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MiR-133b regulates the expression of CTGF in epithelial-mesenchymal transition of ovarian cancer

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Abstract. – OBJECTIVE: To explore the role of miR-133b in ovarian cancer and to preliminarily elucidate the mechanism of miR-133b in epithelial-mesenchymal transition (EMT) of ovarian cancer.

PATIENTS AND METHODS: MiR-133b was detected in ovarian cancer specimens, and the relationship of miR-133b with each pathological index and clinical index of ovarian cancer was analyzed. The action targets of miR-133b in ovarian cancer were analyzed systematically and studied deeply via the target validation and cell function validation. Finally, the possible reasons of ovarian cancer metastasis were analyzed through the molecular regulation mechanism in EMT of ovarian cancer.

RESULTS: The miR-133b level in ovarian cancer was significantly lower than in normal ovarian tissues and benign ovarian tumors ($p<0.05$). The level of miR-133b in ovarian cancer was related to differentiated degree and lymphatic metastasis. Dual-luciferase assay indicated that connective tissue growth factor (CTGF) was the target gene regulated by miR-133b. Reverse transcriptase-polymerase chain reaction (RT-PCR) as well as Western blot results proved that the expression level of E-cadherin representing the epithelial cell phenotype was increased, while the expression level of vimentin representing the mesenchymal cell phenotype was decreased. Transwell assay confirmed that the migration and invasion abilities of ovarian cancer cells declined after transfection with miR-133b plasmid. After co-transfection with miR-133b and CTGF overexpression plasmids, RT-PCR and Western blotting proved that the expression level of E-cadherin representing the epithelial cell phenotype was decreased, while the expression level of vimentin representing the mesenchymal cell phenotype was increased; transwell assay confirmed that the cell migration and invasion abilities were increased after co-transfection.

CONCLUSIONS: The results of this study showed that miR-133b may serve as a new molecular marker of EMT of ovarian cancer, and act as a molecular marker of differentiated degree and lymphatic metastasis of ovarian cancer.

Key Words:
MiR-133b, Ovarian cancer, CTGF, Invasion, Migration.

Introduction

Ovarian cancer is a kind of common gynecological malignant tumor, whose fatality rate ranks first in gynecological malignant tumors. Due to its hidden onset and lack of effective early diagnosis method, patients have been in the advanced stage mostly and lost the operation opportunity when diagnosed; besides, the conventional treatment means are less effective[1,2]. Therefore, it is urgently needed to explore effective early diagnosis methods, which rely on the deep understanding of pathogenesis of ovarian cancer and thorough research on relevant pathogenic genes to a large extent. At present, there is lack of typical serum tumor markers for detecting ovarian cancer, so tumor detection means with high sensitivity and specificity are needed to improve the diagnosis and treatment levels of ovarian cancer[3]. The main cause of death of ovarian cancer is the tumor metastasis; epithelial-mesenchymal transition (EMT) of tumor cells is the key to tumor metastasis. EMT refers that the basic structure of epithelial tissues of cells with epithelial phenotype disappear and the cell polarity and tight junction are lost under the stimulation of a variety of factors, which are manifested as the intracellular skeletal component rearrangement, acquisition of migration capacity and transformation into cells with mesenchymal phenotype.

Connective tissue growth factor (CTGF) has a wide range of biological characteristics and plays important roles in cell growth, differentiation, adherence and movement, tumor growth and other biological processes. In malignant tumors, CTGF regulates the occurrence, development, angiogenesis, invasion and metastasis of tumors

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through the interaction with extracellular matrix (ECM). At present, it has been confirmed that CTGF is a downstream molecule of transforming growth factor-β (TGF-β) signal pathway in EMT, and the activation of TGF-β pathway can increase the expression of CTGF[4,5]. In addition, the expression of CTGF can also be increased by activating Wnt/β-catenin signal pathway. The increase of CTGF expression enhances the proliferation and migration capacities of pancreatic cancer cells, also regulating the expressions of EMT-related markers, which can also enhance the adhesion capacity of gastric cancer cells as well as promote the occurrence of EMT in gastric cancer.

