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Overexpression of miR-32 inhibits the proliferation and metastasis of ovarian cancer cells by targeting BTLA

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Abstract. – OBJECTIVE: The purpose of this study was to explore the role of microRNA-32 (miR-32) in ovarian cancer and the possible underlying mechanism.

PATIENTS AND METHODS: Ovarian cancer tissues were collected from 100 patients diagnosed with ovarian cancer in our hospital. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to detect the expression levels of miR-32 and its target gene in ovarian tissues. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was conducted to detect the proliferation of ovarian cancer cells. Meanwhile, the transwell and wound healing assays were used to evaluate the migration and invasion abilities of ovarian cancer cells, respectively. Bioinformatics (including TargetScan, miRDB, and microRNA) were used to predict the target genes of miR-32. Furthermore, The Dual-Luciferase reporter gene assay was performed to verify the binding relationship.

RESULTS: MiR-32 was significantly downregulated in both ovarian cancer tissues and cells. The overexpression of miR-32 significantly inhibited the proliferation, migration, and invasion of ovarian cancer cells. B and T lymphocyte associated (BTLA) was screened out as a target gene of miR-32. Furthermore, BTLA could counteract the effects of miR-32 on ovarian cancer cells.

CONCLUSIONS: Acting as a suppressor gene, miR-32 inhibited the malignant behaviors of ovarian cancer cells by regulating its target gene BTLA.

Key Words:
MicroRNA-32 (miR-32), Ovarian cancer, B and T lymphocyte associated (BTLA).

Introduction

Ovarian cancer is one of the most common malignant tumors of the reproductive system, seriously threatening women's life. The incidence of ovarian cancer ranks 6th among female tumors. Meanwhile, its mortality ranks first among gynecological tumors, with at least 120,000 deaths worldwide annually[1]. Due to its deep anatomical location, early diagnosis of ovarian cancer is difficult. Nearly 75% of patients have already accompanied by pelvic metastasis or other distant metastasis when diagnosed, whose 5-year survival rate is only 30%[2]. Therefore, a deeply understanding of the development mechanism of ovarian cancer cells, and searching for molecular markers for effective inhibition of ovarian cancer metastasis, as well as new strategies for gene targeted therapy, are urgent problems which need to be solved currently.

Current studies have indicated that miRNAs play extremely important regulatory roles in the biological processes. Meanwhile, they can participate in a variety of tumor-related signaling pathways[3-6]. This is closely related to tumor production, proliferation, invasion, migration, and other processes[7-9]. Thus, the role of miRNA in tumorigenesis has gradually made it a potential target for tumor detection and treatment[10,11]. MiR-32 is closely associated with the occurrence and development of malignant tumors. Li et al[12] have found that the overexpression of miR-32 can significantly inhibit the proliferation and colony formation capability of non-small-cell lung cancer cells. Moreover, it can impair its migration and invasion by regulating the epithelial-mesenchymal transition (EMT) phenotype. Liu et al[13] have also confirmed that miR-32 suppresses the malignant behaviors of HeLa cells. However, whether miR-32 is involved in the pathogenesis of ovarian cancer has not been fully elucidated.

In this study, the expression of miR-32 in ovarian cancer tissues and adjacent normal tissues, as well as SKOV3 and IOSE80 cells was first detected. Subsequently, miR-32 mimics or miR-32 inhibitor was transfected into SKOV3 cells to explore the effects of miR-32 on cell proliferation and metastasis. Our findings might provide theoretical support for the treatment of ovarian cancer.

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Patients and Methods

Tissues

This study was approved by the Ethics Committee of the Ninth People's Hospital of Suzhou. Signed written informed consents were obtained from all participants before the study. 100 paired ovarian cancer tissues and adjacent normal tissues were collected from patients who received treatment in our hospital from June 2016 to October 2018. All tissue specimens were removed by experienced surgeons. No patient received radiotherapy or chemotherapy before surgery.

