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MiR-655-3p inhibited proliferation and migration of ovarian cancer cells by targeting RAB1A

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Abstract. – OBJECTIVE: To investigate the potential effect of microRNA-655-3p (miR-655-3p) on the development of ovarian cancer (OC) and its relevant mechanism.

PATIENTS AND METHODS: Expression level of miR-655-3p in OC tissues was detected. The potential target gene of miR-655-3p was firstly predicted online and subsequently verified by luciferase reporter assay and Western blot. *In vitro* effects of miR-655-3p on SKOV3 cells were determined as well.

RESULTS: Low expression of miR-655-3p in OC was confirmed by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Ras-related protein Rab-1A (RAB1A) was a direct target of miR-655-3p in OC and was negatively regulated by miR-655-3p. Further, the effects of miR-655-3p/RAB1A axis on cell proliferation, metastasis ability, and EMT activation were emphasized.

CONCLUSIONS: Our research emphasized the suppressor function of miR-655-3p in OC. By targeting RAB1A, miR-655-3p played a tumor suppressor role in OC. We affirmed the beneficial effects of miR-655-3p in OC cells for the first time, thus providing an experimental basis for the treatment of OC.

Key Words:
MicroRNA-655-3p (miR-655-3p), Ovarian cancer (OC), Ras-related protein Rab-1A (RAB1A), Proliferation, Metastasis.

Introduction

Ovarian cancer (OC) is a common gynecological malignancy. Epithelial OC accounts for the majority of OC cases, and there are over 200,000 new cases of epithelial OC every year around the world[1]. The mortality rate of OC ranks the top among those of all gynecological malignant tumors. Due to the lack of specific symptoms, OC is difficult to be detected at early stage[2,3], and it tends to be discovered at an advanced stage, seriously threatening female health. Therefore, exploring crucial genes related to the occurrence and development of OC can lay a foundation for further investigating its pathogenesis.

Micro ribonucleic acid (miRNA) is a non-coding single-stranded RNA, composed of 22 nucleotide molecules. It regulates the post-transcriptional translation through the specific binding to the 3'untranslated region (3'UTR) of the target messenger RNA (mRNA)[4]. Studies[5-7] on miRNA developed rapidly, which is a key regulator in the differentiation, proliferation, cycle regulation, and apoptosis of cells and participates in most of the physiological processes. MiRNA plays dual roles as an oncogene or a tumor-suppressor gene[7,8]. Additionally, even slight changes in miRNA expression might affect the progression of malignancies[9]. Jansson and Lund[10] revealed that miRNA can be utilized to intervene in the molecular mechanisms of malignancies, thereby providing an approach for treating cancers.

MicroRNA-655-3p (miR-655-3p) is an important component of the microRNA regulatory network. Lately, it has been certified[11-14] that miR-655-3p exerts critical regulatory effects on many diseases, but the effects of miR-655-3p on the OC have not been researched yet. This work aims to detect the functions of miR-655-3p in the occurrence and progression of OC, so as to provide new ideas and theoretical basis for the clinical treatment and prevention of OC.

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Patients and Methods

Ovarian Cancer Cases and Cells

A total of 50 pairs of OC tissues and adjacent normal ovarian tissues that surgically resected in Obstetrics and Gynecology Department of our hospital from July 2016 to September 2017 were collected as the study groups. Forbidden of preoperative chemotherapy and radiotherapy must be emphasized in all enrolled patients. After resection, the tumor tissue samples were immediately put into liquid nitrogen. This study was approved by the Ethics Committee of The People's Hospital of China Three Gorges University.

Human ovarian serous papillary cystadenocarcinoma cell line (SKOV3) and ovarian epithelial cell line (OEC) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). OC SKOV3 cells were cultured in the medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin, in an incubator containing 5% CO_2 at 37°C. About 80% cell fusion, cells were digested for the passage using trypsin.

