RAB27A Promotes the Proliferation and Invasion of Colorectal Cancer Cells

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Abstract

Colorectal cancer (CRC) is the most commonly diagnosed form of cancer worldwide. Though significant advances in prevention and diagnosis, CRC is still one of the leading causes of cancer-related mortality globally. RAB27A, the member of RAB27 family of small GTPases, is the critical protein for intracellular secretion and was reported to promote tumor progression. However, it is controversial for the role of RAB27A in CRC progression, so we explored the exact function of RAB27A in CRC development in this study. Based on the stable colon cancer cell lines of RAB27A knockdown and ectopic expression, we found that RAB27A knockdown inhibited SW480 colon cancer cell proliferation and clone formation, whereas ectopic expression of RAB27A in RKO colon cancer cells facilitated cell proliferation and clone formation, indicating that RAB27A is critical for colon cancer cell growth. In addition, our data demonstrated that the migration and invasion of colon cancer cells were suppressed by RAB27A knockdown, but promoted by RAB27A ectopic expression. Therefore, RAB27A was identified as an onco-protein in mediating CRC development, which may be a valuable prognostic indicator and potential therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality and the fourth most frequently diagnosed cancer worldwide, accounting for 10.2% of all new cancer cases, and 9.2% of all cancer deaths^[1, 2]. It has been reported that men are more susceptible than women to the disease, and most of these cases occur in individuals above 65 years of age^[3, 4]. Though different risk factors were found to be associated with CRC development, more than two thirds of CRC arise from environmental and genetic factors. Poor dietary habits, alcohol and tobacco consumption, red meat, age, obesity and lack of physical activity are found to play a key role in the pathogenesis of CRC^[5, 6]. Despite significant advances in prevention and diagnosis, CRC is still one of the most deadly cancers worldwide^[7, 8]. CRC arises when colonic epithelial cells acquire a series of genetic or epigenetic mutations that increase cell growth and survival^[9-11]. Therefore, identification of new genes associated with CRC development is important for early diagnose and treatment.

Rab proteins are a family of small monomeric Ras-like GTPases, which is composed of more than 70 mammalian members^[12]. Rab27 is the member of Rab proteins family, which consists of two isoforms, including RAB27A and RAB27B^[13]. As RAB27 proteins are essential for vesicle exocytosis and exosome release, which is critical for regulating the tumor microenvironment, Rab27 recently has been reported to be involved in regulating cancer development^[14, 15]. It has been reported that RAB27A facilitates breast cancer progression by promoting cancer cell invasion and metastasis^[16]. In melanoma, RAB27A has been identified to promote tumor growth by increasing vesicular trafficking and exosome secretion^[14, 17, 18]. Moreover, RAB27A acts as the downstream of NFκB signaling pathway to promote colon cancer cells stemness via up-regulation of cytokine secretion^[19]. In addition, the level of RAB27A is negatively correlated with grade progression and worse prognosis in all grades of gliomas^[20]. These studies

demonstrate that RAB27A acts as the onco-protein in promoting human cancer development. In consistent with the viewpoints, RAB27A highly expresses in colon cancer tumor compared with the adjacent normal tissues, and RAB27A high expression is positively associated with advanced tumor node metastasis (TNM) stage^[21]. However, RAB27A was also found to be down-regulated in the primary tumor tissues of CRC, comparing with the adjacent normal tissues^[22]. Moreover, low expression of RAB27A in tumor tissues exhibited a significant correlation with TNM stage^[22]. The role of RAB27A in these two reports is completely contrary, so it is controversial for the role of RAB27A in mediating CRC progression.

In this study, we explored the exact role of RAB27A in CRC development based on the establishment of stable colon cancer cell lines of RAB27A knockdown and ectopic expression. We found that cell proliferation, clone formation, migration and invasion was suppressed by RAB27A knockdown in SW480 colon cancer cells, but promoted by RAB27A ectopic expression in RKO colon cancer cells. Therefore, our results demonstrate RAB27A acts as the onco-protein and plays a key role in promoting CRC development.

Results

Establishment of RAB27A knockdown and ectopic expression cell lines.