Cells Culture

Human SKOV3 and IOSE80 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 μg/mL streptomycin, and 1000 U/mL penicillin in an incubator with 5% CO_2 at 37°C. The cells were digested with trypsin and passaged when cell density reached 80% to 90%.

MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

After transfection, SKOV3 cells were digested into single-cell suspension and seeded into 96-well plates. 150 μL of complete medium containing 3×10^3 cells was added to each well. 4 replicate wells were set for each group. 50 μL of premixed miRNAs in each group was added to 96-well plates, followed by culture in a 37°C, 5% CO_2 incubator. After every 24 hours, 20 μL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 4 hours of incubation at 37°C with 5% CO_2. Next, MTT solution was sucked out and 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by shaking horizontally for 15 minutes. The absorbance value at the wavelength of 490 nm was measured. Finally, the number of cells was represented by the absorbance value.

Wound Healing Assay

The transfected cells for 48 hours in the above three groups were digested and seeded into 24-well plates. Then, the cells were incubated in an incubator with 5% CO_2 at 37°C overnight. After 24 hours, a 10 μL tip was used for scratching in the plates. Next, the cells were washed and cultured with complete medium. After 48 hours, the cells were observed and photographed.

Transwell Assay

The cells were trypsinized, collected, and re-suspended in serum-free RPMI-1640 medium. 2×10^4 cells were seeded into the transwell chamber and cultured at 37°C for 24 hours. Subsequently, the chambers were removed, and the cells were wiped with a cotton swab. Next, the cells in the chamber were fixed with 1% polyformic acid for 10 min, washed with Phosphate-Buffered Saline (PBS) three times, and stained with hematoxylin for 5 min. Finally, the cells passed through the filter membrane were observed under a microscope. Five fields of view were randomly selected for each sample. The number of migrating or invading cells was counted.

Target Gene Verification Assay

Databases, including TargetScan, miRDB, and microRNA, were used to predict the potential relationship between miR-32 and BTLA. In brief, HEK293T cells (1.5×10^5 cells/well) were plated into 24-well plates and transfected with miR-32 mimics or negative control, respectively. After 48 hours of cultivation, the cells in each group were detected according to the instructions of Dual-Luciferase assay system (Promega, Madison, WI, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA in tissues, and SKOV3 and IOSE80 cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, complementary deoxyribose nucleic acid (cDNA) was synthesized according to the instructions of PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references for miR-32 and BTLA, respectively. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The expression level of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method. The primer sequences used in this study were as follows: BTLA, F: 5'-TCCAGGAAGCCGCCGACTC-3', R: 5'-ACTCGGACTGTCCGAGCGA-3'; miR-32, F: 5'-GCGTAACACATGCCTGCGGACTG-3', R: 5'-GATAGTGTCGAGGAACG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R:

5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

After washing with pre-cooled PBS for three times, the cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing 1% phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China). The protein concentration was determined by the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The total protein (25 μg protein per lane) was separated by 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto 0.45 mm polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were then blocked in 5% bovine serum albumin (BSA) for 1 hour. Next, the membranes were incubated with primary antibodies and corresponding secondary antibodies at 4°C for 24 h and at 37°C for 2 h, respectively. The protein labeled bands were detected in the dark, and the images were analyzed with the Scion Image Software. The ratio of target protein activity to GAPDH seemed as protein expression level.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The measurement data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered as statistically significant.

Results

MiR-32 Was Down-Regulated in Ovarian Cancer Tissues and SKOV3 Cells

To investigate whether miR-32 was associated with ovarian cancer development, we first tested its expression level in ovarian cancer tissues and adjacent normal tissues. The results showed that miR-32 expression in ovarian cancer tissues was significantly lower than that of adjacent normal tissues (Figure 1A). Besides, miR-32 expression in SKOV3 cells was significantly downregulated when compared with IOSE80 cells (Figure 1B). These results indicated that miR-32 might play an important role in the pathogenesis of ovarian cancer.