Transfection

Cells were inoculated into a 24-well plate at 1×10^4 cells/well, and serum-free medium was replaced when the cell density reached over 80%. MiR-655-3p mimics (100 mmol/L), negative control (NC) (100 mmol/L) or LV-Ras-related protein Rab-1A (RAB1A) (2 μg) was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. At 6 h after the transfection, fresh medium was replaced for subsequent cell culture.

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

SKOV3 cells were inoculated into a 6-well plate at 1×10^6 cells/L. After culturing for 36 h, the total RNA was extracted from the cells in accordance with the instruction in TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were measured using an ultraviolet spectrophotometer. The cDNA diluted in SsoFast EvaGreen reagent (Bio-Rad, Hercules, CA, USA) at 1:10 were utilized for quantitative Polymerase Chain Reaction (qPCR) measurement, with U6 as the internal reference. Primer sequences used in this study were as follows: miR-655-3p, F: 5'-CAATCCTTACTCCAGCCAC-3', R: 5'-GTGTCTTAAGGCTAGGCCTA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Luciferase Reporter Assay

In TargetScan, miRDB, and microRNA websites, it was found that RAB1A is the target gene of miR-655-3p. The binding sequence of miR-655-3p at the 3'-end of RAB1A was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA),

When cells reached about 80% density in a 24-well plate, they were co-transfected with the wild-type RAB1A 3'UTR (pGL3-Rab1A-3'UTR) or the mutant-type RAB1A 3'UTR (pGL3-Rab1A-3'UTR-mut), with miR-655-3p mimics or NC using Lipofectamine 2000, respectively. After transfection for 48 h, luciferase activity was determined according to the instructions of the dual-luciferase reporter assay kit (Solarbio, Beijing, China). Results were represented as the activity ratio of firefly luciferase and Renilla luciferase, which reflected the relative luciferase intensity of each group.

Western Blot (WB) Analysis

200 μL of protein lysis buffer was added per well to lyse for 30 min on ice, followed by centrifugation at 12,000 r/min for 10 min in Eppendorf (EP) tubes. The supernatant was taken to measure the concentration of proteins *via* bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The same volume of protein sample was taken from each group, added with Sodium Dodecyl Sulfate (SDS) loading buffer, mixed evenly and boiled for 10 min to denaturalize proteins. Then Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted, and the proteins in the gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by the wet transfer method. After that, the PVDF membranes were sealed in 5% skimmed milk at room temperature, followed by incubation with the primary antibody (diluted at 1:1,000) at 4°C overnight and the secondary antibody for 1 h at room temperature on the second day. The membranes were washed by Tris Buffered Saline-Tween (TBST) for 3 times, followed by color development using enhanced chemiluminescence (ECL) developer. Finally, gel imaging equipment was used to observe and photograph the results that were expressed as the ratio of optical density of the target band to that of internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Cell Proliferation

SKOV3 cells were inoculated into a 96-well plate at 1×10^4 cells/well and transfected after the cell density reached 70-80%. At 0 h, 24 h, 48 h, and 72 h after the transfection, each well was added with 5 μg/μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (Sigma-Aldrich, St. Louis, MO, USA) and placed in an incubator for 4 h of culture. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added for terminating the reaction. Absorbency value (A) was determined at 490 nm using an enzyme-labeled instrument, and cell proliferation was normalized to that of the blank well.

Cell Migration Assays

Transwell chamber was placed in a 24-well plate with Matrigel (50 μL, 0.2 μg/μL) pre-coated at 37°C for 15 min. OC cells were digested, centrifuged and prepared for cell suspension at 2.5×10^4 cells/mL using the serum-free medium. The cell suspension was added into the upper chamber of transwell at 200 μL/well, while 600 μL of medium containing 10% FBS was added into the bottom chamber, followed by culture in an incubator at 37°C. Cells were fixed in formaldehyde and stained with crystal violet for 10 min, and then, cells on the internal membrane were slightly wiped off using cotton swaps. Finally, the number of cells passing through the filter membrane was counted under a microscope. The experiment was repeated for 3 times.

