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Original Article

MiR-361-5p decreases the tumorigenicity of epithelial ovarian cancer cells by targeting at RPL22L1 and c-Met signaling

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Abstract: A large number of studies have shown that miRNAs are important regulators of epithelial-to-mesenchymal transition (EMT) and are associated with metastasis in epithelial ovarian cancer (EOC). MiR-361-5p has been shown to play pivotal roles in tumorigenesis and metastasis; however, a role for miR-361-5p in EOC has not been reported. In this study, we found that miR-361-5p was significantly down-regulated in EOC tissues and cell lines. In addition, over-expression of miR-361-5p inhibited the migration and invasion of EOC cells in vitro. MiR-361-5p influenced the expression of the EMT-associated proteins by upregulating the epithelial marker E-cadherin and downregulating the mesenchymal markers, N-cadherin and vimentin. Further studies identify miR-361-5p directly targeted Ribosomal L22-like1 (RPL22L1) and c-Met. Moreover, miR-361-5p repressed the Akt/mTOR pathway after c-Met inhibition. Reintroduction of RPL22L1 and c-Met reversed miR-361-5p-induced EMT suppression. Consistently, inverse correlations were also observed between the expression of miR-361-5p and RPL22L1 or c-Met in human EOC tissue samples. Taken together, miR-361-5p inhibited the EMT progression in EOC cells by targeting RPL22L1 and c-Met/Akt/mTOR signaling.

Keywords: miR-361-5p, epithelial ovarian cancer, epithelial-mesenchymal transition, ribosomal protein L22 like 1

Introduction

Ovarian cancer is the fifth leading cause of cancer-related mortality among women and remains the most lethal gynecological malignancy in China [1-3]. Most ovarian cancers are epithelial, and treatment usually includes cytoreductive surgery followed by chemotherapy [4]. Unfortunately, the majority of patients who respond to primary chemotherapy later experience relapse. Therefore, identification of novel diagnostic markers and therapeutic targets is crucial.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate the expression of genes implicated in fundamental biological processes such as proliferation differentiation, and apoptosis [5-7]. With increasing research, it is now becoming obvious that many miRNAs are misregulated in a variety of tumors and influence the development and progression of cancer, including ovarian carcinoma [8, 9]. It has been reported that miR-361-5p is involved in several malignant processes, including tumor proliferation, epithelial-mesenchymal transition (EMT), and angiogenesis [10-13]. However, the effect of miR-361-5p on ovarian cancer EMT has not been reported. Thus, we investigated whether miR-361-5p contributes to the metastatic behavior of ovarian cancer cells.

Epithelial-mesenchymal transition (EMT) is a vital process by which epithelial cells lose their polarity and are converted to a mesenchymal phenotype [14]. RPL22L1 was first identified in a screen for 14-3-3ε binding partners in mice brains [15] and has been identified as a novel prognostic marker in maintaining the aggressive phenotype of ovarian cancer and in triggering cell metastasis by inducing EMT [16]. The membrane receptor c-Met (Mesenchymal epithelial transition factor), also known as hepatocyte growth factor receptor is essential for tumor progression, invasion, and metastasis in cancer patients [17]. However, whether miR-361-5p participates in EOC c-Met signaling regulation remains uncertain.

The present study investigated the regulatory role and implications of aberrant expression of miR-361-5p in epithelial ovarian cancer (EOC). We provided evidence that miR-361-5p could impede migration, invasion and EMT of SKOV3 and OVCAR3 cells by targeting the RPL22L1 and c-Met/Akt/mTOR pathways. It may be a possible therapeutic target of ovarian cancer.

Materials and methods

Patients and tissue samples

A total of 52 EOC specimens were collected from patients who underwent surgery at the Department of Obstetrics and Gynecology, the First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China. Written informed consent was obtained from each patient, and the use of clinical specimens was approved by the Institutional Ethics Committee.