Currently, there are a lot of studies on the regulation of a variety of tumor via micro ribonucleic acid (miRNA). As a kind of noncoding single-stranded RNA molecule, miRNAs play corresponding roles in the regulation of transcription function of target genes. More and more evidence shows that miRNAs play a key part in tumor proliferation, progression and stress response[6]. MiRNAs, represented by miR-1, miR-133 and miR-208, can affect the structure and movement capacity of tumor cells through inhibiting the tumor gene translation[7]. However, the possible regulating effect of miR-133 in ovarian cancer remains unclear.

This research aimed to explore the importance of miR-133b in ovarian cancer and to preliminarily elucidate the mechanism of miR-133b in EMT of ovarian cancer, so as to provide new ideas and experimental clues for clinical early detection and tumor targeted therapy, which has important theoretical and practical significance.

Patients and Methods

Clinical Specimen Collection

A total of 65 cases of cancer tissues of patients histopathologically diagnosed as ovarian cancer in PLA 463 Hospital from January 2014 to May 2017 were collected (ovarian cancer group), and 32 cases of normal ovarian epithelial tissues (normal group) and 39 cases of benign ovarian epithelial cyst tissues (benign group) were selected as controls. After excision of the above tissues, they were stored in the ultra-low temperature refrigerator at -80°C. This study was approved by the Ethics Committee of PLA 463 Hospital. Signed written informed consents were obtained from all participants before the study.

Detection of miRNA Expression via RT-PCR

MiR-133b expression was detected *via* Synergy Brands (SYBR) green method. U6 was used as the internal reference, and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using the quantitative PCR instrument. The qRT-PCR system was prepared for all complementary DNA (cDNA) samples, as follows: 2 μL reverse transcription product, 10 μL×10 PCR buffer, 0.4 μL of each PCR specific primer (10 μmol/L), 1.0 μL deoxy-ribonucleoside triphosphate (dNTP) (2.5 mmol/L) (Austin, TX, USA), 0.6 μL $MgCl_2$ solution, 0.2 μL 0.5 U Taq polymerase (Austin, TX, USA), and 0.2 μL 2×ROX Reference Dye. The water was added till the total volume was 8 μL. Reaction conditions: activation of Taq polymerase at 95°C for 3 min; 40 PCR cycles (95°C for 15 s; 60°C for 20 s; 72°C for 20 s; 78°C for 20 s). After the amplification reaction, the mixture was slowly heated from 60°C to 99°C. The miRNA specific primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The miRNA level in tissues was calculated using the $2^{-\Delta Ct}$ method. ΔCt = Ct (miRNA)-Ct (U6); three parallel tubes were set up for each sample. Primer sequences: miR133b: CTTTGGTCCCCTTCAACCA (F), GTGCAGGGTCCGAGGT (R).

Evaluation Indexes of Clinical Specimens

The miR-133b expressions in ovarian tissues were compared among normal group, benign group and ovarian cancer group. According to the mean value of miR-133b level in normal group, the miR-133b level in ovarian cancer group was divided into high-level group (> normal group) and low-level group (≤ normal group), and the clinicopathologic features (age, clinical stage, differentiation degree, tissue type and lymph node metastasis) in different groups were analyzed.

Cell Culture and Plasmid Transfection

SKOV3 cells were obtained from ATCC (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) with 10% fetal bovine serum (FBS) (Waltham, MA, USA) under the condition of 5% CO_2 at 37°C. After the stable passage for 2-3 generations, cells in logarithmic growth phase were taken for the experiment. MiR-133b and CTGF overexpression plasmids were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). SKOV3

cells were cultured in the 6-well plate (Corning, Corning, NY, USA) and then transfected using Lipofectamine when the cell fusion degree reached 70-80% according to the instructions of Invitrogen Lipo2000 (Carlsbad, CA, USA). Next, cells were placed into the cell incubator overnight, the medium was replaced on the next day, and relevant detection was performed after infection for 24-48 h.

Western Blot Assay

After treatment, radioimmunoprecipitation assay (RIPA) cell lysis buffer was added to extract the total protein, and the concentration of protein was determined via bicinchoninic acid (BCA) method. 12% polyacrylamide gel electrophoresis was performed under 120 V for 1.5 h. Then, the protein was transferred to polyvinylidene difluoride (PVDF) membranes and blocked using nonefat milk at room temperature for 1 h; the primary antibody was then applied for incubation at 4°C overnight; at the second day, the membrane was incubated in the secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h, followed by exposure using enhanced chemiluminescence (ECL) method. The CTGR, E-cadherin, vimentin as well as glyceraldheyde 3-phosphate dehydrogenase (GAPDH) primary antibodies, were purchased from Cell Signaling Technology (CST, Danvers, MA, USA).

