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MiR-9 accelerates epithelial-mesenchymal transition of ovarian cancer cells *via* inhibiting e-cadherin

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Abstract. – OBJECTIVE: To investigate the influence of micro-ribonucleic acid (miR)-9 on epithelial-mesenchymal transition (EMT) of ovarian cancer cells by targeted inhibition on E-cadherin (CDH1).

PATIENTS AND METHODS: The human ovarian cancer cells were cultured and miR-9 was repressed by inhibitors and overexpressed by miRNA mimics. The expression of EMT-related proteins was measured *via* Western blotting (WB). The action target of miR-9 was determined through the dual-luciferase reporter gene assay. The changes in protein levels were detected using WB.

RESULTS: The expression of miR-9 was markedly up-regulated in ovarian cancer tissues, that is, the expression level of serum miR-9 in ovarian cancer patients was higher than that in control group. After the inhibition of miR-9, the expression level of epithelial indicator CDH1 was increased, while that of interstitial indicator Vimentin was decreased. MiR-9 contained a complementary site in the 3'-untranslated region (UTR) of CDH1 messenger RNA (mRNA) and the mRNA and protein expressions of CDH1 in the cells were down-regulated obviously by miR-9 overexpression.

CONCLUSIONS: MiR-9 promotes the EMT of ovarian cancer cells through the targeted inhibition on CDH1.

Key Words:
MiR-9, E-cadherin, Ovarian cancer, Epithelial-mesenchymal transition.

Introduction

Epithelial ovarian cancer is one of the leading causes of death from gynecologic diseases among women, with a 5-year survival rate lower than 40%. Most patients have been in the advanced stage and have extensive greater *omentum* metastasis when diagnosed[1]. However, the molecular mechanism of the metastasis still remains unclear. Therefore, it is very important for the diagnosis and treatment of ovarian cancer to better understand the mechanism. In spite of such conventional diagnostic methods as pelvic examination, transvaginal ultrasonography, and serum CA125, they are not practical for the early stage and early diagnosis of the disease. Various genes and signaling pathways play vital roles in the pathogenesis of ovarian cancer, many of which can serve as the molecular targets for treatment, but there is no efficacious therapeutic method capable of extending the overall survival of the patients at present. Meanwhile, it has been found that some non-coding molecules, such as micro ribonucleic acids (miRNAs), can effectively regulate gene expressions, which, as molecular biomarkers, have been studied in different kinds of cancers, including ovarian cancer.

As a category of evolutionarily conserved small non-coding RNAs, miRNAs can regulate the gene expression by matching with the 3'-untranslated region (UTR) of target messenger RNA (mRNA) to guide the post-translational inhibition[2]. MiRNAs exert crucial effects in multiple biological processes such as cell cycle regulation, differentiation, cell apoptosis, angiogenesis, cancer initiation, and progression[3]. Numerous studies[1-4] have manifested that miRNAs act as important molecules in functional regulation, pro-oncogenic genes or anti-onco-

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genic genes in a variety of tumors. Reports have discovered that miR-9 can trigger the malignant phenotype of many cancers as a metastasis-associated miRNA, resulting in progression and poor prognosis of cancers, including breast cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, colorectal cancer, and melanoma. Research[5] on gynecologic tumors has revealed that miR-9 expression is down-regulated in endometrial carcinoma and clear cell carcinoma.

Previous researches[6] have verified that miR-9 is correlated with the prognosis of ovarian cancer and the sensitivity to cisplatin. However, the specific action mechanism of miR-9 has not been clarified yet and needs to be confirmed by further studies. Epithelial-mesenchymal transition (EMT) is a multi-lineage trans-differentiation program that enables the tumor cells to acquire related phenotypes to malignant tumors, which is of important significance for tumor invasion and metastasis. Recently, several works have proven that miRNAs may be involved in the EMT process of tumors.

In the present work, it was found that miR-9 was up-regulated in primary ovarian cancer tissues. Therefore, it was conjectured that miR-9 probably participated in the occurrence, development, and metastasis of ovarian cancer by regulating E-cadherin (CDH1).

