondrial pathway. Because Caspase3 is involved in the development and regulation of apoptotic cell death,, we next detected Caspase3 expression. Transfection of siRab9 robustly increased the expression of Caspase3 (Figure 4D–F).

**Rab9 affects the activation of the PI3K/AKT signaling pathway in breast cancer cells**

To explore the molecular mechanism of Rab9 on inhibition of tumor cells, we evaluated the role of siRab9 on the PI3K/AKT pathway in both MCF7 and MDA-MB-231 cells. The PI3K pathway is involved in many cellular processes and is abnormally activated in a variety of tumors, including breast cancer. Our results showed that, in the siRab9 transfection group, there was no change in expression levels of AKT or mTOR, but the phosphorylation of p-AKT and p-mTOR was significantly decreased (Figure 4G), resulting in a decreased ratio of p-AKT/AKT (Figure 4H and I). These results proved that knockdown of Rab9 suppressed the phosphorylation of AKT and mTOR. Moreover, p-P70/S6K/P70/S6K level was also inhibited by the transfection of siRab9 (Figure 4G–I). To further confirm this conclusion, we overexpressed Rab9 in these two breast cancer
cells and investigated the role of Rab9 on the expression of PI3K/AKT pathway members. Our data demonstrated that the overexpression of Rab9 significantly increased the expression of p-AKT and p-mTOR (Figure 4G). The ratio of pAKT/AKT and p-mTOR/mTOR increased (Figure 4H and I). As expected, p-P70/S6K/P70/S6K was also increased (Figure 4G–I). These results indicated that the GTPase activity of Rab9 plays an important role in the phosphorylation of PI3K/AKT pathway members.

Transcription factor XBP1 is a key regulator of Rab9

According to the promo and Gene Card database, the promoter of Rab9 has a binding region for transcription factor XBP1 and the binding sequence is AGTCAT, suggesting that the expression of Rab9 may be regulated by the transcription factor XBP. To confirm this interaction between Rab9-promoter and XBP1, we performed biluciferase reporter assay as described in the Section “Materials and methods”. The results showed a significant increase in luciferase levels after co-transfection of XBP1 with Rab9-promoter (Figure 5A). This indicated that XBP1 could bind with the Rab9 promoter. To verify the regulatory effect of XBP1 on Rab9, we first introduced interference of the XBP1 mRNA with siXBP1 in the MCF7 cell line. The mRNA levels of XBP1 could be significantly decreased by the siXBP1, and the overexpression of Rab9 was not able to overcome this reduction (Figure 5B). These results showed that XBP1 could be successfully downregulated.

Figure 3 Rab9 promotes migration and invasion in breast cancer cells. (A and B) Transwell assay was used to detect cell migration and invasion. (C–F) The results showed that Rab9 overexpression facilitated cell migration and invasion, while knockdown of Rab9 inhibited cell migration and invasion.

Notes: Compared with siNC: *P<0.05; **P<0.01. Compared with O-nc: #P<0.05; ##P<0.01.
Figure 4 Rab9 inhibits apoptosis by activation of the AKT pathway. (A–C) The apoptosis of MCF7 and MDA-MB-231 cells was analyzed using cell flow-cytometry. (D–F) Apoptosis-related proteins were detected by Western blot. GAPDH served as a loading control. (G–I) Expression of AKT signaling pathway members was detected by Western-blot.

Notes: The values of the band intensity represent the densitometry estimation of each band normalized to GAPDH (compared with siNC: *P<0.05; **P<0.01; ***P<0.001 compared with O-nc: #P<0.05; ##P<0.01).
Figure 5 Transcription factor XBP1 is a key regulator of Rab9. (A) Bi-luciferase reporter assay as described in the Section “Materials and methods”. The luciferase level was significantly increased after co-transfection of XBP1 with Rab9-promoter. (B) The mRNA level of XBP1 could be significantly decreased by the siXBP1, and the overexpression of Rab9 was unable to overcome the reduction of XBP1 mRNA. (C) CCK8 OD curves showed that knockdown of XBP1 significantly inhibited the proliferation of MCF7 cells and that overexpression of Rab9 partly recovers this decrease. (D–F) Transwell assay showed the effect of XBP1 on the migration and invasion of MCF7 cells. (G and H) Western blot results showed XBP1 affected AKT and P70/S6K via Rab9.

Notes: Compared with sinc: *P<0.05; **P<0.01; compared with O-nc: #P<0.05; ##P<0.01.