Cell lines and cell culture

The human ovarian cancer cell lines (SKOV3, OVCA429, A2780, OVCAR3, Caov3 and COV-644) and normal Human Ovarian Surface Epithelial (HOSE) cells were purchased from the China Center for Type Culture Collection (CCTCC). The SKOV3, OVCA429, A2780, and OVCAR3 cell lines were cultured in RPMI-1640 medium. Caov3 and COV644 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, MD, USA). HOSE cells were cultured in a medium containing 1:1 mixture of MCDB 105 and M199 medium (Sigma). All media were supplemented with 10% fetal bovine serum (FBS) and 1% (wt/vol) antibiotic (penicillin/streptomycin). The cells were cultured at 37°C in a 5% CO_2 atmosphere in a humidified incubator.

Oligonucleotides and transfection

mimic-NC, miR-361-5p, inhibitor-NC and miR-361-5p-in were purchased from Ribobio (Guangzhou, China) and transfected with Lipofectamine2000 Reagent (Invitrogen) following the manufacturer's protocol in SKOV3 or OVCAR3 cells at a final concentration of 50 nM.

Migration and invasion assay

Migration and invasion assays were performed using the transwell system (24-well, 8 μm pore size; BD falcon) according to the instruction manuals. Briefly 5-10 × 10^4 cells were seeded into the upper chambers of the transwell with a certain medium containing no FBS, and the lower chambers were filled with culture media containing 5% FBS as a chemoattractant. The chambers were incubated at 37°C for 4-48 hours depending on the cell line type. Successfully translocated cells were fixed by methanol for 15 minutes, stained with 0.2 crystal violet for 30 minutes, and counted under a light microscope.

Luciferase reporter assay

The 3'UTR of RPL22L1 and c-Met and mutated controls were cloned and inserted into the pMIR-GLOTM Luciferase vector (Promega, USA). miRNA mimics were then transfected into SKOV3 or OVCAR3 cells containing wild-type or mutant 3'UTR pMIR-GLOTM plasmids. After cell lysis, the luciferase activity was measured using the Luciferase Reporter Assay System (Promega Corporation Madison, WI, USA) according to manufacturer's protocol.

Quantitative real-time PCR

Total RNA was extracted from cultured cells with Trizol reagent (Ambion) according to the manufacturer's protocol. First-strand cDNA was generated through reverse transcription via RevertAidTM Reverse Transcriptase (Thermo ScientificTM Life Technologies, NY, USA). Primer sequences used in the experiments were as follows: RPL22L1 forward 5'-AGAAGGTTAAAGTCAATGG-3', reverse 5'-ATCACGAAGATTGTTCTTC-3'; E-cadherin forward 5'-CTGCTGCAGGTCTCCTCTTG-3', reverse 5'-TGTCGACCGGTGCAATCTTC-3'; N-cadherin forward 5'-ACAGTGGCCACCTACAAAGG-3', reverse 5'-CCGAGATGGGGTTGATAATG-3'; Vimentin forward 5'-AAGGCGAGGAGAGCAGGATT-3', reverse 5'-GGTCATCGTGATGCTGAGAAG-3'; GAPDH forward 5'-ATCACTGCCACCCAGAAGAC-3', reverse 5'-TTTCTAGACGGCAGGTCAGG-3'; The expression levels of miRNA were detected by hydrolysis Probes miRNA assays (Applied Biosystems) and normalized to U6 small nuclear RNA. The 2-ΔΔCt method was adopted and applied to calculate the relative quantities of subject genes. qRT-PCR was performed according to MIQE guideline standards. All reactions were performed in triplicate.