MiR-32 Was Highly Expressed in SKOV3 Cells After Transfection With MiR-32 Mimics

To further explore the role of miR-32, SKOV3 cells were transfected with 100 nmol miR-32 mimics and 200 nmol miR-32 inhibitors, respectively. After 48 hours, the RNA was extracted in each group and qRT-PCR assay was performed. As shown in Figure 2A, the expression level of miR-32 was remarkably upregulated in miR-32 mimics group compared with the blank control group. However, the expression level of miR-32

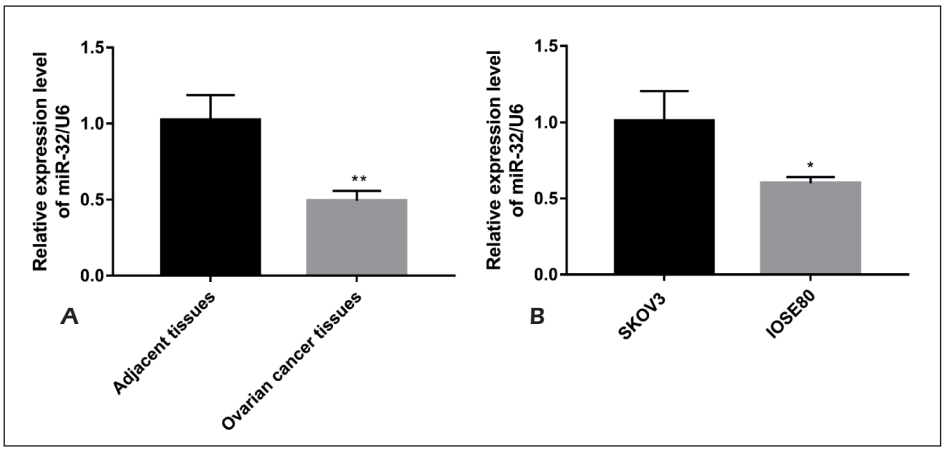

Figure 1. Concentrations of serum NO and ET-1 in the three groups of rats. a: $p<0.05$ *vs.* control group, and b: $p<0.05$ *vs.* model group. Figure 1. The expression level of miR-32 in ovarian cancer tissues and cells. **A**, MiR-32 expression level was downregulated in ovarian cancer tissues when compared with adjacent normal tissues. **B**, MiR-32 expression level was downregulated in SKOV3 cells when compared with normal ovarian IOSE80 cells. (*$p<0.05$, **$p<0.01$).