Invasive and Migration Assays

Cells in the miR-133b group, miR-133b and CTGF co-transfection group and negative control (NC) group were seeded into the upper transwell chamber (pretreated with Matrigel), and then 500 μL 20% fetal bovine serum (FBS) medium were applied into the lower chamber (Millipore, Billerica, MA, USA) for incubation for 48 h before crystal violet (0.1%) staining. In the migration assay, the transwell plate was not covered with matrigel, and the remaining methods and grouping were the same as above. Under the microscope (BX-42, Olympus, Tokyo, Japan), the number of cells passing through the matrix gel and basement membrane was counted under the five high-power fields (×200); the average was taken.

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing. Measurement data were presented as "mean ± SD" and the *t*-test was used. x^2 test was used for the comparison of enumeration data. $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-133b Level in Ovarian Cancer Tissues

The miR-133b level in ovarian cancer group was lower than those in normal group and benign group; the differences were statistically significant ($p<0.05$). No significant difference was seen in miR-133b level between normal group and benign group ($p>0.05$) (Figure 1).

Relationship Between miR-133b Level in Ovarian Cancer and Clinicopathological Features

The level of miR-133b with low degree of differentiation and lymphatic metastasis was lower than that with high degree of differentiation without lymph node metastasis; the difference was statistically significant ($p<0.05$) (Table I). Other clinicopathological features, as age, clinical stage and histological type, had no relationship with miR-133b expression.

Target Validation of miR-133b and Target Gene CTGF in Ovarian Cancer

The target genes of hsa-miR-133b were searched in the Target Scan, Pictar and other miRNA database webs, the data were comprehensively analyzed and the CTGF gene was further selected for validation. The CTGF expression was

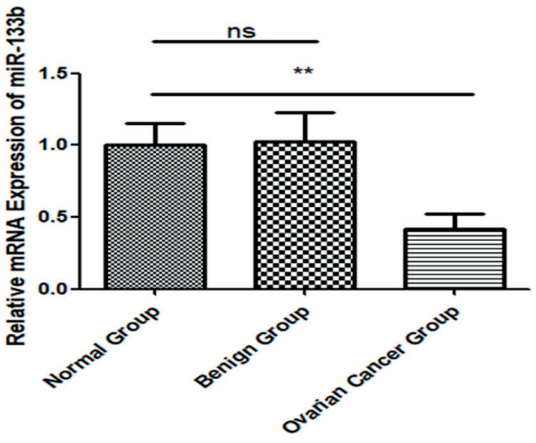

Figure 1. Expression of miR-133b in normal ovarian tissues, benign ovarian tumors, and ovarian cancer via RT-PCR. $^{**}p < 0.01$; ns: none significant.

examined via RT-PCR after the transient transfection with miR-133b mimic as well as inhibitor. The results demonstrated that CTGF expression in mimic group and inhibitor group were significantly decreased and increased compared with that in NC group (Figure 2A); there were significant differences ($p<0.05$), which were consistent with the theoretical values. The results of Western blot showed that the expressions of miR-133b target gene CTGF in mimic group and inhibitor group were significantly decreased and increased compared with that in NC group after transient transfection with miR-133b mimic and inhibitor (Figure 2B). In addition, dual-luciferase reporter gene assay revealed that miR-133b mimic had no significant effect on the activity of luciferase with miR-133b/CTGF mutant 3'UTR fragment inserted, but decreased the activity of luciferase with miR-133b/CTGF 3'UTR fragment inserted (Figure 2C), and the difference was significant. At the same time, NC group had no significant effect on the activity of luciferase with miR-133b/CTGF 3'UTR fragment inserted, suggesting that CTGF is a target gene regulated by miR-133b.