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. All *p*-values were two-sided and $p<0.05$ were considered significative and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

MiR-655-3p Expression Reduced in OC Tissues and Cell Lines

Differentially expressed miRNAs in tumor cells exert certain biological functions. Upregulated miRNAs in tumors usually serve as oncogenes, and conversely, those downregulated ones may be tumor-suppressor genes.

In the 50 pairs of OC and matched para-cancerous tissues enrolled in this experiment, we found that the expression level of miR-665-3p in OC tissues was at a distinct inferior position (Figure 1A). At the cellular level, we compared expression change of miR-665-3p in SKOV3 cells and

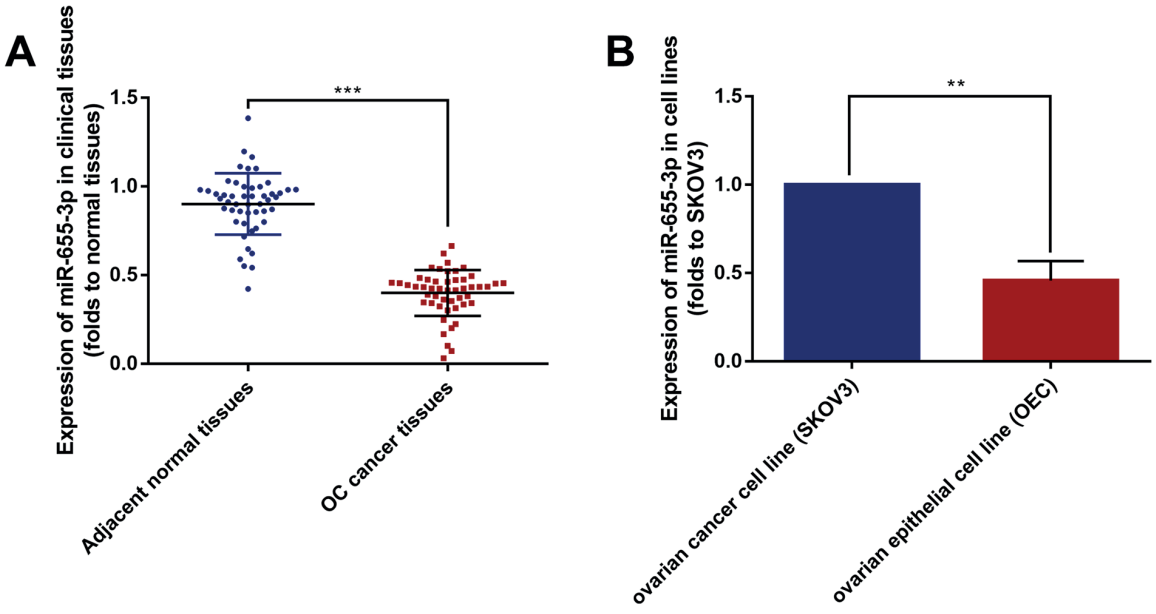

Figure 1. The expressions of miR-655-3p in ovarian cancer tissue samples and ovarian cancer cell. ***A,*** Difference in the expression of miR-655-3p between OC tissues and corresponding adjacent normal ovarian tissues (**$p<0.01$). ***B,*** The expression of miR-655-3p in OC cell line (SKOV3) and ovarian epithelial cell line (OEC) (***$p<0.001$).

OEC cells. QRT-PCR results indicated that the expression of miR-665-3p in OC cells was also in a trough, which was consistent with clinical specimens (Figure 1B). But whether miR-665-3p was a true "guardian" required further experimental research.

RAB1A was a Direct Target of MiR-655-3p in OC Cell

The transfection efficiency was confirmed in qRT-PCR experiments, in which transfection of miR-665-3p mimics increased the expression of miR-665-3p in SKOV3 cells successfully (Figure 2B).