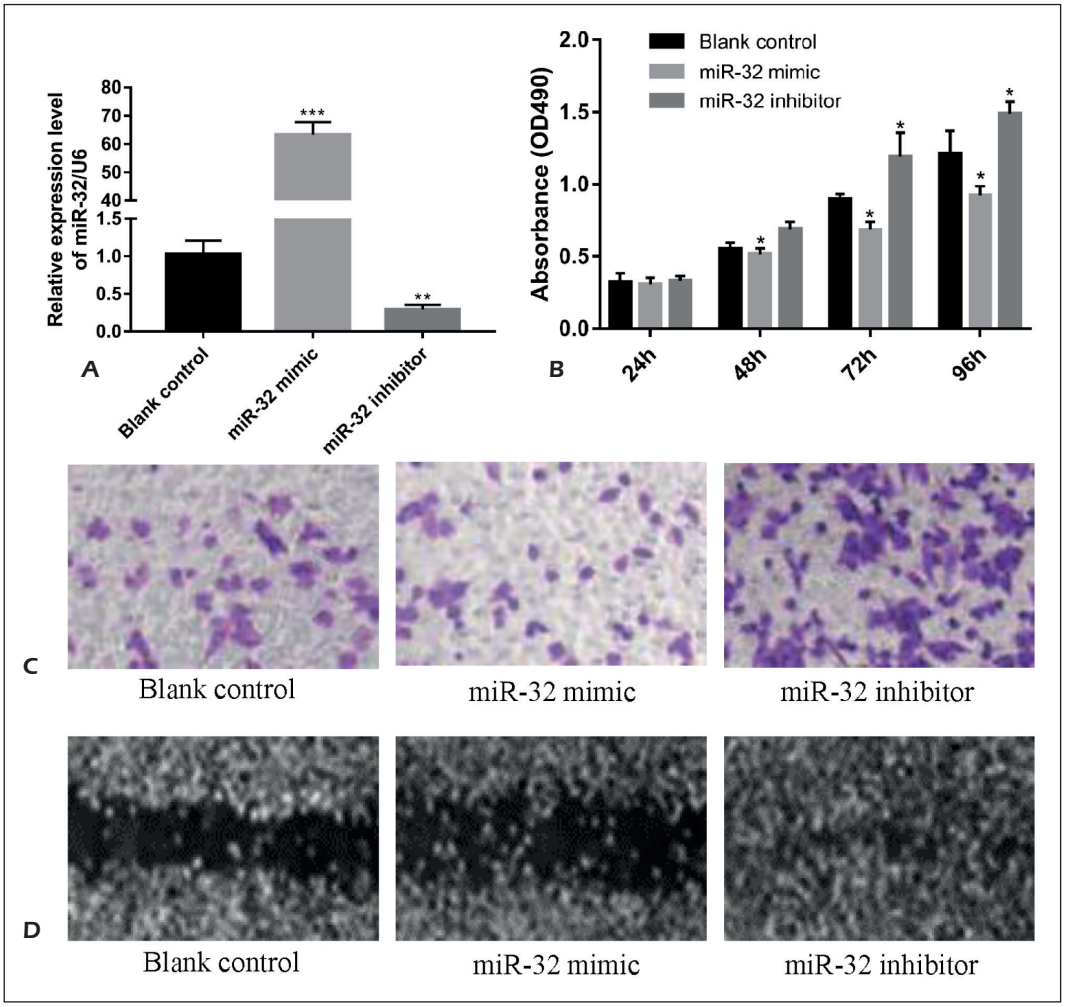

Figure 2. Effects of miR-32 on the proliferation, migration, and invasion of SKOV3 cells. **A**, The expression changes of miR-32 after transfection with mimics or inhibitor. Upregulation of miR-32 in SKOV3 cells inhibited cell proliferation (**B**), migration (**C**) (magnification x 40), and invasion **(D)** (magnification x 40) capabilities. (*p<0.05, **p<0.01, ***p<0.001).

in inhibitor group was significantly downregulated. These results suggested that miR-32 were successfully transfected into cells, which was the basis of subsequent cellular experiments.

MiR-32 Inhibited SKOV3 Cell Proliferation, Migration, and Invasion

After the establishment of SKOV3 cells with high expression of miR-32, MTT, wound healing, and transwell assays were performed to explore the effects of miR-32 *in vitro*. The results demonstrated that the upregulation of miR-32 significantly inhibited the proliferation, migration, and invasion of SKOV3 cells (Figure 2B). However, remarkably promoted cell proliferation, migration, and invasion were observed in miR-32 inhibitor group (Figures 2C, 2D). The results were consistent with our hypothesis, suggesting that miR-32 could inhibit the proliferation and distant metastasis of ovarian cancer cells.

BTLA Was a Direct Target Gene of MiR-32

Databases, including TargetScan, miRDB, and microRNA, were used to predict the direct target genes of miR-32. BTLA was finally screened out as the candidate. As shown in Figure 3A, miR-32 was predicted to bind to the 558-565 segment of the 3'-UTR of BTLA. Therefore, we constructed the Luciferase reporter vectors for performing the Luciferase reporter gene assay. The results found that miR-32 could remarkably reduce the Luciferase activity of the wild

type BTLA3'-UTR. However, no significant changes were observed in the Luciferase activity of mutant BTLA3'-UTR. These results indicated that miR-32 could directly bind to the 3'-UTR of BTLA (Figure 3B).