To determine the exact function of RAB27A on CRC progression, we constructed some plasmids to down-regulate or up-regulate RAB27A expression. As shown in Figure 1A, the protein levels of RAB27A were significantly lower in 293T cells transfecting with RAB27A shRNA plasmids than that in the negative control group. In addition, transfection of Flag-tag RAB27A significantly up-regulated RAB27A expression in 293T cells (Fig. 1B). Therefore, RAB27A shRNA plasmids and Flag-tag RAB27A plasmid are efficient in mediating RAB27A knockdown and ectopic expression, respectively. Then, SW480 colon cancer cells were transfected with RAB27A shRNA plasmid, and RKO colon cancer cells were transfected with Flag-tag plasmids. G418 sulfate (geneticin) was used to select cell clones that stably expressed neo genes. The levels of RAB27A mRNA and protein were significantly lower in RAB27A knockdown SW480 cells than that in the negative control SW480 cells (Fig. 1C and E). In contrast, RAB27A mRNA and protein level significantly increased in RKO cells stably transfected with Flag-tag RAB27A plasmids, comparing with the negative control RKO cells (Fig. 1D and F).

In conclusion, we established the colon cancer cell lines stably down-regulating or up-regulating RAB27A expression by using RAB27A shRNA or Flag-tag RAB27A plasmids.

RAB27A is essential for colon cancer cell growth.

To explore the function of RAB27A on colon cancer proliferation, CCK-8 reagent assay kit was used to measure the growth of RAB27 knockdown and the negative control SW480 cells. As shown in Figure 2A,

OD values were significantly smaller in RAB27A knockdown SW480 cells than that in the negative control cells, indicating that RAB27A knockdown inhibits SW480 cells growth. In contrast, the OD values in RKO cells stably expressed Flag-tag RAB27A were significantly bigger than that in the negative control groups (Fig. 2B), suggesting that RAB27A ectopic expression promotes RKO cells growth. In addition, we also determined the effect of RAB27A on cell clone formation by using soft agar colony formation assay. As shown in Figure 3A and B, the areas of cell clone in RAB27A knockdown SW480 cells were significantly smaller than that in the negative control SW480 cells, whereas cell clone areas in RKO cells with stable RAB27A expression were significantly larger than that in the negative control group (Fig. 3C and D), indicating that RAB27A is necessary for clone formation of colon cancer cell cells.

Collectively, our data demonstrate that RAB27A is essential for colon cancer cell growth.

RAB27A is involved in mediating the migration and invasion of colon cancer cells.

By using the stable cell lines of RAB27A knockdown and ectopic expression, we also explored the effect of RAB27A on cell migration and invasion by using the 24-wells Transwell chamber with or without Matrigel. As shown in Figure 4A and B, the migrated cell number in RAB27A knockdown SW480 cells was significantly smaller than that in the negative control, indicating that RAB27A plays a critical role in mediating SW480 colon cancer cell migration. Furthermore, we found that RAB27A ectopic expression promoted RKO cell migration (Fig. 4C and D), confirming the critical role of RAB27A in mediating colon cancer cell migration. In addition, we also found that RAB27A knockdown inhibited SW480 cell invasion (Fig. 5A and B), whereas RAB27A overexpression facilitated RKO cell invasion (Fig. 5C and D), suggesting that RAB27A is also necessary for colon cancer cell invasion.

In conclusion, RAB27A is essential for colon cancer cell migration and invasion.

Discussion

RAB27A was firstly identified as an oncogenic factor in melanoma, which has been found to promote melanoma cell proliferation via activating ERK signaling pathway^[23]. Following studies demonstrate that RAB27A also promotes melanoma cell invasion and metastasis by facilitating the secretion of pro-invasive exosomes^[14]. Moreover, RAB27A enhances breast cancer cell invasiveness and metastasis potential through facilitating the secretion of insulin-like growth factor II^[16]. In this study, we found that the proliferation, migration and invasion of colon cancer cells were significantly suppressed by RAB27A knockdown, but promoted by RAB27A ectopic expression. Therefore, our data demonstrate that RAB27A plays an oncogenic role in CRC progression. It has been reported that RAB27A promotes CRC tumor formation and growth via facilitating the secretion of VEGF and TGF- β , which improves the self-renewal of colon cancer stem cells^[19]. In addition, ectopic expression of RAB27A abrogates the inhibitory effects

of miR-582-5p on colon cancer cell proliferation and invasion^[24]. Together with our data, these studies demonstrate RAB27A acts as an onco-protein in the procession of CRC development.

