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Original Research

miR-409-3p inhibits the proliferation and migration of human ovarian cancer cells by targeting Rab10

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Abstract: Ovarian cancer is a leading cause of gynecological cancer-related mortality. It has been reported that miR-409-3p is involved in the proliferation and migration of cancer cells. However, the role of miR-409-3p in ovarian cancer has not been well studied. The present study aimed to investigate the functional role of miR-409-3p in the pathogenesis of ovarian cancer, and its potential mechanism. It was found that the expression levels of miR-409-3p in 6 ovarian cancer tissues were upregulated. Through proliferation, migration and colony formation assays, it was revealed that overexpression of miR-409-3p inhibited the proliferation and migration of ovarian cancer cells. It was predicted from bioinformatics assays that the complementary binding sites were within miR-409-3p and Rab10. It was also demonstrated that the downregulation of the expression of Rab10 reversed the miR-409-3p downregulation-induced abnormal proliferation of ovarian cancer cells. These results suggest that miR-409-3p expression can be used as a predictive marker for the prognosis of ovarian cancer. Thus, the miR-409-3p/Rab10 axis may be a novel therapeutic target for ovarian cancer.

Key words: Ovarian cancer; miR-409-3p; Rab10.

Introduction

Ovarian cancer is one of the common gynecological malignancies, It ranks the top three among the causes of death of female gynecological tumors in China and the fifth in the world.The causes of high mortality are difficulty in early diagnosis, limited treatment and easy recurrence, which pose a serious threat to women's health (1, 2). Due to a lack of specific early symptoms of ovarian cancer, the disease is not easily detected at the onset stage. Thus, most patients are at the advanced stage of ovarian cancer at the time of diagnosis, and since the current treatment methods for the disease are poor, the 5-year survival is less than 30 % (2). Although a good prognosis may be achieved in some ovarian cancer patients through surgery, a large number of ovarian cancer patients do not benefit from surgical intervention (3). Therefore, studies on the pathogenesis of ovarian cancer, and the identification of new molecular markers/targets for its early diagnosis and treatment are of considerable interest to clinicians.

MicroRNAs (miRNAs) are a class of non-coding RNAs that range in size from 18 to 22 nucleotides (4). Through complete or incomplete complementary pairing with the 3' end of the non-coding region of the target gene mRNA, a miRNA may enhance or inhibit the translation of the target mRNA, thereby regulating gene expression at the post-transcriptional level (5). MicroRNAs (miRNAs) are involved in several important life processes such as growth, differentiation, cell proliferation, apoptosis, invasion, migration, and metabolism (6). In terms of molecular biology, miRNA has attracted more attention in studies on the occurrence, development and prognostic factors of ovarian cancer, suggesting that miRNA is related to the occurrence, development, metastasis, drug resistance and recurrence of ovarian cancer (20). For example, the miRNA-214, the miRNA-200-a and significant expression of miRNA-100, may be associated with the progress of ovarian tumors (21), the high expression of miRNA-205 is associated with the transfer of ovarian cancer (22), the miRNA - 600 inhibits OVCAR ovarian cancer - 3 cell migration and invasion (23). miRNAs play a variety of roles in ovarian cancer, the miRNA research results and the diagnosis, treatment and prognosis of ovarian cancer are closely linked. It may have some theoretical value for its early diagnosis and treatment. One of the miRNAs that have received considerable attention in recent years is miR-409-3p (7, 8). Studies have shown that miR-409-3p is downregulated in various cancers of the digestive system (9), and the urinary system (10). However, not much is known about the role of miR-409-3p in ovarian cancer, and the mechanism associated with this role. In the present study, RT-PCR was used to determine the expression level of miR-409-3p in ovarian cancer tissues and cells, and *in vitro* cell experiments were used to determine the effect of the miR-409-3p expression on cell proliferation and apoptosis.

Rab proteins are small GTPases belonging to the Ras protein family, consisting of about 200 amino acids, including the conserved G domain and highly variable N-terminal and C-terminal (24). In recent years, studies

have shown that Rab protein is closely related to tumor, especially plays an important role in tumor cell proliferation, migration, signal transduction and other aspects (25). At present, more than 60 human Rab proteins are known (11). One of these proteins i.e. Rab10, a member of the Rab-GTPase family, is involved in tumorigenesis and vesicle transport (12). Studies have shown that Rab10 is correlated with the occurrence and development of osteosarcoma, liver cancer and other tumors (26,27), and the present study was also focused on the possible role of Rab10 as a pathogenic factor in ovarian cancer, and as a direct target of miR-409-3p.