Patients and Methods

Cell Culture and Transfection

Human ovarian cancer ES-2 cell lines purchased from the Chinese Academy of Sciences (Shanghai, China) were cultured in an incubator containing a Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and 5% CO_2 at 37°C.

The miR-9 mimics/negative control (NC) mimics and miR-9/NC inhibitors were synthesized by GenePharma (Shanghai, China). The cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol.

Clinical Samples

The ovarian cancer samples and adjacent normal tissues were collected *via* surgery from our hospital. All the ovarian cancer samples and adjacent normal tissues were confirmed histologically and pathologically. This research was approved by the Ethics Committee of The First Affiliated Hospital of Yangtze University. Written consent was obtained from all the patients or their principals.

Reverse Quantitative Transcription-Polymerase Chain Reaction (qRT-PCR)

The cells were collected at the end point of each experiment, washed with phosphate-buffered saline (PBS) twice, and lysed with QIAzol reagent (Invitrogen, Carlsbad, CA, USA) to separate the total RNA. After that, the level of miR-9 in the total RNA was quantified *via* real-time PCR using TaqMan miRNA qPCR kit and primer/probe set (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The results were normalized by U6 through relative quantification. Primer sequences used were as follows: CDH1, F: 5'-CGAGCGAGCCTCGCCGTGTAGCTGC-3', R: 5'-GCAAGGTGTCTCACGACCAA-3'; microRNA-9, F: 5'-GCCTGTAACACATCCTCCGACTCG-3', R: 5'-AGGTAATGCTGTCGTGAGTCG-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blotting (WB)

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Yeasen, Shanghai, China). The protein sample was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were then transferred

Table I. Primer sequences.

Gene	Forward primer sequences	Reverse primer sequences
CDH1	5'-TTCCCTGCGTATACCCTGGT-3'	5'-GCCATCTCTTGCTCGAAGTCC-3'
MiR-9	5'-CTGGAAAGAGGGATGC-3'	5'-CCTGGCTCCTACTTGGC-3'
GAPDH	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

to polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibody of CDH1 (1:500; catalog number: 89017313; Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C. The membranes were then washed with Tris-Buffered Saline and Tween 20 (TBST) and followed by the incubation of secondary antibody at room temperature for 1 h. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL). The ImageJ image analysis software was utilized to calculate the grayscale value of the bands on the images developed, which was used for statistical analysis.

Dual-Luciferase Reporter Gene Assay

On the first day of the experiment, the cells appropriately selected according to the specific experiment were digested and seeded into a 35 mm culture dish, followed by culture in the incubator with 5% CO_2, and saturated humidity at 37°C overnight. When the cell density was 70%, TurboFect Transfection Reagent (Fermentas, Vilnius, Lithuania), plasmid complementary deoxyribonucleic acid 3 (pcDNA3)/enhanced green fluorescence protein (EGFP)-CDH1-3′-UTR, and pCDNA3/EGFP-CDH1-3'-UTR mutant (kindly provided by Tianjin University) were used. 293T cells were co-transfected with pCDNA3/pri-miR-9 or miR-9 control vector or control oligonucleotide in a 24-well plate. Moreover, the cells were transfected with reporter vector pCDNA3/EGFP-CDH1-3'-UTR or pcDNA3/EGFP-CDH1-3'-UTR mutant the next day. All the transfection experiments were repeated for three times.

High-Throughput Screening for MiRNA Expression Difference

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract the total RNA from the ovarian cancer tissue samples and paired adjacent normal ovarian 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. The difference in miRNA expression level between ovarian cancer tissues and paired adjacent normal ovarian tissues manifesting a fold change >2 was considered to be significant.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple 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

High-Throughput Screening for MiRNAs

The expressions of miRNAs in the cancer tissues and adjacent normal tissues of three patients with ovarian cancer were screened *via* high-throughput miRNA chip. 112 miRNAs were found to be differentially expressed in the cancer tissues and adjacent tissues ($p\leq0.05$, fold change ≥2.0) (Figure 1). It was further discovered in bioinformatics analysis that miR-9 was down-regulated markedly in the ovarian cancer tissues.