Effects of miR-133b and CTGF on mRNA and Protein Expressions of EMT Markers

The expressions of EMT markers, E-cadherin and vimentin, after transfection with miR-133b plasmid and co-transfection with miR-133b plasmid and CTGF overexpression plasmid, are shown in Figure 3. The results demonstrated that the expression levels of E-cadherin and vimentin in cells transfected with miR-133b were increased and decreased, respectively, compared with those in NC group, and the differences were statistically significant ($p<0.05$). Comparing to those in NC group, E-cadherin and vimentin expressions in CTGF co-transfection group were decreased and increased, respectively, and the differences were significant ($p<0.05$).

Detection of Effects of miR-133b and CTGF on Cell Migration and Invasion Capacities via Transwell Assay

As shown in Figure 4A, the number of SKOV3 cells passing through the basement membrane after transfection with miR-133b was decreased compared with that in NC group, and the difference was statistically significant ($p<0.05$), suggesting that the cell migration capacity is decreased after transfection with miR-133b. The number of cells passing through the basement membrane after co-transfection with miR-133b and CTGF was increased compared with that in miR-133b group, and the difference was statistically significant ($p<0.05$), indicating that the cell migration capacity is increased after co-transfection with miR-133b and CTGF. As shown in Figure 4B, compared with that in NC group, the number of SKOV3 cells passing through the invasion membrane in miR-133b group was significantly decreased, and the difference was statistically significant ($p<0.05$), indicating that the cell invasion capacity is decreased after transfection with miR-133b. The number of cells passing through the invasion membrane in co-transfection group was increased compared with that in miR-133b

Table I. Relationship between miR-133b level in ovarian cancer and clinicopathological features.

Clinicopathological Parameter	miR-133b		p
	High expression	Low expression	
Age (years)			0.222
≤50	14	15	
>50	12	24	
Clinical stages			0.567
I+II	11	12	
III+IV	17	25	
Histological type			0.067
Serous	9	25	
Other	15	16	
Differentiated degree			
High+Moderate	21	18	
Low	5	21	
Lymphatic metastasis			
No	15	7	
Yes	11	32	

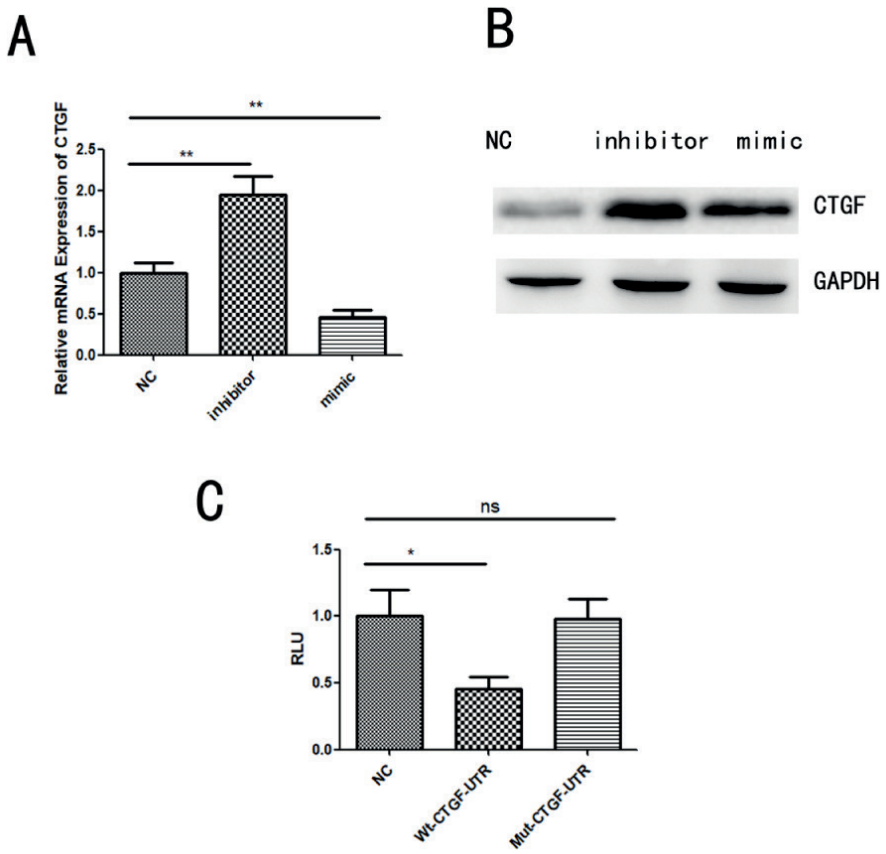

Figure 2. Target validation of miR-133b and target gene CTGF in ovarian cancer cells. ***(A)*** Effect of miR-133b on mRNA expression of CTGF; ***(B)*** Effect of miR-133b on protein expression of CTGF; ***(C)*** Double luciferase to verify the relationship between miR-133b and CTGF. $^{**}p < 0.01$, $^{*}p < 0.05$; ns none significant.

group, and the difference was statistically significant ($p<0.05$), suggesting that the cell invasion capacity is increased after co-transfection with miR-133b and CTGF.