Previous studies have demonstrated that RAB27A was highly up-regulated and acts as a valuable prognostic indicator in various cancers, including hepatocellular carcinoma^[25], gliomas^[20], esophageal squamous cell cancer^[26] and clear cell renal cell carcinoma^[27]. However, it is controversial for RAB27A expression in colon cancer tumor tissues. It has been reported that RAB27A highly expressed in CRC tumor tissues compared with matched adjacent tissues^[21]. Moreover, high level of RAB27A protein positively correlated with certain substantial clinical attributes^[21]. In contrast, RAB27A was also found to be down-regulated in CRC primary tumor tissues compared with the matched adjacent normal tissues, and down-regulation of RAB27A is associated with some clinical features, including advanced TNM stage, distant metastasis, and local recurrence^[22]. Two possible reasons may explain the discrepancy between the two reports. Firstly, these results may be a result of selection bias when collecting enrolled patients, including gender and age. Secondly, RAB27A protein level in tumor and the normal tissues was determined by IHC, which is somewhat subjective in evaluating RAB27A expression.

In summary, this is the first study to explore the exact function of RAB27A on CRC progression based on the stable cell lines of RAB27A knockdown and ectopic expression. We found that the proliferation, migration and invasion of colon cancer cells were significantly suppressed by RAB27A knockdown, but promoted by RAB27A ectopic expression. Therefore, RAB27A is essential for colon cancer development, and may be a valuable prognostic indicator and potential therapeutic target for CRC.

Materials And Methods

Cells and reagents

293T cells, SW480 and RKO colon carcinoma cell lines were purchased from the Byotime Institute of Biotechnology (Jiangsu, China). The cells were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Kangyuan Biology, China). The crystal violet was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation analysis

Enhanced Cell Counting Kit-8 (CCK-8) assay kit (Byotime, China) was used for cell viability test as per the manufacture's instructions. Cells were seeded in 96-well plates at the concentration of 3000 cells per well, and routinely cultured at 37°C overnight. Subsequently, cells were added with CCK-8 reagent (10 µl/well) and incubated at 37°C for 1-2 h. Finally, the absorbance values (OD Values) at 450 nm were measured by a microplate reader (Bio-Tek, USA).

Soft agar colony formation

The low-melting-point agarose (Biowest, Spain) was prepared as 1.2% and 0.6% solution in ddH₂O by using high pressure sterilization. The 0.6% agar/medium base layer was prepared by mixing 1.2% agarose solution with 2-fold DMEM medium at 1:1 (volume), and then added to a 6-well cell culture plate to prevent cell attachment to the plastic base. Cells were collected by trypsinization, counted and then suspended with DMEM media at the density of 1×10^4 cells/ml. The 0.3% agar upper layer was prepared by mixing 0.6% agarose with 2-fold cell medium at 1:1 (volume). Subsequently, 1 ml upper agarose mixed with 100 μ l single-cell suspension ($\sim 1,000$ cells) were added to each well and solidified at room temperature. Cells were cultured at 37°C in a humidified incubator containing 5% CO₂ for 1 month, and cell clones were visualized by microscopy (4 $\times$). At least three random fields were selected and the cell clone area was calculated.

Transwell cell migration and invasion assay

Cells transfected with either RAB27A knockdown or ectopic expression plasmids were collected and resuspended in DMEM. For cell migration assay, 4×10^4 cells were seeded in the upper well of the Transwell chamber (Corning, USA). And for cell invasion assay, 8×10^4 cells were seeded in the chamber (Corning, USA). Meanwhile, 600 μ l DMEM containing 20% fetal bovine serum was added to the lower well of the chamber. The cells were routinely cultured for 48 h, after which cells on the upper-side of the membrane were cleaned out and cells on the underside were stained with 800 μ l crystal violet for 30 mins. At least three random fields were selected for cell counting.