Verification of Clinical Samples

Another 45 pairs of cryopreserved fresh specimens of ovarian cancer tissues and adjacent tissues were extracted by means of surgery and the aforementioned results of chip screening were verified *via* quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). It was shown that the expression level of miR-9 in the cancer tissues was significantly lower than that in adjacent tissues in control group ($p\leq0.05$) (Figure 2A). In the meantime, it was also proven through qRT-PCR that the expression level of serum miR-9 in the ovarian cancer patients declined compared with that in control group ($p\leq0.01$) (Figure 2B).

Impacts of MiR-9 on EMT

The expression levels of EMT-related indicators in ovarian cancer cells were measured after the cells were transfected with miR-9 inhibitors and miR-9 mimics separately. The results manifested that after inhibition on miR-9, the expression level of epithelial indicator CDH1 was decreased, while that of interstitial indicator Vimentin was increased ($p\leq0.01$) (Figure 3).

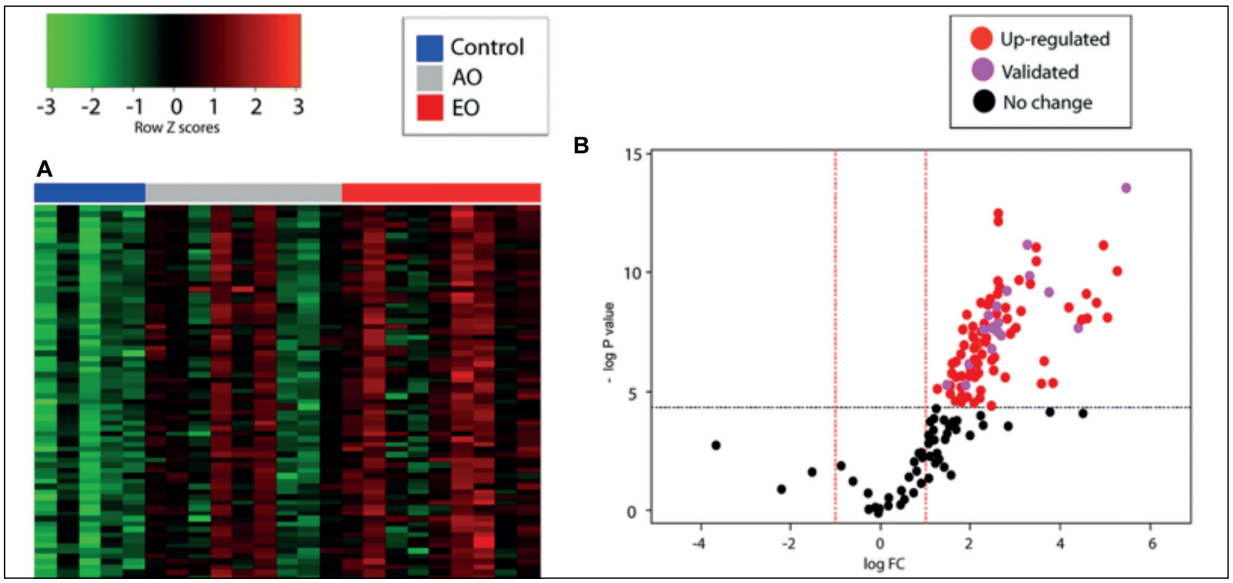

Figure 1. Expression levels of miRNAs in the cancer tissues and adjacent normal tissues of three patients with ovarian cancer screened *via* high-throughput miRNA chip.