The target gene of miR-655-3p was predicted using online prediction software, and it was found that RAB1A might be the potential target of miR-655-3p (Figure 2A). To verify whether RAB1A was the direct target of miR-655-3p, the luciferase reporter gene plasmids containing the wild-type and mutant-type RAB1A 3'UTRs were established. The luciferase activity was detected *via* dual-luciferase reporter assay to demonstrate the regulatory relationship between the RAB1A and miR-655-3p. The results showed that miR-655-3p could significantly inhibit the luciferase activity in cells transfected with the wild-type Rab1A-3'UTR plasmid, but it had no effect on that in cells transfected with the mutant-type Rab1A-3'UTR plasmid (Figure 2C).

MiR-655-3p Decreased the Expression Level of RAB1A

Dual-luciferase reporter assay confirmed the regulation of RAB1A by miR-655-3p in SKOV3 cells. Western blot results further identified that overexpression of miR-655-3p in SKOV3 cells

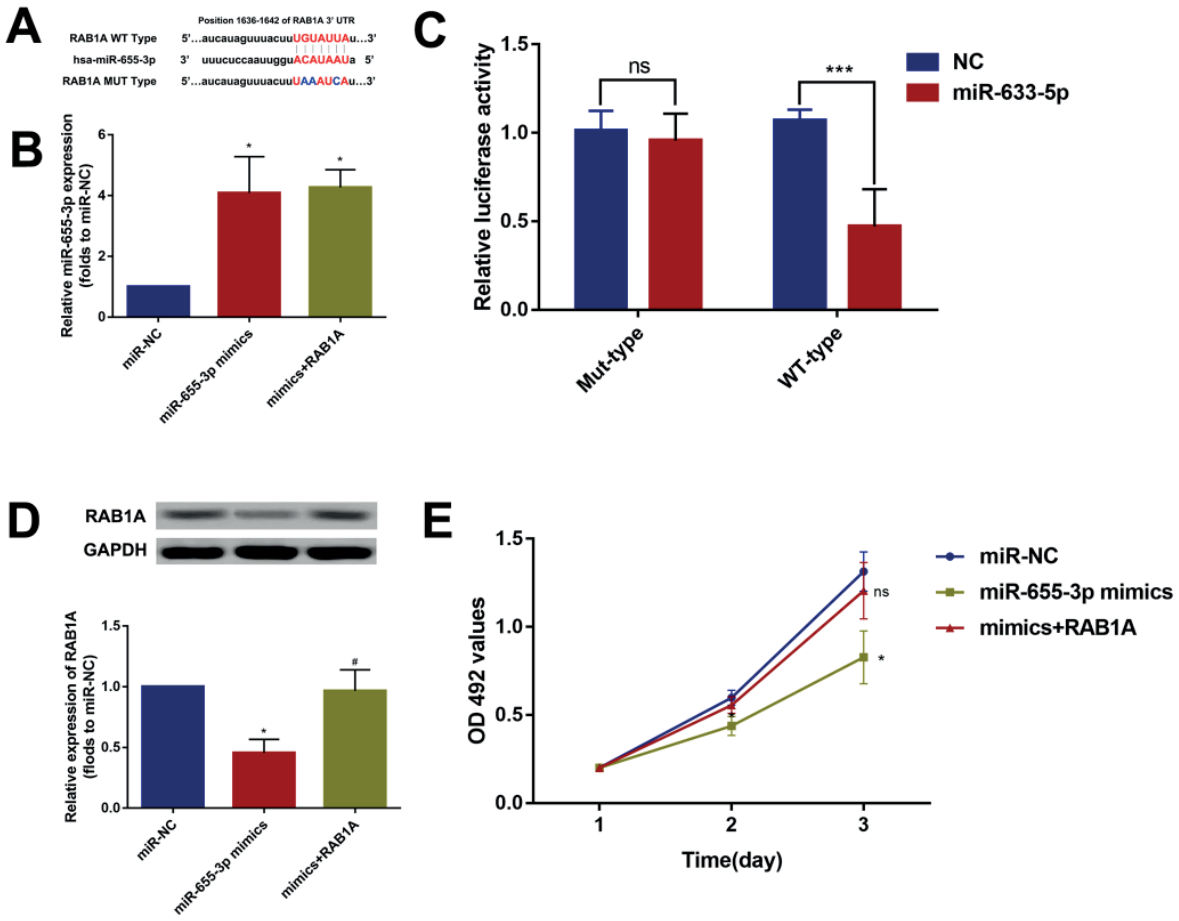