Western blot analysis

Cells were lysed and protein concentration was determined using the BCA Assay Kit (Thermo

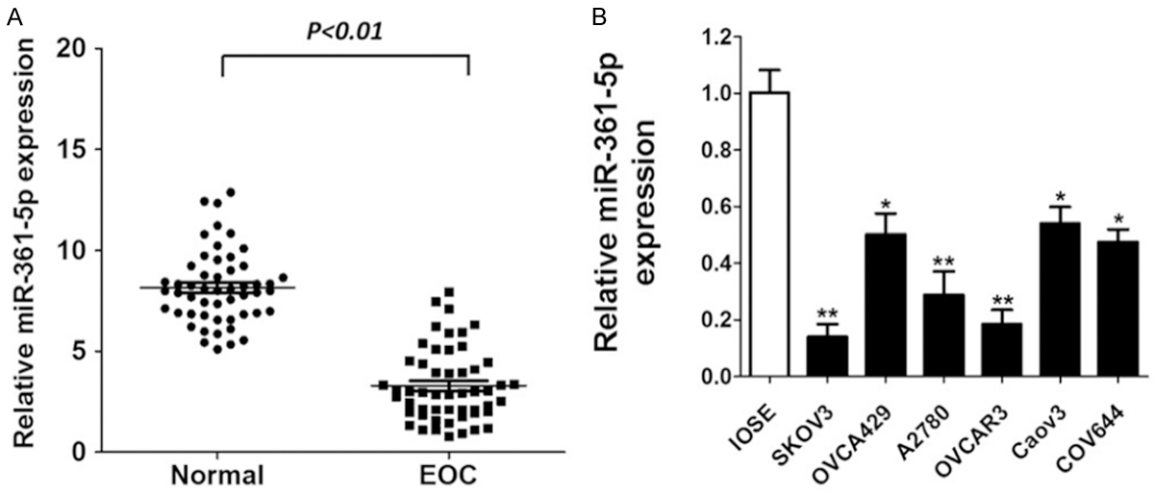

Figure 1. miR-361-5p was down-regulated in EOC tissues and cell lines. A. miR-361-5p expression was determined by Real-time PCR and normalized against an endogenous control (U6) in 52 pairs of human ovarian cancer tissues (EOC) and adjacent non-tumor tissues (Normal). Each bar represents the mean of three independent experiments. ***P < 0.01*. B. Real-time PCR analysis of relative miR-361-5p expression in non-tumorigenic epithelial cell line HOSE and various tumor cell lines.

Scientific). Cellular proteins were extracted and separated in 4-10% Tris glycine/SDS-polyacrylamide gels and electrotransferred to ECL nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated with specific antibodies. All of the antibodies were purchased from Cell Signaling Technology Company, and the GAPDH protein was used as the endogenous control.

Statistical analysis

All statistical analyses were performed using the SPSS13.0 software (SPSS Inc., Chicago, IL), and data were expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant. Differences in the mean of samples were analyzed using the Student's t test or one way ANOVA test with selected comparison using Tukey's HSDpost-hoc test or Wilcoxon signed-rank test. The correlation between miR-361-5p and RPL22L1, and c-Met was determined by Spearman's rank test. The experiments were repeated at least in triplicate.

Results

MiR-361-5p is significantly down-regulated in EOC tissues and cells

To determine the level of miR-361-5p expression in EOC, we compared its expression in 52 pairs of human EOC tissues and pair-matched adjacent non-tumor tissues by Quantitative real time PCR. We found that miR-361-5p expression was down-regulated in all ovarian cancer samples when compared to the benign ovarian tissue lesions (**Figure 1A**). We also tested the expression of miR-361-5p by qRT-PCR in various ovarian cancer cell lines with the non-tumorigenic epithelial cell line HOSE as a control. Consistently, the expression levels of miR-361-5p in all six ovarian cancer cell lines tested were significantly reduced at different degrees compared to the HOSE cells (**Figure 1B**). Taken together, these data suggest that miR-361-5p is down-regulated in ovarian cancer, which may contribute to ovarian cancer pathogenesis.