Materials and Methods

Cell lines and culture conditions

Ovarian cancer cell lines (SKOV3 and OVcar) and normal ovarian cell line (IOSE80) were purchased from the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China. The cells were cultured at 37°C in RPMI-1640 medium (without HEPES) containing 10 % fetal bovine serum, 1% double-antibody, and Taxol (800 ng/ml) in an incubator with 95 % air and 5 % CO_2 (Mlbio, Shanghai, China) (13).

RNA extraction and qRT-PCR

Total cellular and tissue RNA were extracted with TRIZOL reagent (Thermo Fisher, USA). TaqMan probes (Applied Biosystems, USA) were used to quantify miRNAs. The reactions were performed in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min, with GAPDH as an internal control. The primer sequences used were: miR-409-3p: forward 5′-GAATGTTGCTCGGTGA-3′; reverse 5′-GTGCAGGGTCCGAGGT-3′; Rab10: forward 5′-GGGAATTCATGGAGGCCATCTGGCTGTAC-3′, reverse: 5′-CGGGATCCCTAGCACAA ACATCTCCTCTC-3 ′; GAPDH: forward 5 ′-GCACCGTCAAGGCTGAGAAC-3′; reverse 5′-ATGGTGGTGAAGACGCCAGT-3'.

Western blotting

The SKOV3, OVcar and IOSE80 cells were washed twice with ice-cold PBS and centrifuged at 12000 g for 10 min at 4 °C. Total proteins were isolated from the cells using cell lysis buffer (Thermo Fisher, MA, USA). The proteins were subjected to 10 % SDS–PAGE and transferred to PVDF membranes. Next, the membranes were incubated with 0.5% bovine serum albumin for 1 h at room temperature, followed by rinsing with PBS. Thereafter, the membranes were incubated overnight with primary antibodies (diluted 1:1000) at 4°C. Then, the membranes were rinsed with PBS and incubated with secondary antibody at room temperature for 1-2 h. Finally, the bands were evaluated with scanning densitometry through enhanced chemiluminescence (Thermo Fisher, MA, USA). The GAPDH gene served as a loading control. The protein bands were quantified using Image J Software (14).

Cell viability assay

The SKOV3, OVcar and IOSE80 cells were seeded in 96-well plates, each at a density of 1 x 10^3 cells per well. Cell viability was determined at the outset, and daily for three days, using MTT assay. The absorbance of the formazan solution in each well was read at 570 nm and recorded.

Plasmid construction and luciferase reporter assay

The 3'-UTR sequence of Rab10 was searched from the NCBI, as well as the 3'-UTR of Rab10 that contained the presumed miR-409-3p binding sites (https://www.targetscan.org). The pMIR-Rab10-3'-UTR plasmid was constructed using pMIR-REPORT Luciferase NC (15).

Colony formation assay

Colony-formation assays were used to determine the effect of Rab10 on colony formation in SKOV3 cells. Lentivirus-infected SKOV3 cells were seeded at a low density (1000 cells/well) in 6-well plates and cultured for 10-14 days. The colonies were stained with 0.1% crystal violet and quantified using Image J software (National Institute of Health, Bethesda, MD).

Plasmid construction and siRNA interference

Rab10 knockdown was accomplished by transfecting the cells with siRNA. Rab10 and control siRNA were synthesized by Synthgene (China). Moreover, the control plasmid (pCMV6) and overexpression plasmid (pCMV6-Rab10) were products of Synthgene (China).

Statistical analysis

The results are expressed as mean ± standard deviation of the mean of three independent experiments. Statistical comparisons were done using Student's t-test. Differences with $p < 0.05$ were considered statistically significant.

Results

Rab10 was highly expressed in ovarian cancer tissues and cell lines

As shown in Figure 1A, Rab10 levels were significantly increased in the ovarian cancer tissues, when compared to normal tissues. Moreover, Figure 1B shows that Rab10 expression was significantly upregulated in the two ovarian cancer cell lines when compared to IOSE80 cells. These results suggest that Rab10 is ovarian cancer -related protein.

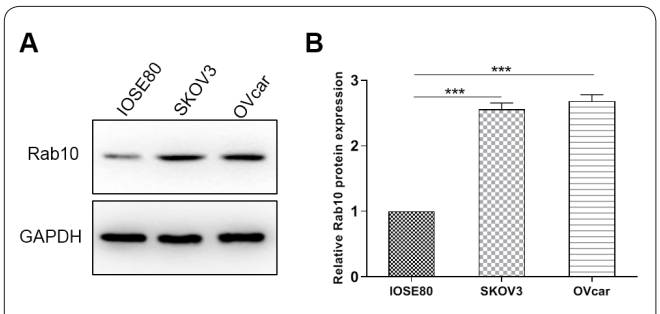

Figure 1. Detection of Rab10 in ovarian cancer cell lines. (A) WB analysis the expression of Rab10 protein in ovarian cancer cell lines. (B) Quantify the protein bands of the Rab10 protein. Compare to IOSE80 group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t*-test).