Figure 2. RAB1A is a direct and functional target of miR-655-3p. ***A,*** Diagram of putative miR-655-3p binding sites of RAB1A. ***B,*** Transfection efficiency was determined by qRT-PCR. ***C,*** Relative activities of luciferase reporters. ***D,*** The protein expressions of RAB1A detected by WB assay in OC cells after different treatment, ***E,*** The proliferation of OC cell detected by MTT after different treatment. Data were presented as means ± standard deviations (*p<0.05, ***p<0.001 *vs.* NC group; #p<0.05 *vs.* Mimics group).

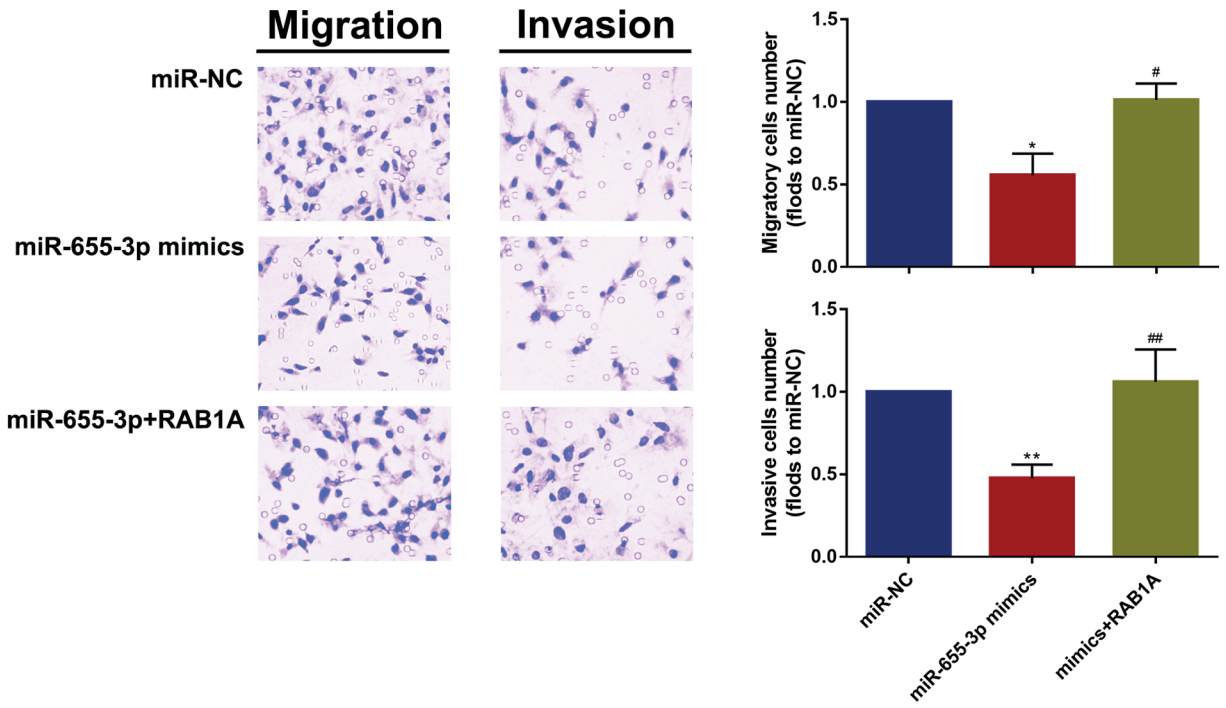

Figure 3. MiR-655-3p inhibited the invasion and migration of OC cell. The invasion and migration of OC cell post-transfection were analyzed using transwell assay and detected by microscope (× 200) (*p<0.05, **p<0.01 *vs.* NC group; #p<0.05 *vs.* Mimics group).

downregulated RAB1A expression. The negative regulation of miR-655-3p on RAB1 in OC was a focus of our experiment (Figure 2D).