Next, we detected the effect of XBP1 on the proliferation of MCF7 cells with CCK8 analysis. The OD curves showed that, at 72 hours post-transfection, knockdown of XBP1 significantly inhibited the proliferation of MCF7 cells, and the overexpression of Rab9 could only partly overcome this decrease (Figure 5C).

Transwell assay detected cell migration and invasion after transfection, and the results showed that siXBP1 robustly inhibited the migration and invasion of MCF7 cells, while the overexpression of Rab9 could significantly reverse this inhibition caused by siXBP1 (Figure 5D–F).

Because knockdown of Rab9 can inhibit the AKT signaling pathway, we hypothesize that XBP1 reduction will also suppress the AKT signaling pathway due to its regulatory effect on the Rab9 promoter. Results of Western blot analysis showed that the levels of phosphorylated form of AKT and P70/S6K were significantly decreased by siXBP1, and the overexpression of Rab9 could significantly reverse the inhibition of the AKT pathway caused by siXBP1 (Figure 5G and H). These above results indicated that XBP1 is a key regulator of Rab9 and further confirmed that Rab9 play important roles in the tumorigenesis of breast cancer.
Discussion

Breast cancer is the most frequent malignancy in the world, and a very common type of non-epidermal cancer.1 In 2017, breast cancer displayed the highest incidence among female cancers worldwide.22 Rab9 is a small GTPase localized in the TGN and late endosomes3 that functions in the recycling of mannose-6-phosphate receptors (MPRs) and the promotion of autophagy and lysosome biogenesis.23

In this study, we investigated the effect of Rab9 in the tumorigenesis of breast cancer. For the first time, we reported the Rab9 facilitated the proliferation, migration, and invasion on human breast cancer cell lines MCF7 and MDA-MB-231 cells and elucidated the underlying mechanisms. The overexpression or knockdown of Rab9 affects apoptosis of both MCF7 and MDA-MB-231 cells via the AKT pathway. In addition, we found that transcription factor XBP1 is a key regulator of Rab9. Knockdown of XBP1 can suppress the increased effects of overexpressed Rab9 on breast cancer, which further confirmed the conclusion that silencing of Rab9 can inhibit breast cancer cells. Our results reflected a potential pathologic role of Rab9 in breast cancer, and this is the first time it has been reported that knockdown of Rab9 or XBP1 could inhibit the proliferation and migration of breast cancer cell MCF7 and MDA-MB-231 and promote their apoptosis.

Our Western blot results showed that the expression of Bcl family proteins changed. BAX (BCL2 associated X) is a pro-apoptotic protein belonging to the Bcl2 family.24 During our research, we found that transfection of siRNA Rab9 increased the expression levels of Active Caspase-3 and the ratio of Bax/Bcl-2. Bim can only neutralize certain members of the pro-survival Bcl-2 sub-family to induce apoptosis.17 The knockdown of Rab9 significantly increased the expression of Bim in both MCF7 and MDA-MB-231 cells. These data suggest that reduction of Rab9 triggers the mitochondrial pathway of apoptosis by affecting Bcl-2 family members.

To further elucidate the role of Rab9 in breast cancer cells, we studied the PI3K pathway members. We discovered that knockdown of Rab9 suppressed the activation of the phosphoinositide PI3K pathway in MCF7 and MDA-MB-231 cells. Rab9 knockdown decreased AKT and mTOR phosphorylation which occurred upstream of PI3K; however, AKT and mTOR were not changed. The P70/S6K was also decreased by knockdown of Rab9. These data prove that Rab9 promotes proliferation, migration, and apoptosis inhibition via the AKT pathway, suggesting that Rab9 is a potential target for breast cancer treatment.

In addition, our results first reported that transcription factor XBP1 is a key regulator of Rab9. XBP1 interaction with Rab9 and knockdown of XBP1 could also inhibit the proliferation and migration of breast cancer cells by the suppression on Rab9. And, without XBP1, the increasing effects of Rab9 overexpression on breast cancer were lost. These results further confirmed the conclusion that silencing of Rab9 can inhibit breast cancer cells.

Above all, we prove that Rab9 promotes proliferation, migration, and apoptosis inhibition via XBP1 and the AKT pathway. Our findings indicate the role Rab9 plays in the tumorigenesis of breast cancer and its regulation by XBP1. These results suggest that miRNA of Rab9 and XBP1 can be considered potential breast cancer inhibitors in therapy. The specific mechanism of Rab9 and cancer conventional therapies requires further investigation in the future.

Disclosure

The authors report no conflicts of interest in this work.

References