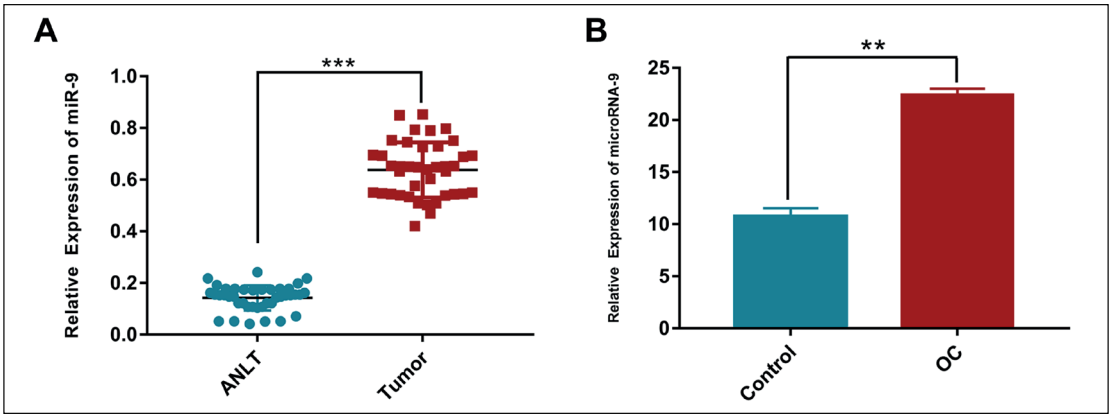

Figure 2. ***A,*** Expression level of miR-9 in the ovarian cancer tissues detected *via* qRT-PCR, ***B,*** Expression level of serum miR-9 in patients with ovarian cancer detected *via* qRT-PCR.

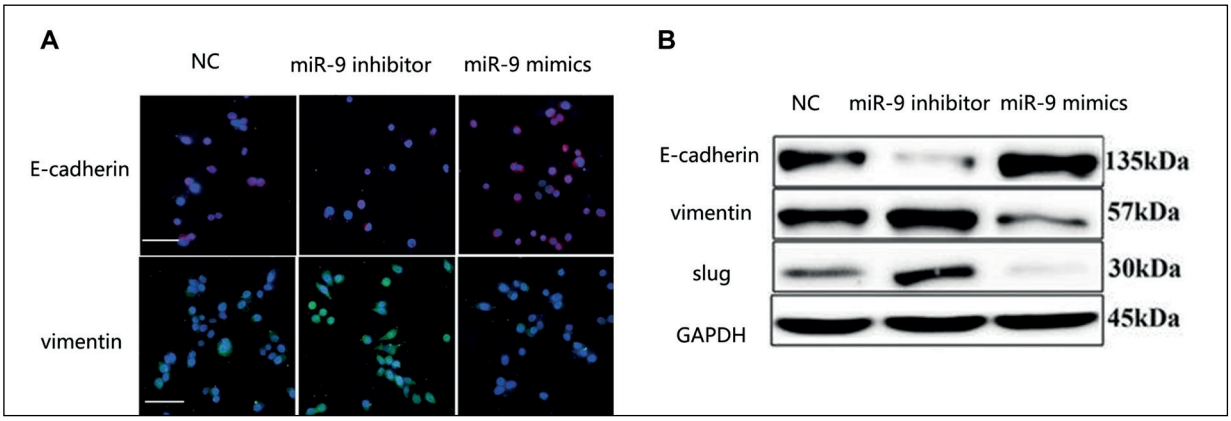

Figure 3. ***A,*** Expressions of EMT-related indicators in cells displayed by immunofluorescence (magnification ´ 40), ***B,*** EMT-related proteins detected *via* WB. After inhibition on miR-9, the expression level of epithelial indicator CDH1 is decreased, while that of interstitial indicator Vimentin is increased ($p \leq 0.05$).