Western blotting

The cells were lysed with IP buffer and protease inhibitors, and the protein concentration was determined by BCA assay (Beyotime, China). The proteins (30 μ g per lane) were separated on a 12% polyacrylamide gel and transferred onto a PVDF membrane. Then the membrane was blocked with 5% non-fat milk at room temperature for 1 h and incubated with RAB27A monoclonal antibody (1:1000, Santa Cruz, USA), β -actin monoclonal antibody (1:5000, proteintech, USA) and flag polyclonal antibody (1:1000, MBL, China) at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibody, the proteins were detected by ELC reaction system and gel-imaging system (Tanon, China).

Real-Time PCR (qPCR)

Total RNA of the RAB27A interference and ectopic expression cells were extracted by RNA extraction Kit (BioTeKe, China), and then reverse-transcribed into cDNA by *EasyScript* First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China) as per the manufacturer's instructions. Real-time PCR was performed using BeyoFast™ SYBR Green qPCR Mix (Beyotime, China) and CFX96 Touch™ Real-Time PCR

System (Bio-Rad). The primer sequences were shown in Table 1. β -actin was used as control for normalization and $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of RAB27A.

RAB27A knockdown

The DNA fragments encoding shRNA targeting Rab27A were cloned into the GV102 vector. The constructed RAB27A interference plasmids and control plasmid were subsequently transfected into 293T cells for 48 h. Total protein was extracted and western blotting was performed to select the plasmid with the highest interference efficiency. For stable transfection, SW480 cells were transfected with selected interference plasmid and control plasmid, then G418 was used for resistance screening. The sequences of RAB27A shRNA and control shRNA were shown in Table 2.

RAB27A ectopic expression

Human RAB27A cDNA with Flag-tag sequence was cloned into the GV362 vector to express Flag-tag RAB27A and the plasmids were transfected into 293T cells. To get the stable cell lines that express Flag-tag RAB27A, RKO cells were transfected with Flag-tag RAB27A plasmids, and G418 was used to screen cells with high level of Flag-tag RAB27A expression.

Statistical analysis

GraphPad prism 7 software was used to analyze the data and construct statistical graphs. If variance was homogeneous, statistical significance was analyzed by performing a two-tailed Student's t-test, otherwise, the t'-test was used for comparison between two groups. $P < 0.01$ was defined as significantly different. All experiments were repeated at least three times, and the data are expressed as the mean $\pm$ s.d. from representative experiments.

Declarations

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Author contributions statements

(I) Conception and design:W.X.; (II) Provision of study materials:Q.L.,H.Z. and W.D.; (III) Collection and assembly of data:N.G.,Y.H.,Z.Z.,H.Z.,F.Z. and Qiu.L.; (IV) Data analysis and interpretation:J.Y. and Q.L.; (V) Manuscript writing: All authors; All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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Tables

Table 1

The primers used to amplify cDNA in Real-Time PCR

Gene name	Forward	Reverse
Human RAB27A	5'-ACAGCGTTCTTCAGAGATGCTATGG -3'	5'- TCTGCGAGTGCTATGGCTTCCT-3'
Human Actin	5'- ACTCTTCCAGCCTTCCTTCC-3'	5'- CGTACAGGTCTTTGCGGATG-3'

Table 2

The sequences of RAB27A shRNA and control gene

shRNA	RAB27A shRNA sequences
shRNA-1#	5'- GATCCCgcTGCCAATGGGACAAACATACTCGAGTATGTTTGTCCCATTGGCAGCTTTTTGGAT- 3' 3'- GGCGACGGTTACCCTGTTTGTATGAGCTCATACAAACAGGGTAACCGTcgAAAAACCTATCGA- 5'
shRNA-2#	5'- GATCCCcaGGAGAGGTTTCGTAGCTTACTCGAGTAAGCTACGAAACCTCTCCTGTTTTTGGAT- 3' 3'- GGGTCCTCTCCAAAGCATCGAATGAGCTCATTGATGCTTTGGAGAGGacAAAAACCTATCGA- 5'
shRNA-3#	5'- GATCCCccTGTGCATTTGAATTGTATACTCGAGTATACAATTCAAATGCACAGGTTTTTGGAT-3' 3'- GGGGACACGTAAAACTTAACATATGAGCTCATATGTAAAGTTTACGTGTccAAAAACCTATCGA- 5'
Con shRNA	5'-GATCCCTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCCGAGAATTTTTGGAT-3' 3'-GGAAGAGGCTTGACAGTGCAGAGCTCTGCACTGTGCAAGCCTCTTAAAAACCTATCGA-5'

Figures

Figure 1

Identification of RAB27A knockdown and ectopic expression.