Inhibitory effects of miR-361-5p on cell migration, invasion and EMT in EOC cells

To investigate the potential function of miR-361-5p in EOC tumorigenesis, we selected the SKOV3 and OVCAR3 cell lines among the six cell lines that we tested, to conduct further functional studies. We first transfected miR-361-5p, miR-361-5p-in or NC into EOC cells in order to reveal the gain-of-function effect or lose-of-function effect on the migration and invasion of the EOC cell lines. The transwell migration assay showed that the restoration of miR-361-5p significantly inhibited the migration capacity in both SKOV3 and OVCAR3 cells (**Figure 2A**). On the contrary, downregulating miR-361-5p expression using an inhibitor sig-

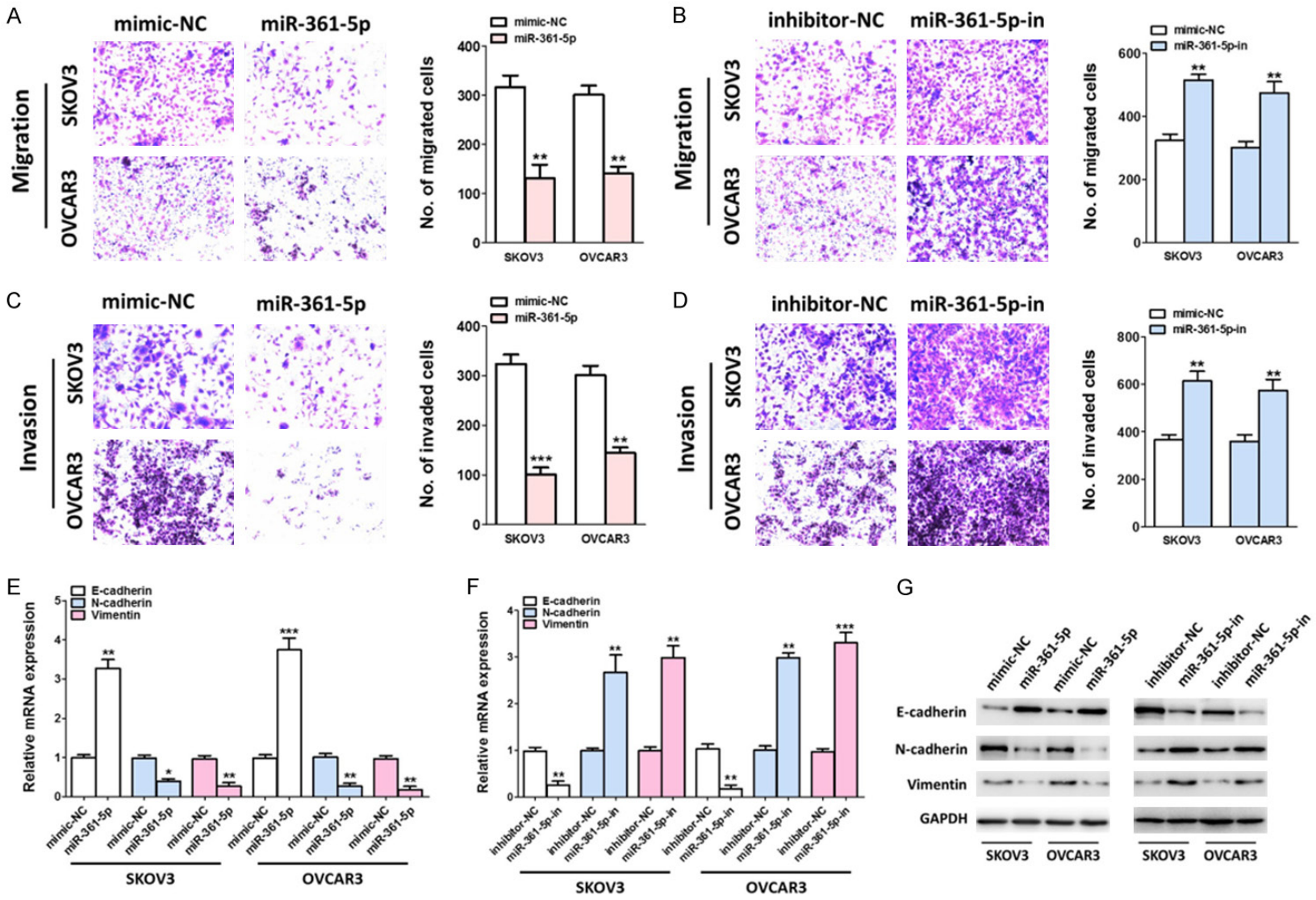

Figure 2. Ectopic expression of miR-361-5p suppresses EOC cell migration and invasion and regulates EMT related gene expression. A and B. Effects of ectopic expression of miR-361-5p on the migration of SKOV3 and OVCAR3 cells. C and D. Effects of ectopic expression of miR-361-5p on the invasion of SKOV3 and OVCAR3 cells. The cell number of migrated cells were counted in randomly selected fields and presented in the bar graph (means ± SD; *$P < 0.05$; **$P < 0.01$, Student's t test). E and F. Real-time PCR analysis of E-cadherin, N-cadherin, and Vimentin in SKOV3 and OVCAR3 cells, in response to mimic-NC, miR-361-5p, inhibitor-NC or miR-361-5p-in. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. G. Western blot analysis of E-cadherin, N-cadherin, and Vimentin in SKOV3 and OVCAR3 cells, in response to mimic-NC, miR-361-5p, inhibitor-NC or miR-361-5p-in. GAPDH was used as loading control.