Furthermore, we detected BTLA expression in SKOV3 cells transfected with miR-32 mimics or miR-32 inhibitor. The results showed that both the mRNA and protein expression levels of BTLA significantly decreased in the miR-32 mimics group. However, BTLA expression level was significantly upregulated in the cells transfected with miR-32 inhibitor (Figures 3C, 3D, 3E). Taken all, these results revealed that BTLA was a direct target gene of miR-32.

Effects of BLTA on The Role of MiR-32

Next, we further explored whether high expression of BLTA could counteract the effects of miR-32 on SKOV3 cells. After pre-transfected with miR-32 mimics, the BLTA gene plasmids were transfected into SKOV3 cells. The results showed that the high expression of BTLA could significantly resist the anti-cancer effect of miR-32. This was proved by enhanced cell proliferation (Figure 4A), migration, and invasion (Figures 4B, 4C). All these findings demonstrated that BTLA could antagonize the effects of miR-32 on ovarian cancer cells.

Discussion

Ovarian cancer is one of the most common malignant tumors of the female reproductive system, with the highest mortality. The survival rate of patients is far from satisfactory due to the lack of effective early diagnosis and treatment methods.

Notably, studies on ovarian cancer have found that multiple microRNAs can influence its occurrence, pathological progression, and metastasis by regulating the expression of the target genes. Furthermore, it is closely related to chemothera-