(A) and (B). RAB27A knockdown and overexpression in 293T cells. Cells were transfected with RAB27A shRNA and Flag-tag RAB27A, respectively. Then, western blotting was used to determine RAB27A and Flag. Actin was used as a loading control. (C), (D), (E) and (F). Identification of RAB27A knockdown or overexpression in colon cancer cells. SW480 cells were transfected with RAB27A shRNA or Con shRNA plasmids, and RKO cells were transfected with Flag-tag RAB27A or the negative control plasmids. G418 was used to select cell clones stably expressed neo genes. The mRNA level of RAB27A was measured by using real time PCR, and the protein level of RAB27A was determined by western blot. Actin was used as a loading control. The level of RAB27A mRNA was normalized by comparing mRNA level in all groups with that in the negative control groups. All the relative levels of RAB27A mRNA are shown as the mean $\pm$

s.d. of three separate experiments (n=3) and they were analyzed by a two-tailed t-test. All the western blot analyses were repeated independently three or more times, with representative images were shown.
** $P<0.01$.

Figure 2

The effect of RAB27A on the proliferation of colon cancer cells.

(A). RAB27A knockdown suppresses the proliferation of SW480 cells. The RAB27A knockdown and the negative control SW480 cells were seeded in 96 well plates, and cell proliferation was measured by using CCK-8 assay kits. (B). RAB27A overexpression promotes RKO cell proliferation. The RAB27A overexpression RKO cells and the negative control cells were seeded in 96 well plates, and cell proliferation was measured by using CCK-8 assay kits. The optical density (OD) values (%) are shown as the mean $\pm$ s.d. (n=3), and analyzed by a two-tailed t-test. ** $P<0.01$.

Figure 3

The effect of RAB27A on clone formation of colon cancer cells.

(A). and (B). RAB27A knockdown inhibits clone formation of SW480 cells. RAB27A knockdown and the negative control SW480 cells were mixed with the melting agarose gel and plated in 6-well plates for 4 weeks, and cell clones were determined by microscopy (40 $\times$). (C). and (D). RAB27A overexpression promotes clone formation of RKO cells. The RAB27A overexpression and the negative control RKO cells were mixed with melting agarose gel and plated in 6-well plates for 4 weeks, and the cell clones were determined by microscopy (40 $\times$). All the experiments were repeated three times independently, and more than three fields in each group were observed, and the representative images are shown. The area of cell clones was measured by using image J software, and were analyzed by a two-tailed t-test. ** $P<0.01$.

Figure 4

The effect of RAB27A on colon cancer cell migration.

(A) and (B). RAB27A knockdown suppresses the migration of SW480 cells. RAB27A knockdown and the negative control SW480 cells were seeded in the Transwell for 48 h, and the migrated cells were stained with the crystal violet. (C) and (D). RAB27A overexpression facilitates RKO cell migration. RAB27A overexpression cells and the negative control cells were seeded in the Transwell for 48 h, and the migrated cells were stained with the crystal violet. Cell migration was determined by microscopy (200 $\times$),

and more than three fields in each group were observed, then representative images are shown. All the migrated cells are shown as the mean $\pm$ s.d. of three separate experiments (n=3) and they were analyzed by a two-tailed t-test. **** $P < 0.01$.**

Figure 5

The effect of RAB27A on colon cancer cell invasion.

(A) and (B). RAB27A knockdown suppresses SW480 cell invasion. RAB27A knockdown and the negative control SW480 cells were seeded in the Transwell with overspreaded Matrigel for 48 h, and the invaded cells were stained with the crystal violet. (C) and (D). RAB27A overexpression facilitates RKO cell invasion. RAB27A overexpression and the negative control RKO cells were seeded in the Transwell overspreaded with Matrigel for 48 h, and the invaded cells were stained with the crystal violet. Cell invasion was determined by microscopy (200 $\times$) and more than three fields in each group were observed, then the representative images are shown. All the invaded cells in the images were calculated and shown as the mean $\pm$ s.d. of three separate experiments (n=3) and they were analyzed by a two-tailed t-test. **** $P < 0.01$.**

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