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MiR-125a-5p inhibits EMT of ovarian cancer cells by regulating TAZ/EGFR signaling pathway

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Abstract. – OBJECTIVE: To investigate the influences of micro ribonucleic acid (miR)-125a-5p on epithelial-mesenchymal transition (EMT) of ovarian cancer cells by regulating the transcriptional co-activator with PDZ-binding motif (TAZ)/epidermal growth factor receptor (EGFR) signaling pathway.

PATIENTS AND METHODS: The human ovarian cancer cells were cultured, and miR-125a-5p was repressed by inhibitor and overexpressed by miRNA mimics. The expression of EMT-related proteins was measured via Western blotting (WB). The action target of miR-125a-5p was determined through a dual-luciferase reporter gene assay. The changes in protein levels were detected via WB.

RESULTS: MiR-125a-5p was down-regulated remarkably in ovarian cancer tissues. The expression level of serum miR-125a-5p in patients with ovarian cancer was lower than that in control group. After inhibition on miR-125a-5p, the expression level of E-cadherin, an epithelial indicator, was decreased, while that of Vimentin, an interstitial indicator, was increased. MiR-125a-5p contained a complementary site in the 3'-untranslated region (UTR) of TAZ messenger RNA (mRNA). The expressions of TAZ mRNA and protein in cells were down-regulated markedly after the overexpression of miR-125a-5p. The expressions of EGFR, phosphorylated EGFR (p-EGFR) and p-Akt were up-regulated in the cells transfected with miR-125a-5p mimics and those transfected with miR-125a-5p mimics overexpressing TAZ.

CONCLUSIONS: MiR-125a-5p can inhibit the EMT of ovarian cancer cells by regulating the TAZ/EGFR signaling pathway.

Key Words:
MiR-125a-5p, TAZ/EGFR, Ovarian cancer, Epithelial-mesenchymal transition.

Introduction

Ovarian cancer has the highest mortality rate among all the gynecological cancers, accounting for 4% of the deaths of female cancer patients. Histologically, there are 5 types of ovarian cancer, of which epithelial ovarian cancer (EOC) is the most lethal, accounting for about 90% of the cases reported[1, 2]. Due to the lack of early biomarkers and atypical symptoms, EOC becomes the fifth major cause of cancer death among women around the world. Although some key driver genes and core signaling pathways have been elaborated recently, there are still many unknowns in a better understanding of the molecular biological mechanism of EOC, including the discovery of new tumor suppressor genes (TSGs). High-grade serous ovarian cancer (HGSOC) is the most important (70% of EOC patients) and lethal histologic subtype. Extensive cytoreductive surgery combined with platinum-based chemotherapy is the standard therapy for most of the patients at present, but the responses of the HGSOC patients to treatment and prognosis are different. Lately, several studies including The Cancer Genome Atlas (TCGA) project have obtained the complete genome map of HGSOC and discovered some prognosis-related molecular biomarkers.

Epithelial-mesenchymal transition (EMT) is a primary transition process of the early ovarian tumor into invasive and metastatic malignancy, and the loss of epithelial features and acquisition of mesenchymal features promote the invasiveness of ovarian cancer[3]. Therefore, EMT is described as a crucial event of cancer progression and metastasis, and it has aroused extensive attention in the research on ovarian cancer metastasis[4]. EMT is usually triggered by the abnormality of signaling pathways, of which the transforming growth factor-beta (TGF-β) pathway is regarded as the main inducer[5,6]. The kinase phosphorylation of TGF-β receptor II (TβRII) activates TβRI, further phosphorylating Smad2 and Smad3. Then, the

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phosphorylated Smad2 and Smad3 can form a trimer with Smad4 that leads to molecular changes to a large extent through the transcription factor family[7].

The Hippo pathway is an important tumor suppressor pathway, and transcriptional co-activator with PDZ-binding motif (TAZ) is a key downstream component of the pathway. The activation of the Hippo pathway can result in the inhibition of TAZ phosphorylation and TAZ accumulation in cytoplasm[8]. As a newly presumed oncogene, TAZ is capable of accelerating the incidence and development of the tumor. There is growing evidence that the high expression of TAZ is correlated with the prognosis of multiple human malignancies such as breast cancer, non-small-cell lung cancer (NSCLC) and glioblastoma[9]. The down-regulated TAZ represses the proliferation and development of breast cancer and lung cancer[10]. Previous studies have revealed that TAZ expression is up-regulated in retinoblastoma cells, and such up-regulation is associated with the poor prognosis of glioblastoma patients. TAZ regulates the proliferation of glioblastoma through controlling the expressions of cyclin E and cyclin-dependent kinase 2[11].