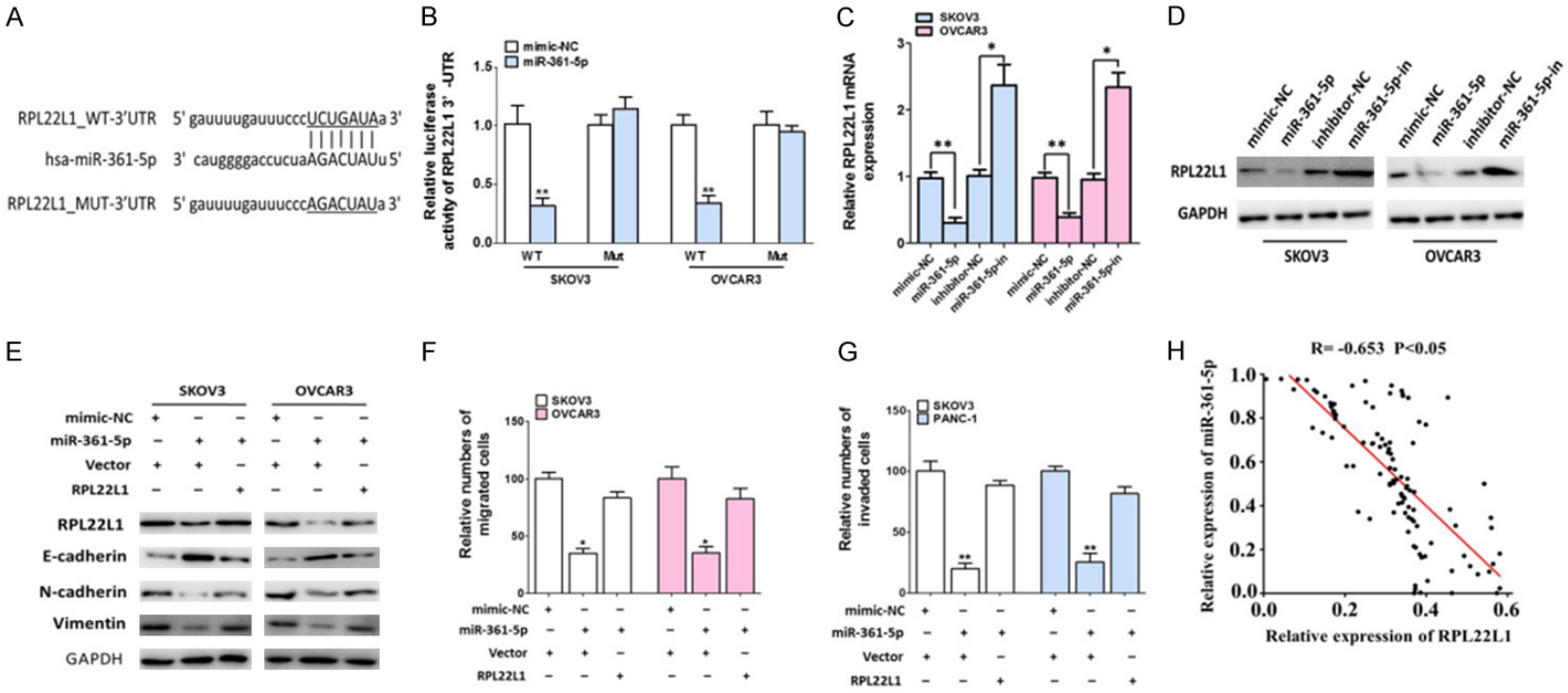

Figure 3. miR-361-5p directly targeting RPL22L1. A. Sequence of miR-361-5p with the putative binding sites of RPL22L1 3'-UTR. B. Luciferase activity of SKOV3 and OVCAR3 cells transfected with plasmids carrying a wild-type or mutant 3'UTR of RPL22L1, in response to miR-361-5p over-expression. C. mRNA levels of RPL22L1 examined by Real-time PCR in SKOV3 and OVCAR3 cells transfected with mimic-NC, miR-361-5p, inhibitor-NC or miR-361-5p-in. *$P < 0.05$ compared with the mimic-NC or inhibitor-NC group. D. Protein levels of RPL22L1 examined by Western blot in SKOV3 and OVCAR3 cells transfected with mimic-NC, miR-361-5p, inhibitor-NC or miR-361-5p-in. GAPDH was used as loading control. E. The protein level of RPL22L1, E-cadherin, N-cadherin and vimentin was detected by Western blot in cells transfected with indicated molecules. F. Cell migration assay to assess the invasion abilities of SKOV3 and OVCAR3 cells transfected with indicated molecule. *$P < 0.05$ compared with control miRNA and vector transfected cells. G. Cell invasion assay to assess the invasion abilities of SKOV3 and OVCAR3 cells transfected with indicated molecule. **$P < 0.01$ compared with control miRNA and vector transfected cells. H. The mRNA expression levels of RPL22L1 correlated inversely with miR-361-5p.