Discussion

MiRNA is a kind of endogenous non-coding RNA, which is widely distributed in the living body. It is a single-stranded structure of about 22-23 nucleotides in length. The mature miRNA can promote the target mRNA cleavage or inhibit the translation process, thus negatively regulating the gene expression at the post-transcriptional level by binding to the mRNA 3'UTR[8,9]. The regulating effect of miRNA is more extensive, playing an important part in cell growth, apoptosis and differentiation processes. MiRNAs are highly conserved among different species, and it has been demonstrated that specific miRNAs can play roles as proto-oncogenes or tumor suppressor genes in the body. Therefore, its abnormal expression plays a vital part in the occurrence and development of tumors. MiRNAs are stable in the body and can tolerate the environments affecting traditional tumor markers, so the detection of abnormal miRNA expression can be used in diagnosis and prognosis prediction of human tumors[10,11].

At first, studies suggest that miR-133b is a kind of myocardium-specific miRNA, which can regulate some key genes involved in regulating myocardial development and muscle signaling pathways in a targeted way, and it plays an important role in myocardial development and functional maintenance[12,13]. Recently, several independent research groups have found that the miR-133b expression is disordered in different tumors and there is a definite tumor suppressive effect. Wong et al[14] detected that miR-133b expression is down-regulated in tongue cancer and it is correlated with the high expression of PKM2. Crawford et al[15] found that miR-133b is expressed lowly in lung cancer tissues and the functional experi-