In this research, it was found that micro ribonucleic acid (miR)-125a-5p was down-regulated prominently in ovarian cancer, and in-depth cytological experiment confirmed that miR-125a-5p bound to the 3'-untranslated region (UTR) of TAZ messenger RNA (mRNA) and suppressed the TAZ expression and the activation of downstream epidermal growth factor receptor (EGFR) signal transduction.

Patients and Methods

Cell Culture and Transfection

Human ovarian cancer SKOV3 cells were purchased from the cell bank of Chinese Academy of Sciences and cultured in an incubator containing a Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 5% CO_2 at 37°C.

The miR-125a-5p mimics/negative control (NC) mimics and miR-125a-5p/NC inhibitor were synthesized by GenePharma (Shanghai, China). The cell transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol.

Clinical Samples

Cryopreserved ovarian cancer samples and corresponding adjacent normal tissues were collected from the Department of Gynecology of our hospital. After the samples were obtained through surgery, they were immediately frozen in liquid nitrogen and stored at -80°C for standby use. All the ovarian cancer samples and corresponding adjacent normal tissues were confirmed histologically and pathologically. This research was approved by the Ethics Committee of our hospital. Written consent was obtained from all the patients or their principals.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cells subjected to different treatments were harvested to extract the total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. 1 μL RNA solution was taken onto a microplate reader to measure the concentration and purity of the total RNA, of which the optical density $(OD)_{260}/OD_{280}$ should be 1.6-1.8. The remaining RNA solution was sub packaged and preserved at -80°C for standby use.

The M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) and 2 μg RNA were used for RT. To evaluate the level of TAZ mRNA, SYBER Green PCR Master Mix (TaKaRa Bio Inc., Otsu, Shiga, Japan) and StepOnePlus 7500 real-time PCR system (Bio-Rad, Hercules, CA, USA) were adopted to analyze the RT products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous control. The changes in mRNA expression level were calculated *via* the $2^{-\Delta\Delta Ct}$ method. According to the manufacturer's protocol, the expression level of miR-125a-5p was evaluated by means of CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) and TaqMan probe (Invitrogen, Carlsbad, CA, USA) (Table I).

Western Blotting (WB)

The cells cultured were lysed in cold lysis buffer, and 100 μg proteins in each kind of lysate were assessed using the standard WB assay as mentioned above. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Next, 40 μg total proteins were separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by in-

Table I. Primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
TAZ	5'-ACACCCTGCAATCTTTCAGACA-3'	5'-GATTCCACTTTGCGT-3'
MiR-125a-5p	5'-CTGGAAACAGAGGGATGC-3'	5'-CCTGGCTCCTCACTTGGC-3'
GAPDH	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

cubation with primary antibodies at 4°C (1:2000, Abcam, Cambridge, MA, USA) and corresponding horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h (1:1000, Beyotime, Shanghai, China). Later, 200 μL chemiluminescence solution was added in drops, followed by color development on a chemiluminescent imaging analysis system. ImageJ image analysis software was applied to calculate the grayscale value of the bands on the images developed, which were used for statistical analysis.

Dual-Luciferase Reporter Gene Assay

On the first day of the experiment, the cells selected appropriately according to the specific experiment were digested and seeded into a 35 mm cell culture dish, followed by culture in the incubator with 5% CO_2 and saturated humidity at 37°C overnight. When the cell density reached 70%, the cells were co-transfected into HEK-293T cells with miR-125a-5p mimics or miR-125a-5p antagomir and psiCHECK2-UTR Vectors (Promega, Madison, WI, USA) using TurboFect Transfection Reagent (Fermentas, Vilnius, Lithuania). After that, the cells were cultured for 24 h, and the cell activation was determined using Dual-Glo Luciferase Assay (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. All the transfection experiments were repeated for three times.