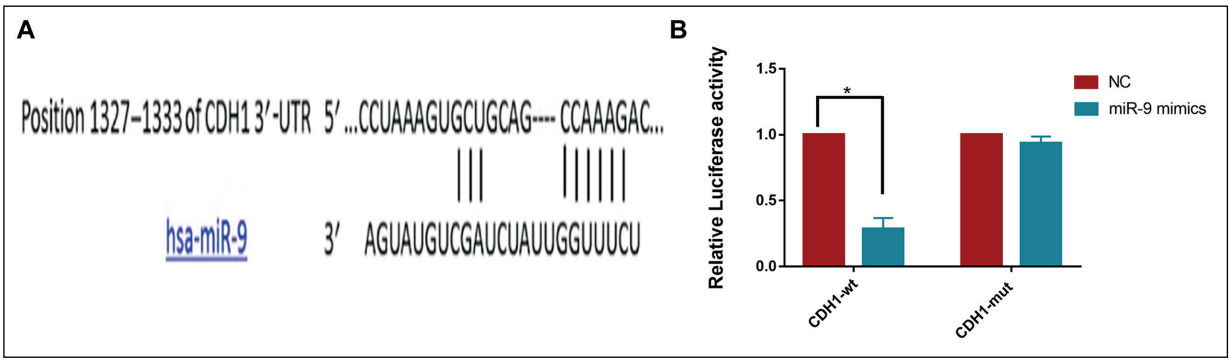

Figure 4. ***A,*** Bioinformatics prediction: miR-9 contains a complementary site in the 3'-UTR of CDH1 mRNA, ***B,*** Luciferase reporter gene assay. The activity of the luciferase reporter genes fusing with the CDH1 3'-UTR-WT is reduced by miR-9 mimics.

Dual-Luciferase Reporter Gene Assay

To further elaborate the molecular mechanism of miR-9 in regulating the EMT in ovarian cancer, the miRNA target prediction program "miRanda" was applied to predict the presumed targets. According to the results, miR-9 contained a complementary site in the 3'-UTR of CDH1 mRNA which encoded CDH1 (Figure 4A). Therefore, it was inferred that CDH1 may participate in the regulatory effect of miR-9. 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 CDH1 3'-UTR-Wild Type (WT) was reduced by 59% by miR-9 mimics, while the luciferase reporter vector containing CDH1 3'-UTR-mutant was not affected by miR-9 mimics (Figure 4B).

CDH1 Was a Direct Binding Target of MiR-9

The mRNA and protein expression levels of CDH1 in the cells were down-regulated prominently by the overexpression of miR-9. There were statistical differences in the expression of CDH1 among different groups after the cells were transfected with miR-9 ($p<0.01$) (Figure 5). In addition, the WB assay revealed that the protein level of CDH1 declined after transfection with miR-9 mimics. All these data illustrated that CDH1 is negatively regulated by miR-9, and the miR-9/CDH1 axis probably plays a predominant role in regulating the EMT of ovarian cancer cells.

Expressions of MiR-9 and CDH1 in Tissues

The correlation between miR-9 and CDH1 in tissues was further testified and it was shown that miR-9 was overexpressed, and CDH1 expression was down-regulated in ovarian cancer tissues. Moreover, the opposite expression trends were detected in adjacent normal tissues (Figure 6).