Knockdown Rab10 inhibited metastasis of ovarian cancer cells

Results in Figures 2A and 2B indicate that Rab10 siRNA visibly reduced Rab10 expression in SKOV3 cells, relative to non-transfected cells. Moreover, the knockdown of Rab10 expression significantly inhibited the migratory capacity of SKOV3 cells (Figures 2D and 2E). Thus, Rab10 knockdown produced a negative effect on ovarian cancer cell proliferation.

Rab10 is a target gene of miR-409-3p

The Rab10 gene was found to contain two putative sites in the 3'-UTR untranslated region (3'-UTR) that matched the miR-409-3p seed region (Figures 3A and 3B). Results from qRT-PCR revealed that the expression level of miR-409-3p was markedly downregulated in the ovarian cancer cells (Figure 3C).

To verify whether miR-409-3p directly regulated Rab10, the expression of Rab10 in cells under conditions of miR-409-3p overexpression and knockdown were determined. As shown in Figure 3D, the expression of Rab10 was significantly upregulated by transfection with anti-miR-409-3p. However, the expression

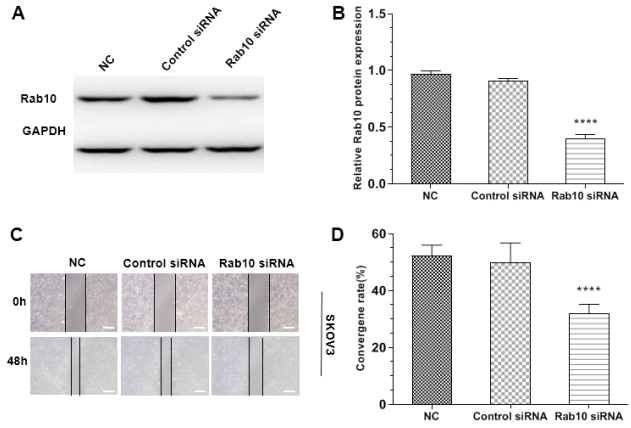

Figure 2. Role of Rab10 in the metastasis of ovarian cancer cells. (A) The expression of Rab10 protein in SKOV3 cells after transfection (WB). (B) Quantify the protein bands of the Rab10 protein. (C, D) Wound-healing analysis of cell migration in SKOV3 cell. Compare to control NC group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t*-test).

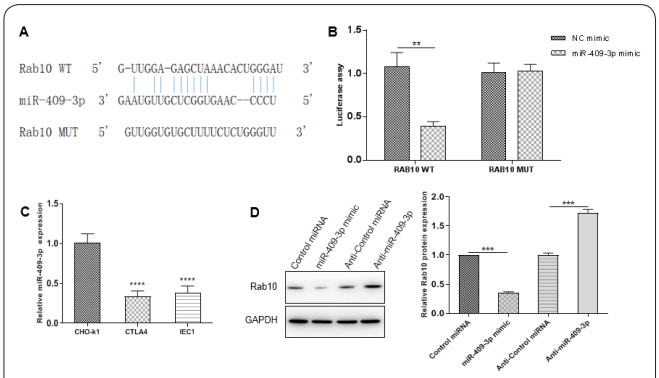

Figure 3. miR-409-3p directly regulates Rab10. (A) Binding side of Rab10 and miR-409-3p. (B) The relative luciferase signal of SKOV3 cells. (C) The mRNA expression levels of miR-409-3p in SKOV3 cells. (D) WB analysis the expression of Rab10 protein in SKOV3 cells after transfection. Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t*-test).