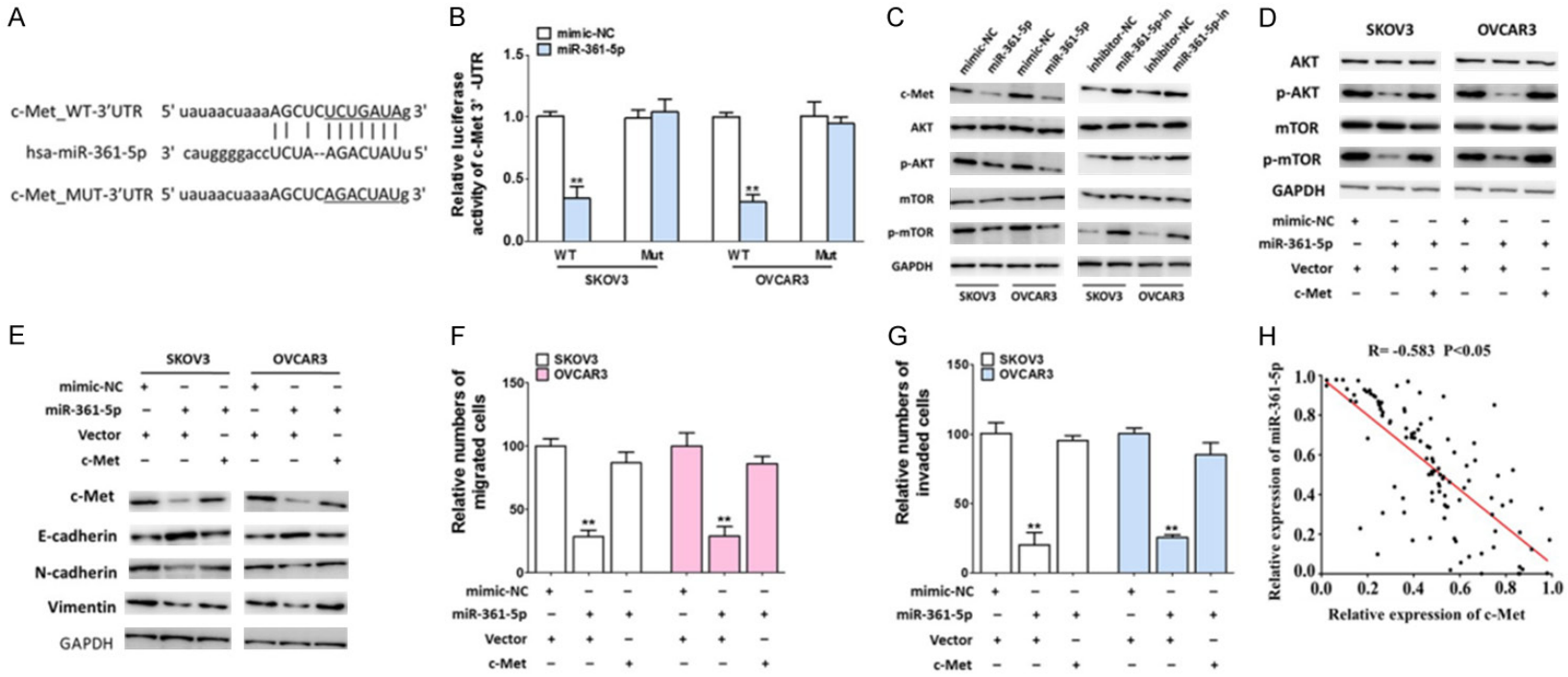

Figure 4. miR-361-5p suppresses EMT via c-Met/Akt/mTOR signaling. A. Sequence of miR-361-5p with the putative binding sites of c-Met 3'-UTR. B. Luciferase activity of SKOV3 and OVCAR3 cells transfected with plasmids carrying a wild-type or mutant 3'UTR of c-Met, in response to miR-361-5p over-expression. C. c-Met, AKT, p-AKT, mTOR and p-mTOR protein expression levels in SKOV3 and OVCAR3 cells transfected with mimic-NC, miR-361-5p, inhibitor-NC or miR-361-5p-in. D. The protein level of AKT, p-AKT, mTOR and p-mTOR was detected by western blot in cells transfected with indicated molecules. E. The protein level of c-Met, E-cadherin, N-cadherin and vimentin was detected by western blot in cells transfected with indicated molecules. F. Cell migration assay to assess the invasion abilities of SKOV3 and OVCAR3 cells transfected with indicated molecule. ***P < 0.01* compared with control miRNA and vector transfected cells. G. Cell invasion assay to assess the invasion abilities of SKOV3 and OVCAR3 cells transfected with indicated molecule. ***P < 0.01* compared with control miRNA and vector transfected cells. H. The mRNA expression levels of c-Met correlated inversely with miR-361-5p.

nificantly enhanced the migratory activity of SKOV3 and OVCAR3 cells (**Figure 2B**). Meanwhile, the transwell invasive assay demonstrated that miR-361-5p restoration significantly repressed the invasiveness of SKOV3 and OVCAR3 cells, whereas inhibiting miR-361-5p expression facilitated EOC cell invasion (**Figure 2C**, **2D**). Since EMT is one of the critical steps of cancer cell metastasis [18], we then examined the mRNA and protein expression levels of EMT markers in mimic-NC-, miR-361-5p-, inhibitor-NC- or miR-361-5p-in- transfected EOC cells. Over-expression of miR-361-5p resulted in an increased expression of E-cadherin and a decreased expression of N-cadherin and Vimentin, while knockdown of miR-361-5p had the opposite effects (**Figure 2E-G**). Collectively, these results support our hypothesis that miR-361-5p suppresses EOC cell migration, invasion and EMT.