Gene Chips

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract the total RNA from the ovarian cancer tissue samples and paired adjacent normal tissues stored at -80°C. Then, Affymetrix Human Gene 2.0 ST microarray was adopted to assess the transcriptional profiles of miRNA and mRNA. The analysis of variance (ANOVA) was applied to evaluate the significant changes in standardized data, and fold changes were analyzed using Partek Genomics Suite software. If the fold change of the difference in miRNA expression levels between ovarian cancer tissues and paired adjacent normal tissues was greater than 2, it was considered to be significant.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA)

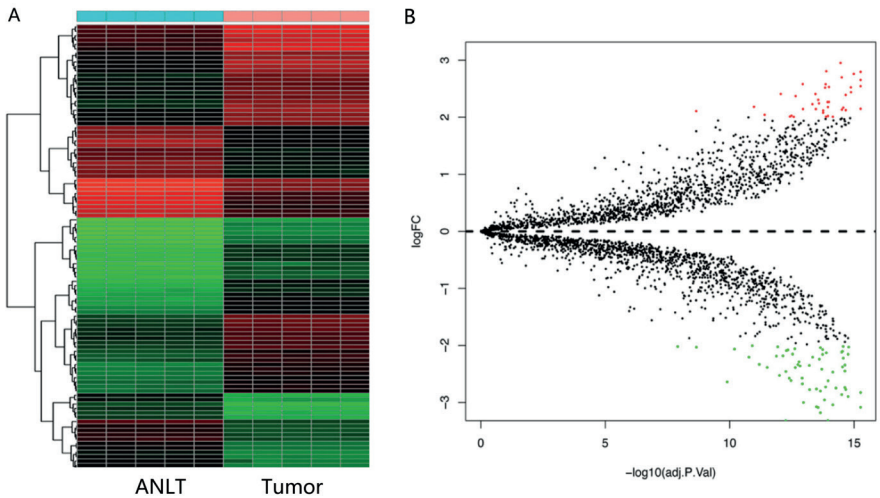

Figure 1. MiRNA transcriptomes in the cancer tissues and adjacent normal tissues of three patients with ovarian cancer screened *via* high-throughput miRNA chip.

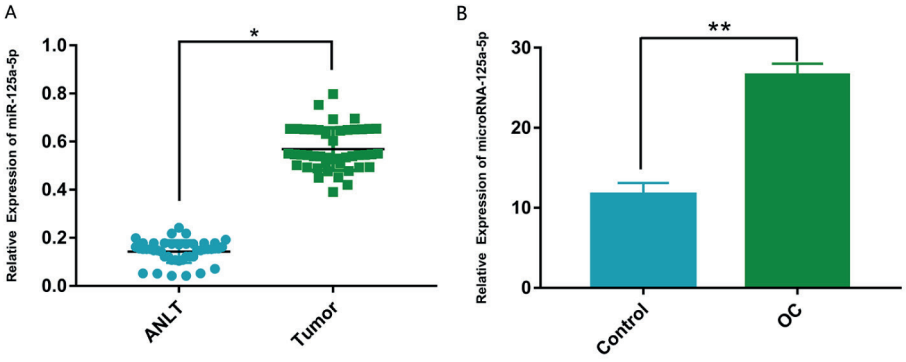

Figure 2. A, Expression level of miR-125a-5p in the ovarian cancer tissues detected *via* RT-qPCR, **B,** Expression level of serum miR-125a-5p in patients with ovarian cancer detected *via* RT-qPCR.

was used for statistical analysis. The *t*-test was adopted for differences between two groups, and comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Bilateral 95% confidence interval was used for all the tests, and $p<0.05$ suggested that the difference was statistically significant.

Results

Chip Screening

To investigate the expression of miRNAs in the patients with ovarian cancer, the high-throughput miRNA chip was utilized to screen the miRNA transcriptomes in the cancer tissues and adjacent normal tissues of three patients with ovarian cancer. It was discovered that 32 miRNAs were differentially expressed in the cancer tissues and adjacent normal tissues ($p\leq0.05$, fold change ≥2.0) (Figure 1). It was further indicated that miR-125a-5p was down-regulated markedly in the ovarian cancer tissues.

Verification of Clinical Samples

Another 40 pairs of cryopreserved freshly resected specimens of ovarian cancer and adjacent tissues were extracted, and the aforementioned results of chip screening were verified *via* Real Time-quantitative PCR (RT-qPCR). It was shown that the expression level of miR-125a-5p in the cancer tissues was markedly lower than that in adjacent tissues in control group ($p\leq0.05$) (Figure 2A). Meanwhile, it was also proven through RT-qPCR that the expression level of serum miR-125a-5p in the patients with ovarian cancer declined compared with that in control group ($p\leq0.01$) (Figure 2B).