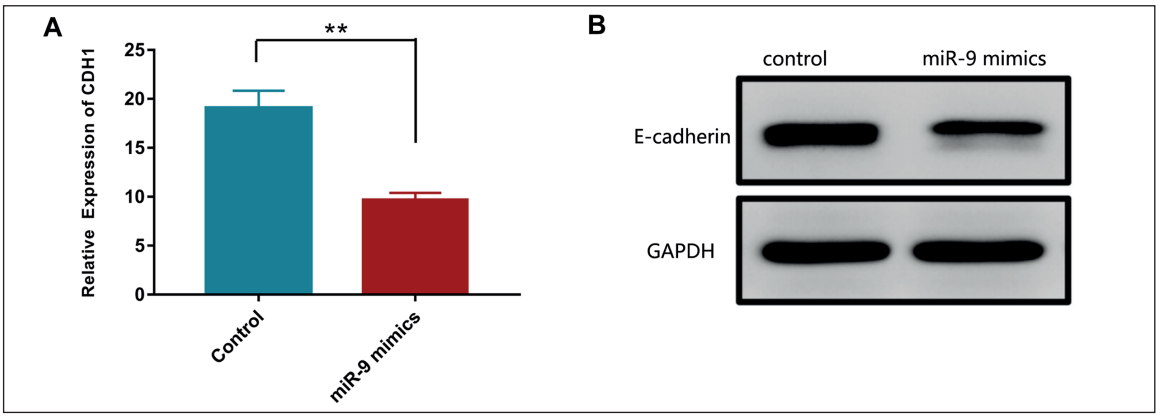

Figure 5. ***A,*** MiR-9 down-regulates CDH1 expression in cells, ***B,*** Expression levels of CDH1 mRNA and protein decline after the overexpression of miR-9 ($p<0.01$).

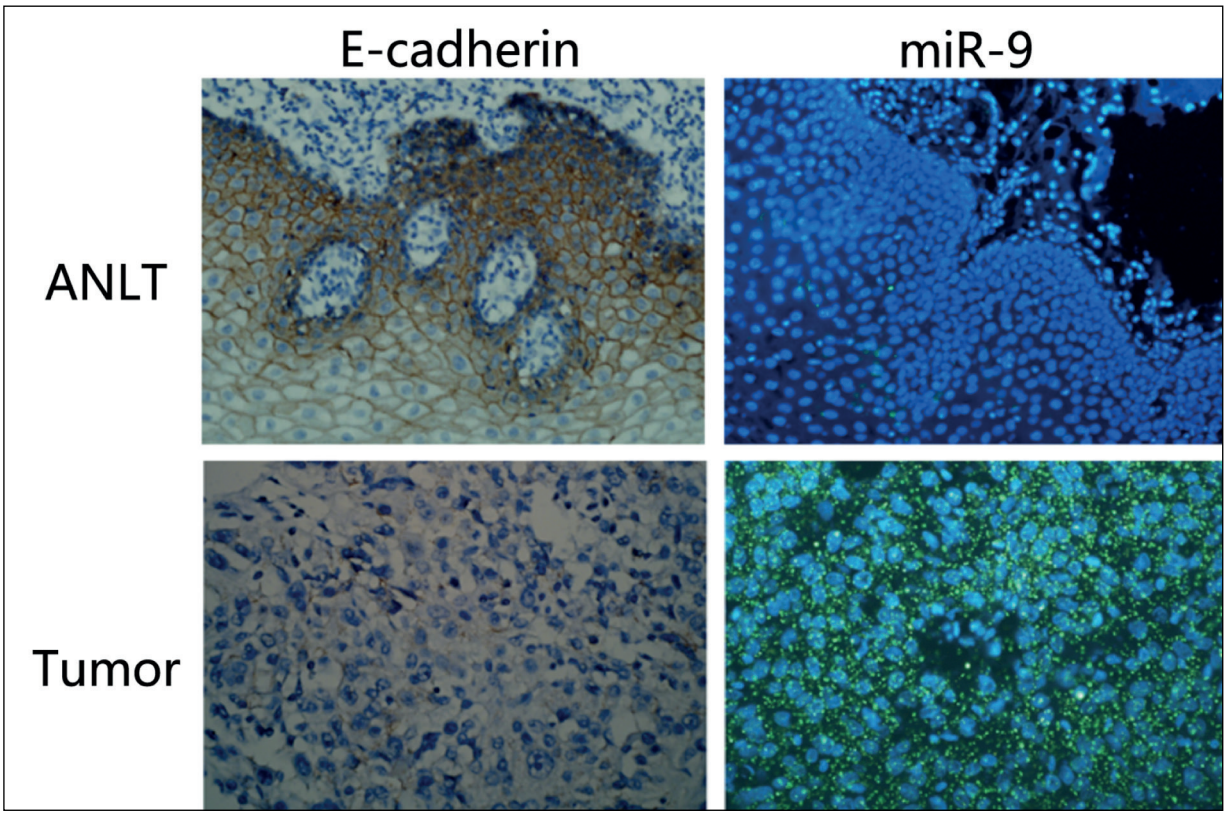

Figure 6. CDH1 staining (IHC staining) and miR-9 staining (MISH staining) in the two groups of ovarian cancer tissues. The results show that CDH1 expression is down-regulated in tumor tissues with overexpressed miR-9 (magnification × 200).