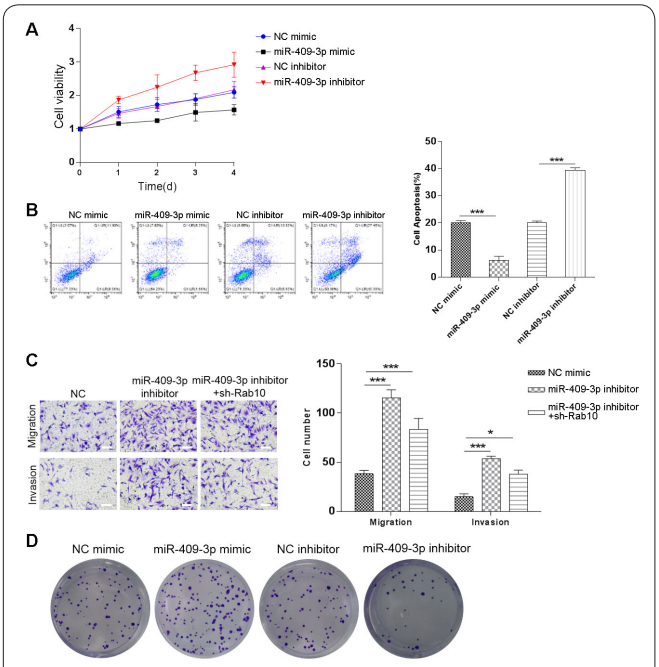

Figure 2. miR-409-3p regulates ovarian cancer cell proliferation by targeting Rab10. (A) Cell viability of SKOV3 cells was measured by MTT assay. (B) Comparison of apoptosis levels of CLTA4 cells after transfection. (C) Transwell migration and invasion assay analyses in SKOV3 cells. (D) Colony formation assay of cell proliferation after SKOV3 cells were transfected with plasmid. Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t*-test).

of Rab10 was significantly downregulated by transfection with miR-409-3p mimics. Results from Western blotting showed that the expression level of miR-409-3p was negatively correlated with that of Rab10.

Involvement of miR-409-3p in the development of ovarian cancer via regulation of Rab10

As shown in Figure 4A, when miR-409-3p was knocked down with miR-409-3p inhibitor, cell viability was significantly decreased, relative to the other three groups. Similarly, the level of apoptosis also increased significantly when miR-409-3p expression levels were suppressed (Figure 4B). In addition, miR-409-3p inhibitors increased the proliferation and migratory capacity of SKOV3 cells. However, co-transfection of miR-409-3p inhibitor with siRab10 counteracted the effect of the miR-409-3p inhibitor on cell proliferation and migration, resulting in significant decreases in cell proliferation and migration, when compared with control (untreated) cells where cell proliferation and migration were increased (Figure 4C). Similar results were obtained in the clone formation experiment, as shown in Figure 4D.

Discussion

Ovarian cancer is one of the most common gynecologic malignancies, and it often presents at an advanced stage (16). The absence of early symptoms and lack of reliable and specific early clinical diagnostic indicators have consistently resulted in the late diagnosis of the disease in 75% of patients (3). This results in poor clinical outcomes, largely due to unsolved problems of metastasis, recurrence and the issue of intrinsic or acquired drug

resistance. Thus, a better understanding of the pathways underlying tumor progression might aid in the design of more effective treatment strategies for ovarian cancer.

The Rab10 protein, a member of the Ras superfamily with guanosine triphosphatase activity, regulates the maintenance of inner membrane transport of eukaryotic cells, natural immunity, and development of the nervous system (15). In particular, increased expression of Rab10 has been seen in various cancers. Upregulated Rab10 expression has been reported in liver cancer patients, relative to the healthy control group (17). In the inflammatory state, the upregulation of Rab10 inhibits the activity of macrophages. The present research has revealed that Rab10 is expressed in ovarian cancer cells and that its upregulation is related to the abnormal expression of miR-409-3p. High expression of Rab10 is associated with poorly-differentiated tumor cells, pathologically advanced tumor, intravascular tumor density, and poor prognosis (18). Studies have shown that Rab10 is overexpressed in esophageal squamous cell carcinoma and its expression level is related to the proliferation and differentiation of esophageal epithelial cells (14). In previous studies, knockdown of miR-107 increased Rab10 expression, leading to abnormal growth of esophageal epithelial cells stimulated by external physical and chemical factors(14). The clone formation experiment revealed that the knockdown of Rab10 led to decreases in proliferation and colony formation capacity, thereby increasing apoptosis *in vitro* and inhibiting tumor growth *in vivo*. Previous studies have reported that the upregulation of Rab10 is related to cancer metastasis (19). Rab10 is a signal pathway mediated by CirRNA-PTN and miR-432-5p, and it has been shown to enhance the invasion and migration of glioma cells (15). Recently, there have been reports that Rab10 plays an important role in the viability of acute leukemia cells. In the present study, it was found that knockdown of Rab10 inhibited the migration and invasion of human ovarian cancer cells. This was confirmed by results from the wound healing test. In effect, the results obtained in this study have revealed the important role of Rab10 in the pathogenesis of ovarian cancer.

This study has revealed that Rab10 is a pathogenic and novel regulatory factor in ovarian cancer. This finding may open new approaches in the treatment of ovarian cancer in the future.

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Conflicts of interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yuko Ohno and Hua Hu; Yanyi Li, Li Chen, Beibei Zhang, Yuko Ohno, Hua Hu collected and analysed the data; Yanyi Li wrote the text and all authors have read and approved the text prior to publication. Yuko Ohno and Hua Hu are the co-corresponding authors.

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