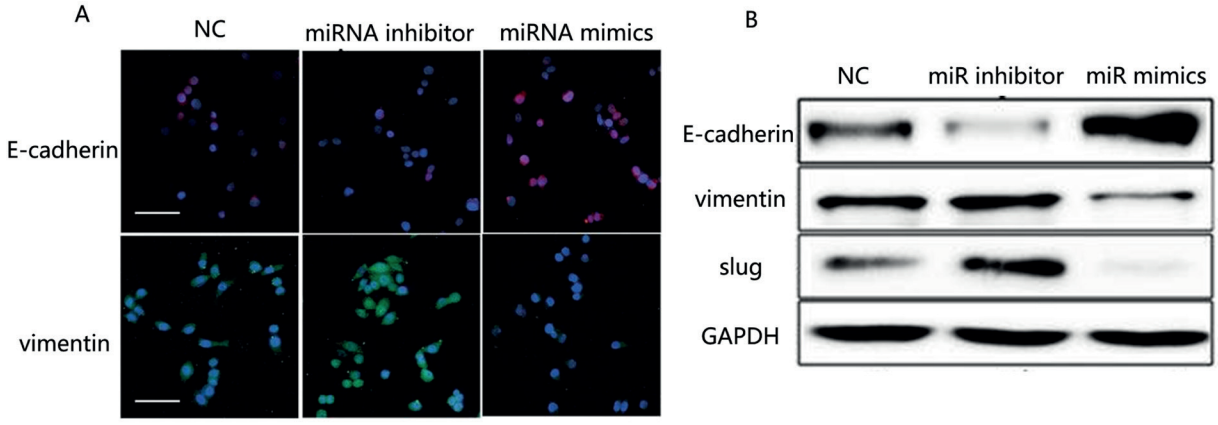

Figure 3. A, Expression of EMT-related indicators in cells displayed *via* immunofluorescence (magnification: 100×), **B,** EMT-related proteins detected *via* WB: after inhibition on miR-125a-5p, the expression level of E-cadherin, an epithelial indicator, is decreased, while that of Vimentin, an interstitial indicator, is increased. ($p\leq0.01$).

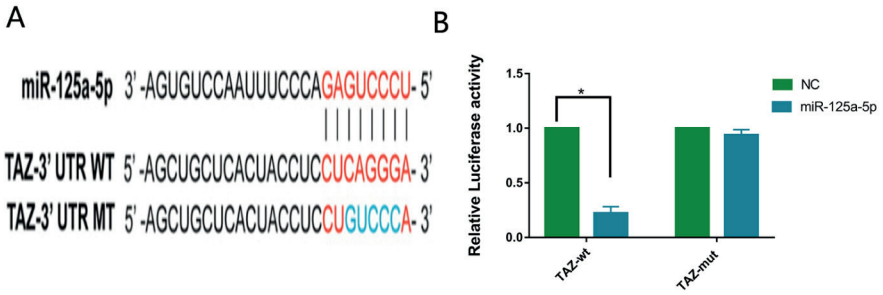

Figure 4. A, Bioinformatics prediction: miR-125a-5p contains a complementary site in the 3'-UTR of TAZ mRNA, **B,** Luciferase reporter gene assay: the activity of the luciferase reporter genes fusing with the TAZ 3'-UTR-Wild Type WT is reduced by miR-125a-5p mimics.

Impacts of MiR-125a-5p on EMT

The expression levels of EMT-related indicators were measured after the ovarian cancer cells were transfected with miR-125a-5p inhibitor and miR-125a-5p mimics separately. The results displayed that after inhibition on miR-125a-5p, the expression level of E-cadherin, an epithelial indicator, was decreased, while that of Vimentin, an interstitial indicator, was increased ($p \leq 0.01$) (Figure 3).

Dual-Luciferase Reporter Gene Assay

To further elaborate the molecular mechanism of miR-125a-5p in regulating EMT in ovarian cancer, "miRanda", a prediction program for miRNA targets, was adopted to predict the presumed targets. It was indicated that miR-125a-5p contained a complementary site in the 3'-UTR of TAZ mRNA (Figure 5A), so it was inferred that TAZ may participate in the regulation of miR-125a-5p. Then, the luciferase reporter gene assay was performed to verify the inference. As shown in Figure 6, the activity of the luciferase reporter genes fusing with the TAZ 3'-UTR-Wild Type (WT) was reduced by 36% by miR-125a-5p mimics, while the luciferase reporter gene vector containing TAZ 3'-UTR-Mutant was not affected by miR-125a-5p mimics (Figure 5B).