Discussion

The regulation on miRNAs makes the occurrence and development of tumors more complex. It was revealed that miR-9 was up-regulated in ovarian cancer tissues and cell lines. The expression level of miR-9 is associated with the proliferative and migratory abilities of ovarian cancer *in vitro* and *in vivo*. It was also manifested in the present study that miR-9 had cancer-specific functions and exerted tumor-promoting effects in ovarian cancer.

The maturate hsa-miR-9 transcript (miR-9) in human is produced by three independent genes, namely miR-9-1, miR-9-2, and miR-9-3, which are located on chromosome 1, 5 and 15, respectively[7]. MiR-9 is highly expressed in nerve tissues, and its expression in brain tumor is higher than that in other histological types of tumors[8]. MiR-9 exhibits the opposite functions in different types of cancers. It is reported that genes of the miR-9 family (miR-9-1, miR-9-2, and miR-9-3) manifest abnormally high methylation in primary tumors with lymph node metastasis compared with those in primary tumors without lymph node metastasis, including colon cancer, lung cancer, breast cancer, and melanoma[9]. These studies[10-11] also illustrate that no expression of miR-9 transcripts is detected in tumors with methylation of miR-9 promoter.

Some investigations[12] have demonstrated that the function of miR-9 is tumor-specific. For example, miR-9 directly targets CDH1 and increases the movement and invasiveness of breast cancer cells. It is well known that the lowered CDH1 level in cancer tissues is related to tumor metastasis and recurrence. However, miR-9 can suppress the tumor in gastric cancer[13]. RAB34, GRB2 and nuclear factor-kappa B1 (NF-κB1) have been determined as potential targets of miR-9, all of which are capable of mediating the tumor-promoting effect of miR-9[14]. The deletion of miR-9 can promote the migration of neuronal cells by regulating the cytoskeletal proteins. According to the present study results, the reduced miR-9 expression

can accelerate the cell movement by forming more filipodia and processes, and increase the expression of cytoskeletal proteins involved in cell movement.

The role of miR-9 is mediated by the EMT of ovarian cancer cells. In the EGFP-CDH1-3'-UTR reporter gene assay, it was discovered that the intensity of EGFP was decreased after miR-9 overexpression, while the mutation of the miR-9 binding sites protected the cells from the influences of miR-9. The above results indicated that miR-9 is able to directly bind to the 3'-UTR of CDH1 and repress its expression in ovarian cancer cells. It was also revealed that the expression level of miR-9 was elevated in ovarian cancer tissues in comparison with that in adjacent normal tissues. Snail1, an effective inhibitory factor of CDH1 expression, is a downstream target of NF-κB, and down-regulated miR-9 can lead to the up-regulation of CDH1 in ovarian cancer. The role of miR-9 can be reversed by the constitutive expression of active NF-κB1. These data indicate that the effects of miR-9 on Snail1 and CDH1 depend on the activation of NF-κB1[15].

MiR-9 influences CDH1 in an environment-dependent manner. It has been illustrated that miR-9 induces the EMT of breast cancer cells and promotes tumor progression in a cell type- and context-dependent manners[16,17]. The opposite effects of miR-9 on CDH1 expression in breast cancer and ovarian cancer possibly result from the direct inhibitory effects of miR-9 on CDH1. In specific tumors, one of the routes may be primarily inhibited, thus leading to an increase or decrease in CDH1 expression in such tumors.

Conclusions

We showed that miR-9 expression is raised during the progression of ovarian cancer, which may be conducive to maintaining the malignant phenotype by sustaining the low levels of NF-κB1, Snail1, and CDH1 in metastatic ovarian cancer cells. The results of the present study confirmed the new mechanism of inhibition on CDH1 expression mediated by miR-9, and they manifested that the functions of miR-9 are cell type- and context-dependent.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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