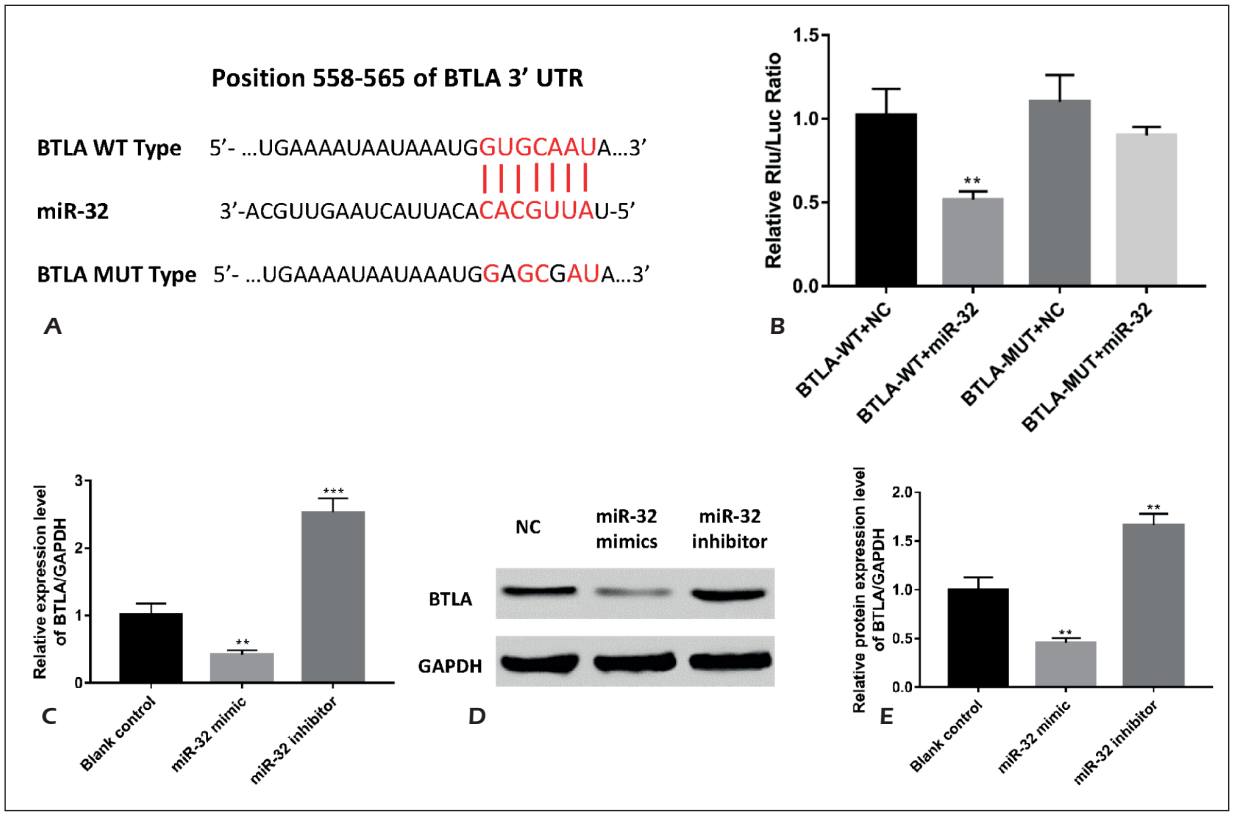

Figure 3. BTLA was a target gene of miR-32. **A**, The binding site of miR-32 and BTLA. **B**, Relative Rluc/Luc ratio. **C**, The mRNA expression level of BTLA in SKOV3 cells after transfection of miR-32 mimics or inhibitor, respectively. **D**, BTLA protein expression level in SKOV3 cells after transfection of miR-32 mimics or inhibitor, respectively. **E**, Qualification of protein expression level of BTLA. (**p<0.01, ***p<0.001).