MiR-655-3p Suppressed Proliferation of OC Cell

MTT assay results showed that overexpression of miR-655-3p inhibited the proliferation of SKOV3 cells. On the third day of the MTT assay, the cell proliferation status began to show a significant difference in the viability of SKOV3 cells between miR-655-3p overexpression group and control group. However, there was no significant difference in cell proliferative capacity between the miR-NC and co-transfected groups (Figure 2E).

MiR-655-3p Inhibited Migration of OC Cell

Invasion and migration are important factors affecting the prognosis of cancer patients, and EMT is an important mechanism for tumor cell invasion and migration.

The impacts of miR-655-3p on the cell migration and invasion were detected by transwell assay. By calculating the number of invading and migrating cells under the microscope, we could intuitively find the limitation of miR-655-3p on OC cell invasion and migration abilities (Figure 3).

Activation of EMT in our experiments was performed by detecting two important molecular markers: the epithelial cell marker E-cadherin and the mesenchymal cell marker N-cadherin. We found that EMT was also inhibited in miR-655-3p mimics group (Figure 4).

However, when the expression of RAB1A was restored, the metastatic ability and EMT activation of OC cells were correspondingly recovered.

Discussion

Since lin4 was discovered in 1993[15] and let7 in 2000[16], researchers started a wave of studies on miRNA. MiRNA widely exists in animals and large numbers of plants and exerts important regulatory effects in these organisms. It binds to the target gene in many ways, but mainly through the complementary pairing of the base, and different binding methods could result in varying regulations. MiRNA is large in number, and each type of miRNA has its own distinctive effect in different

organisms and all tissues and organs. According to the nature of the action, miRNA could be divided into cancer-promoting miRNA and cancer-inhibiting miRNA. Therefore, to find out the mechanism of regulating the occurrence and development of tumors, it was essential to first identify the differentially expressed miRNAs in tumor tissues and the specific expressions in the tumor.

Ras, a small GTPase, is the first proto-oncogene detected in cancer gene studies and ubiquitous in all eukaryocytes[17]. Ras-like protein in rat brain (Rab), as a major sub-family in the Ras superfamily, can be involved in many important processes[18]. Rab protein was discovered as early as in 1983. Rab1A, a member in the Rab family, plays a key role in the vesicle transport of proteins and it bears a very close relationship with protein transport[19]. Chia and Tang[18] have demonstrated that the abnormality of Rab protein is closely associated with the development of tumors[18]. In liver cancer, upregulated RAB1A can significantly enhance the proliferation, invasion, and migration abilities of SK-HEP-1 cells. Conversely, Rab1A silence downregulated the expression of P-S6K1 in the mTORC1 (p-S6K1) pathway, suggesting that RAB1A might regulate the growth changes of cells by affecting the mTORC1 (p-S6K1) pathway[20]. However, different from those in gastric cancer and liver cancer, RAB1A knockout in lung cancer had no influences on the expression levels of the mTORC1 (p-S6K1) and mTORC2 (p-AKT), nor changes the proliferation level of cells[21].

Most (75%) OC patients are accompanied by the progression of the disease and extensive distant metastases[22], and studies have manifested that invasion and migration tend to be closely associated with the poor prognosis of malignancies[23]. Moreover, epithelial-mesenchymal transition (EMT) is recognized as a key step for the invasion and migration of tumors[24]. EMT, known as epithelial-mesenchymal transition, refers to the transformation of the epithelial cells into mesenchymal cells. This process leads to the cytoskeletal reorganization and the loss of cell polarity to the epithelium, thus weakening and even damaging the adhesions between basal membrane cells and strengthening the transmigration and movement abilities of cells. EMT played important parts in multiple physiological and pathological processes, such as embryogenesis, migration, and invasion of the tumor, and drug resistance in chemotherapy[25]. EMT process is a key startup, and its activation is regarded as the characteristics for identifying malignancies[26], in which the crucial molecules include the epithelial cell marker E-cadherin and the mesenchymal cell marker N-cadherin. E-cadherin is a transmembrane glycoprotein maintaining the structural integrity of the epithelium while N-cadherin is a mesenchymal marker closely related to cancer progression[27,28]. In OC cells, the factors inducing EMT normally facilitate the development of OC, while EMT-inhibiting factors hinder the development of the cancer[29].