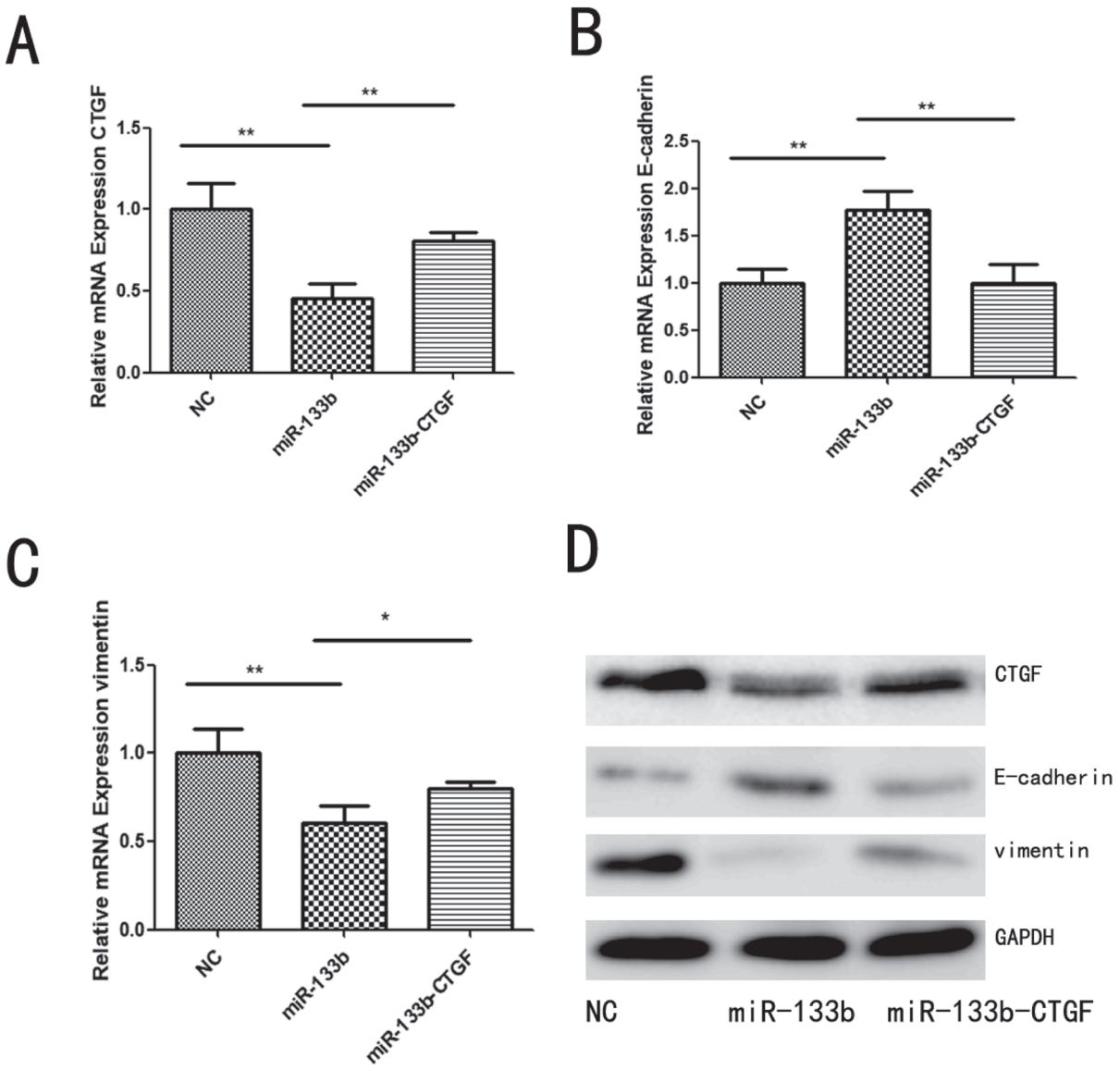

Figure 3. Effects of miR-133b and CTGF on mRNA and protein expressions of EMT markers. ***(A-C)*** RT- PCR revealed that miR-133b increased E-cadherin, but decreased vimentin expression in SKOV3 cells, and these effects could be blocked via CTGF overexpression. ***(D)*** Protein expression of CTGF, E-cadherin and vimentin of miR-133b and CTGF via Western-blot. $^{**}p < 0.01$, $^{*}p < 0.05$.

ments prove that miR-133b attenuates the growth of lung cancer cells through regulating the myeloid cell leukemia-1 (MCL-1) and Bcl-2-like protein 2 (BCL2L2) gene expressions with the effect of cell cycle arrest. Kano et al[16] confirmed that the miR-133b expression is significantly down-regulated in esophageal cancer and has the effects of inhibiting tumor cell growth and invasion through the targeted regulation of Fascin 1 (FSCN1) gene. Besides, the disordered expression of miR-133b in gastric cancer, bladder cancer as well as head-neck tumors, is reported in succession[17-19].

EMT refers to a complex process that the basic structure of epithelial tissues of cells with epithelial phenotype disappears, and the cell polarity and tight junction are lost under the stimulation of a variety of factors, which are manifested as the intracellular skeletal component rearrangement, acquisition of migration capacity and transformation into cells with mesenchymal phenotype[20]. It was found in this study that miR-133b was expressed lowly in ovarian cancer and it was a miRNA with anti-tumor function. After the miR-133b plasmid was constructed, it was introduced into SKOV3