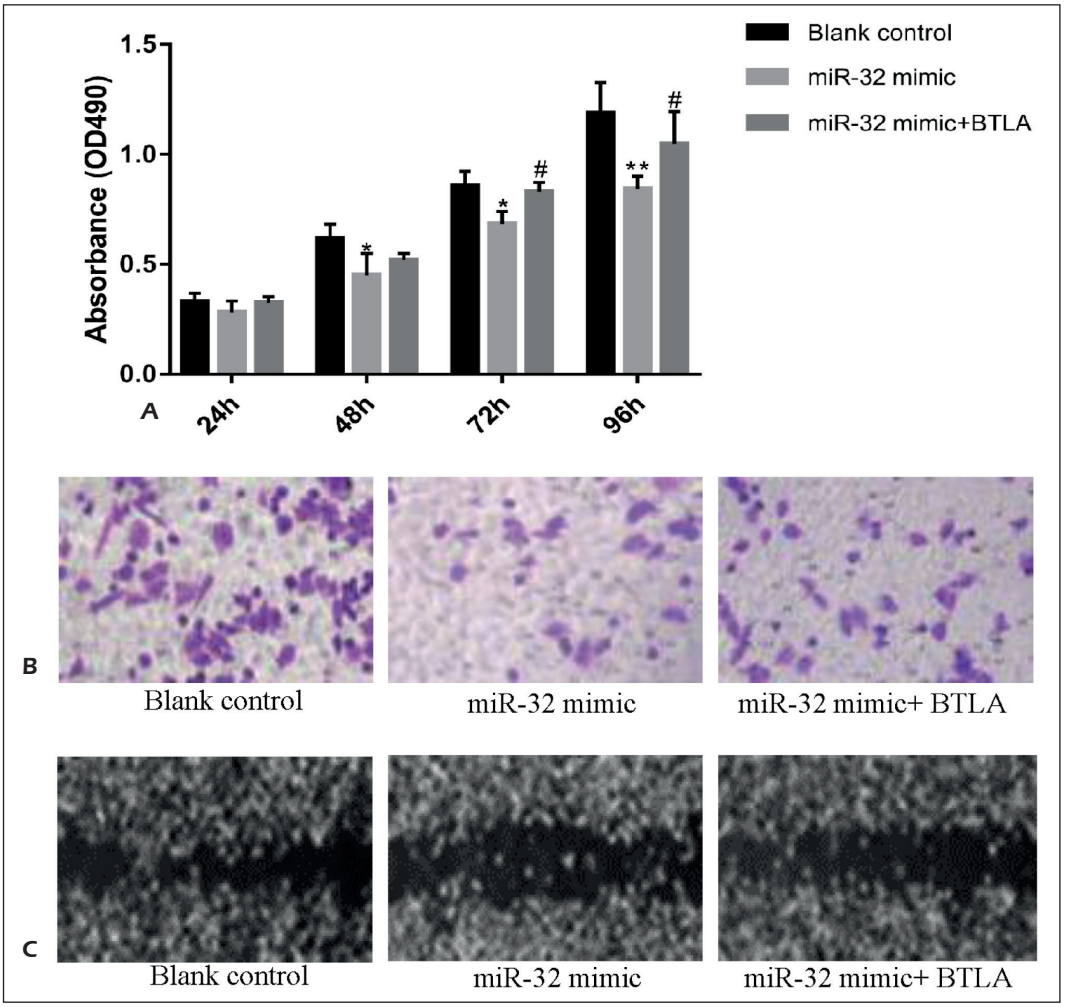

Figure 4. Figure 4. BTLA inhibited the effect of miR-32 on SKOV3 cells. BTLA improved the proliferation **(A)**, migration **(B)** (magnification x 40), and invasion **(C)** (magnification x 40) of SKOV3 cells. (*p<0.05 *vs.*blank control group; **p<0.05 *vs.* blank control group; $^{\#}p$<0.05 *vs.* miR-32 mimic group).

py resistance. Hu et al[14] have found that miR-1180 dramatically accelerates the proliferation and glycolysis of ovarian cancer cells. Wang et al[15] have indicated that miR-30a-5p is downregulated in ovarian cancer and can inhibit cell migration and invasion by downregulating the downstream target genes. Besides, Duan et al[16] have reported that miR-122 inhibits the migration and invasion, EMT, and metastasis of ovarian cancer cells.

MiR-32 has been confirmed to play an important role in non-small cell lung cancer[12,17] and prostate cancer[18]. However, its association with ovarian cancer has never been reported. In view of this, we firstly detected the miR-32 expression in ovarian cancer tissues and adjacent normal tissues. The results found that miR-32 was significantly downregulated in ovarian cancer tissues. Therefore, we speculated that miR-32 could negatively regulate the progression of ovarian cancer. Subsequent MTT, wound healing and transwell assays confirmed our speculation. After the overexpression of miR-32, the proliferation, migration, and invasion capabilities of ovarian cancer cells were significantly inhibited when compared with the negative control group. However, the proliferation and metastasis of ovarian cancer cells were remarkably upregulated in the cells transfected with miR-32 inhibitor.

It is well known that miRNA works by binding to the 3'-UTR of target genes. Therefore, we used websites to predict the direct target gene of miR-32 in ovarian cancer. Finally, BTLA was screened out as the direct target of miR-32. BTLA is a new member of the CD28 family[19], which is

highly expressed in activated T cells, especially Th1 cells[20]. It exerts immunosuppressive effects when interacting with the herpes virus (HVEM), negatively regulating lymphocytes, and inhibiting T cell activation[21]. Recently, numerous studies have reported that BTLA plays an important role in gastric cancer[22], gallbladder cancer[23], and breast cancer[24].

In our study, Dual-Luciferase reporter gene assay found that BTLA was a direct target of miR-32. QRT-PCR and Western blot assay was then performed to verify whether miR-32 could regulate the expression of BTLA. The results showed that the overexpression of miR-32 could significantly inhibit the mRNA and protein expressions of BTLA. Finally, we conducted rescue experiments to explore the underlying mechanism of miR-32 in inhibiting the proliferation and metastasis of ovarian cancer cells. The results indicated that the overexpression of BTLA could reverse the inhibitory effects of miR-32 on ovarian cancer cells. All these findings indicated that BTLA was involved in the anti-cancer effects of miR-32. Therefore, a negative feedback loop was formed between miR-32 and BTLA to regulate the malignant behaviors of ovarian cancer cells.

Conclusions

To sum up, miR-32 was lowly expressed in ovarian cancer cells and could negatively regulate BTLA expression by targeting its specific 3'-UTR region, thereby inhibiting the proliferation, migration, and invasion of ovarian cancer. As a tumor suppressor gene, miR-32 was expected to be a potential biomarker for the diagnosis and treatment of ovarian cancer.

Conflict of Interests

The authors declared that they have no conflict of interests.

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