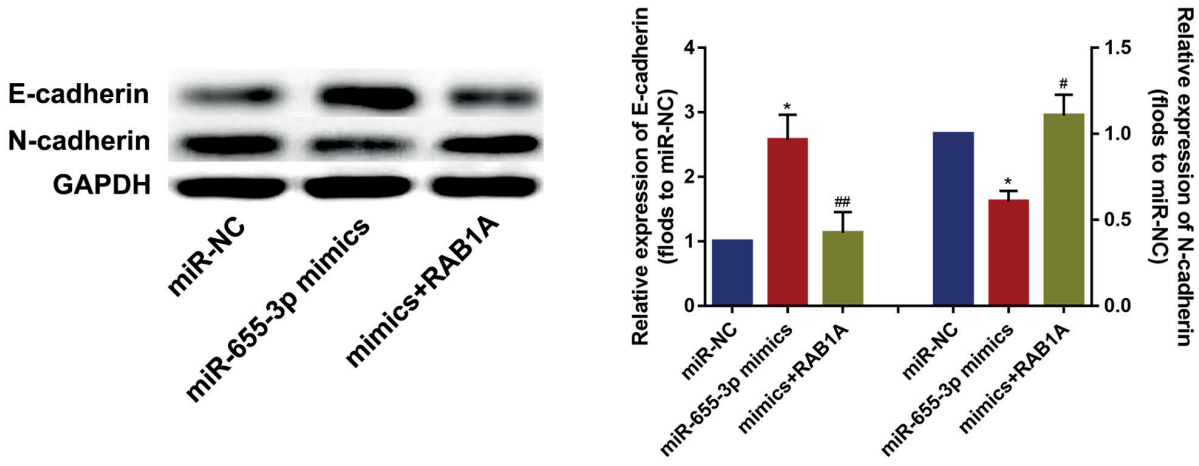

Figure 4. The epithelial-mesenchymal transition (EMT) of OC cells. The EMT associated markers post-transfection were detected by WB assay. Data were presented as means ± standard deviations (*p<0.05 *vs.* NC group; #p<0.05, ##p<0.01 *vs.* Mimics group).

Although studies showed that miR-655-3p is associated with the occurrence, development, invasion, and migration of multiple cancers, no studies have yet focused on its specific effect and mechanism in OC. In this work, differential expression of miR-655-3p in OC tissues and normal tissues was first detected by qRT-PCR, in which, the abnormally low expression of miR-655-3p in OC tissues was evident. The same results were also found at the cellular level. Based on a current study on the abnormal expression of miRNAs in malignant tumor tissues, it was especially important to find the target of miRNA and reveal their interaction. Combined with online prediction software, luciferase reporter, and subsequent WB experiment, the mutual constraint relationship between miR-655-3p and RAB1A in OC gradually emerged. As a downstream target gene of miR-655-3p, limiting the expression of RAB1A by over-expressing miR-655-3p in SKOV3 cells could achieve satisfactory results in controlling cell proliferation and metastasis.

Conclusions

For the first time, we highlighted the regulation of miR-655-3p on OC cells. By targeting to inhibit the expression of RAB1A, miR-655-3p exerted an excellent anti-cancer effect in OC cell line (SKOV3). Proliferation and metastatic ability of SKOV3 cells overexpressing miR-655-3p were controlled, indicating miR-655-3p/RAB1A, as a target for the treatment of OC, might become a feasible and new method of tumor treatment.

Conflict of interest

The authors declare no conflicts of interest.

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