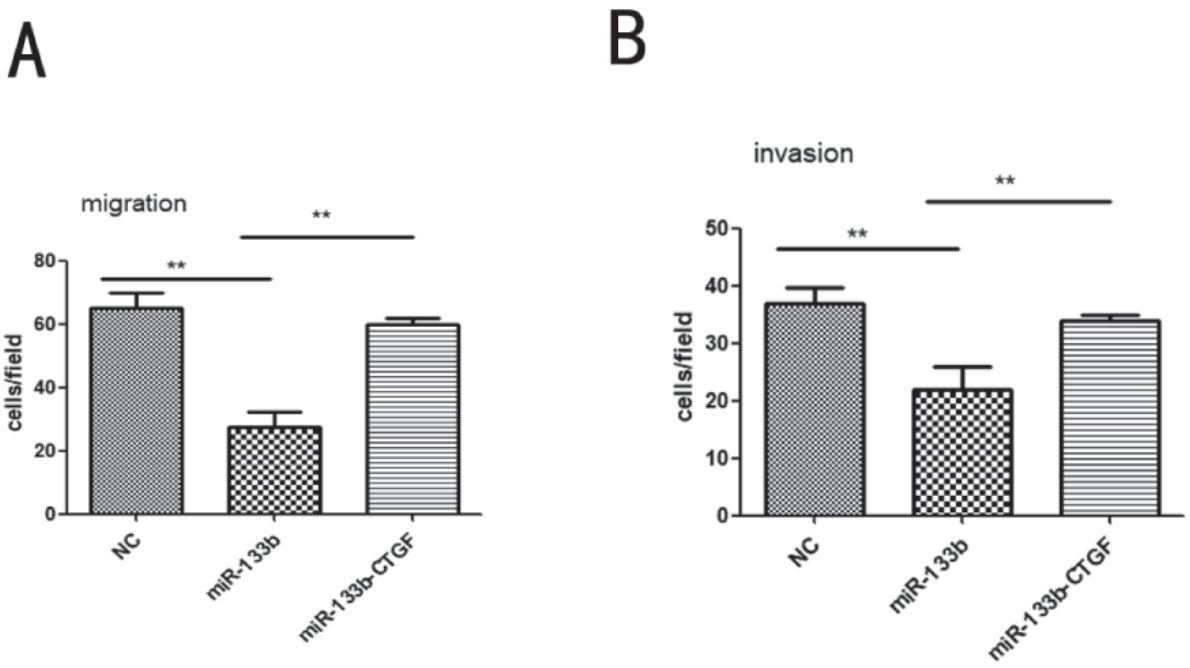

Figure 4. Detection of the effects of miR-133b and CTGF on cell migration and invasion capacities via transwell assay. MiR-133b decreased cell migration ***(A)*** and invasion ***(B)*** abilities of SKOV3 cells, but could be blocked by CTGF overexpression. $^{**}p < 0.01$, $^{*}p < 0.05$.

cells of ovarian cancer. RT-PCR as well as Western blot, proved that the expression level of E-cadherin representing the epithelial cell phenotype was increased, while the expression level of vimentin representing the mesenchymal cell phenotype was decreased. It was confirmed by transwell assay that the invasion, as well as migration capacities of ovarian cancer cells, were decreased after transfusion with miR-133b plasmid. Therefore, miR-133b can inhibit the EMT of ovarian cancer. Considering that miRNA exerts various functions via targeted effect on specific target genes, it is speculated that the mechanism of its regulation of EMT is related to the target gene of miR-133b. Dual-luciferase reporter gene system was used combined with Gene Ontology (GO) classification analysis to screen out the miR-133b target gene closely related to EMT. CTGF has a wide range of biological properties and plays an important role in cell growth, differentiation, adhesion and movement, tumor growth and other biological processes[21]. In addition, it can also be used as storage libraries of vascular endothelial growth factor (VEGF) and other angiogenic factors. In malignant tumors, CTGF, through the interaction with ECM, regulates the occurrence, development, angiogenesis, invasion and metastasis of tumor. It has been confirmed that CTGF is a downstream molecule of TGF-β signaling pathway in EMT[22], and the activation of TGF-β pathway can increase the expression of CTGF[23]. Therefore, the relationship between CTGF and miR-133b was studied deeply, and it was found that CTGF was one of the target genes of miR-133b, and miR-133b could directly regulate the expression of CTGF; the regulating effect of miR-133b on EMT markers of ovarian cancer cells could be blocked by CTGF. It can be seen that CTGF is a key link in the regulation of ovarian cancer EMT via miR-133b, and the miR-133b/CTGF axis can regulate the ovarian cancer EMT. The specific mechanism of regulating EMT needs further study.

Conclusions

The results of this study showed that miR-133b may serve as a new potential molecular marker of EMT of ovarian cancer, and can also be used as a molecular marker of differentiation degree and lymph node metastasis of ovarian cancer. At the same time, this study could help to design target drugs of CTGF and miR-133b, so as to avoid the disadvantages of developing therapeutic targets only based on single pathway. Meanwhile, it gives full play to the unique multi-gene targeting of miRNA, and achieves better treatment results.

Moreover, small-molecular RNA is more likely to be delivered to the target organ, which is of great significance in improving the cure rate of tumors and survival rate of patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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