TAZ Was a Direct Binding Target of MiR-125a-5p

The expression levels of TAZ mRNA and protein in the cells were down-regulated prominently by the overexpression of miR-125a-5p. There were statistical differences in the expressions of TAZ among different groups after the cells were transfected with miR-125a-5p ($p<0.01$) (Figure 5). In addition, WB assay manifested that the protein level of TAZ was lowered after transfection with

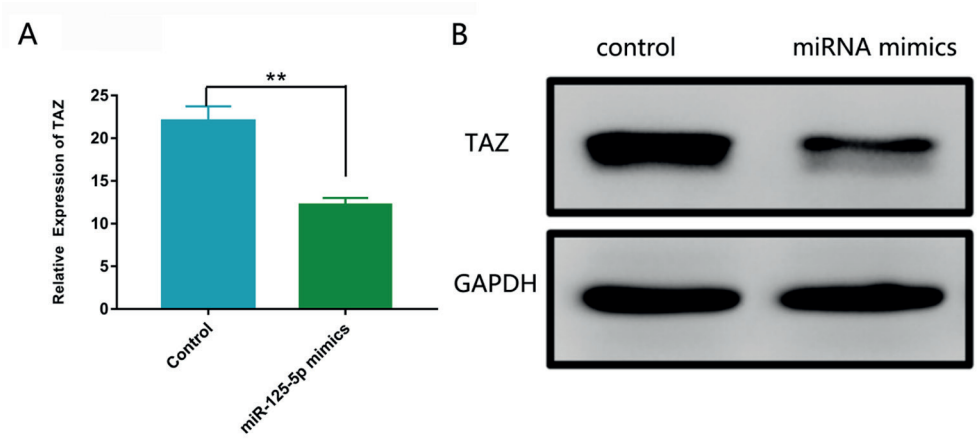

Figure 5. Expression of TAZ in cells down-regulated by miR-125a-5p: the expression levels of TAZ mRNA and protein are decreased after the overexpression of miR-125a-5p ($p<0.01$).

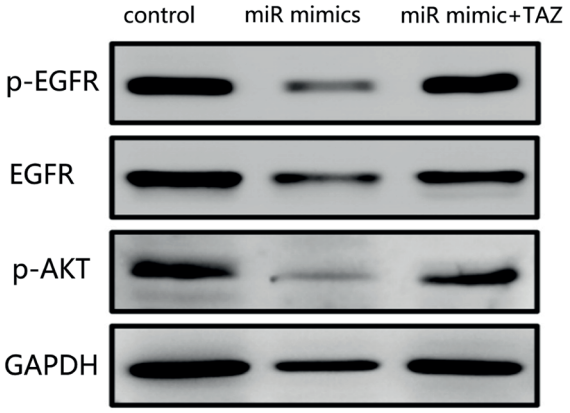

Figure 6. WB analysis: the expressions of EGFR, p-EGFR, and p-Akt were up-regulated in the cells transfected with miR-125a-5p mimics and those transfected with miR-125a-5p mimics overexpressing TAZ ($p<0.01$).

miR-125a-5p mimics. All these data indicated that TAZ is negatively regulated by miR-125a-5p, and the miR-125a-5p/TAZ axis probably plays a dominant role in regulating the EMT of ovarian cancer cells.

MiR-125a-5p Repressed the EMT of Ovarian Cancer Cells Through the EGFR Pathway

The overexpression of EGFR is closely correlated with the progression of various cancers. The EGFR signal promotes the tumor proliferation and progression by activating the downstream phosphatidylinositol 3-kinase (PI3K)/Akt and Ras-ERK signal transduction pathways. Therefore, the expressions of EGFR, phosphorylated EGFR (p-EGFR), and p-Akt proteins in the ovarian cancer cells transfected with miR-125a-5p mimics were analyzed using WB. It was indicated that the expressions of EGFR, p-EGFR, and p-Akt were up-regulated in the cells transfected with miR-125a-5p mimics and those transfected with miR-125a-5p mimics overexpressing TAZ ($p<0.01$) (Figure 6).

Discussion

MiR-125 is a category of miRNAs that have close correlations with the development of cancers. Existing studies have manifested that miR-125a-5p serves as a tumor suppressor in multiple cancers, including lung cancer, breast cancer, and retinoblastoma. However, its function and potential mechanism in ovarian cancer have not been reported yet.

In this research, it was proven that miR-125a-5p repressed the EMT of ovarian cancer cells by directly inhibiting TAZ expression and thereby inactivating EGFR. Then, the screening results through high-throughput miRNA chip showed that there were significantly differentially expressed miRNAs in ovarian cancer and adjacent tissues. The analysis based on the TCGA database revealed that the levels of the differentially expressed miRNAs in ovarian cancer were closely associated with the cTNM stage. It was also found that the expression of miR-125a-5p was down-regulated remarkably in the ovarian cancer tissues among the 40 patients. All these findings demonstrate that miR-125a-5p may be a prognostic indicator of ovarian cancer. Moreover, it was discovered that the overexpression of miR-125a-5p repressed the EMT of ovarian cancer cells, while the knockdown of miR-125a-5p exerted an opposite effect.

According to previous studies[12-14], miR-125a-3p can inhibit the adhesion, proliferation, and migration of prostate cancer and breast cancer by repressing metastasis-associated factors such as Fyn (tyrosine kinase), breast cancer susceptibility gene, and focal adhesion kinase. Furthermore, it suppresses the proliferation, migration, and invasion of NSCLC by targeting metastasis-associated gene 1. In this research, it was discovered for the first time that miR-125a-3p performed its functions by inhibiting TAZ and targeting EGFR. TAZ is a vital functional component of the Hippo pathway which is correlated with the inhibition on tumor proliferation and progression[15,16]. Researches have demonstrated that the up-regulation of TAZ can accelerate the proliferation of retinoblastoma and has a close correlation with the poor prognosis of retinoblastoma patients[17]. Nevertheless, the significance of TAZ overexpression in ovarian cancer still remains unclear. In this report, it was revealed that TAZ was a direct target of miR-125a-5p, and miR-125a-5p bound to the complementary site in the 3'-UTR of TAZ, so as to promote the down-regulation of TAZ expression. In addition, it was identified in the 40 cases of ovarian cancer tissues that the expression of miR-125a-5p had a significantly negative correlation with TAZ. It was also revealed that the activation of the EGFR pathway was restored in the group of miR-125a-5p mimics overexpressing TAZ. These findings manifest that the recovery of

TAZ expression may antagonize the inhibitory effects of miR-125a-5p. In a word, the results of this research indicated that TAZ was a direct target of miR-125a-5p in ovarian cancer, suggesting that the up-regulation of TAZ is caused by the down-regulation of miR-125a-5p. The aforementioned findings also demonstrate that miR-125a-5p is probably a potential prognostic indicator of ovarian cancer.

EGFR is reported to be a key regulatory factor for tumor proliferation, progression, invasion, and metastasis, whose high expression is considered to be associated with the low survival rate of ovarian cancer patients. Besides, some studies[18-20] have indicated that TAZ stimulates the proliferation and tumor formation through the EGFR signal transduction in many cancers. Hence, the activation of EGFR and its downstream signal transduction pathways (RAS-RAF-MEK-ERK and PI3K/Akt) were examined. It was found that the overexpression of miR-125a-5p inhibited EGFR and activated the corresponding downstream signal transduction pathways in ovarian cancer cells, which was consistent with the previous reports, suggesting that miR-125a-5p can inhibit the EMT of ovarian cancer cells by repressing the TAZ/EGFR signaling pathway.

Conclusions

A new mechanism of the miR-125a-5p/TAZ/EGFR signaling pathway in inhibiting the EMT of ovarian cancer cells was elaborated in this research. There was convincing evidence that miR-125a-5p directly targeted TAZ, thus controlling the EMT of ovarian cancer cells by regulating the activation of EGFR. In summary, the research data demonstrate that miR-125a-5p, as an important tumor inhibitor, can repress the EGFR pathway, so the miR-125a-5p/TAZ/EGFR axis may be a potential therapeutic target for ovarian cancer and can be taken as a part of novel treatment strategies for the patients.

Conflicts of interest

The authors declare no conflicts of interest.

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