RPL22L1 is a direct downstream target of miR-361-5p

As the function of miRNAs in tumor development is dependent on inhibiting their target mRNAs by binding to the 3'UTR, it is crucially important to identify the targets of miR-361-5p. On the basis of two major prediction software programs, TargetScan and miRanda, the putative binding site of miR-361-5p in the 3'UTR of RPL22L1 was predicted (**Figure 3A**). In order to validate whether RPL22L1 is the direct target of miR-361-5p, a dual-luciferase assay was used with co-transfection of miR-361-5p and a luciferase reporter plasmid containing a wild-type or mutant 3'UTR of human RPL22L1. EOC cells co-transfected with a miR-361-5p mimic and wild-type RPL22L1 3'UTR showed a significant decrease in luciferase activity (**Figure 3B**). However, there was no detectable change in luciferase activity when the seed region of RPL22L1 was mutated (**Figure 3B**). In addition, we carried out qRT-PCR and Western blotting to confirm that restoration of miR-361-5p resulted in downregulation of RPL22L1 in SKOV3 and OVCAR3 cells. Compared to the control cells, the mRNA and protein expression level of RPL-22L1 was significantly repressed in cells with the miR-361-5p mimic but inversely upregulated in cells with the miR-361-5p inhibitor (**Figure 3C**, **3D**).

It has been reported that RPL22L1 is implicated in EOC progression [16]. Down-regulation of RPL22L1 by miR-361-5p in ovarian cancer prompted us to investigate whether miR-361-5p affects the EMT process by regulating RPL22L1 expression. SKOV3 and OVCAR3 cells were co-transfected with miR-361-5p and either pMIR-GLOTM-RPL22L1 (without 3'-UTR region) or the empty pMIR-GLOTM vector. Although miR-361-5p increased E-cadherin expression and decreased N-cadherin or vimentin expression, RPL22L1 induction markedly rescued these activities by suppressing E-cadherin and promoting N-cadherin or vimentin expression (**Figure 3E**). The cells were then subjected to transwell migration and invasion assays to identify whether RPL22L1 ameliorates miR-361-5p-induced suppressed migration and invasion of EOC cells. Our results indicate that miR-361-5p is an ovarian tumor suppressor to mediate cell migration and invasion (**Figure 3F**, **3G**). Moreover, a Spearman correlation analysis revealed a reverse correlation between miR-361-5p and RPL22L1 expression (**Figure 3H**). Overall, these results suggested RPL22L1 was the potential functional target gene of miR-361-5p.

miR-361-5p suppresses c-Met/Akt/mTOR signaling and EMT through targeting c-Met

To investigate whether c-Met is another target of miR-361-5p, we screened the 3'UTR region of c-Met mRNA using TargetScan and miRanda. We found that the 3'UTR of c-Met mRNA contained a complementary site for the seed region of miR-361-5p (**Figure 4A**). To determine whether c-Met is the direct target gene for miR-361-5p, a dual-luciferase reporter system was used. As shown in **Figure 4B**, transfection of miR-361-5p caused a significant decrease in luciferase activity in cells transfected with the reporter plasmid with the wild type targeting sequence of c-Met mRNA but not the reporter plasmid with the mutant sequence of c-Met. These findings demonstrated that miR-361-5p downregulated c-Met expression by binding to its 3'UTR directly. We next examined whether miR-361-5p could inhibit EMT by targeting the c-Met signaling. Western blot analysis showed that c-Met protein levels were reduced in EOC cells with miR-361-5p mimic and upregulated in cells with miR-361-5p inhibitor (**Figure 4C**). In addition, we found that the levels of both phosphorylated Akt (p-Akt) and phosphorylated mTOR (p-mTOR) decreased in the miR-361-5p mimic EOC cells but elevated in the miR-361-5p inhibited cells (**Figure 4C**). Moreover, restoring c-Met expression counteracted the downregu-

lation of p-Akt and p-mTOR, induced by miR-361-5p (**Figure 4D**).

In order to further investigate whether c-Met cooperates with miR-361-5p in EOC cell EMT regulation, miR-NC or miR-361-5p mimic was co-transfected with c-Met expression plasmid or its related vector respectively into EOC cells. The expression of c-Met, E-cadherin, N-cadherin, and vimentin was verified by western blot analysis. In **Figure 4E**, c-Met restoration decreased E-cadherin expression and increased N-cadherin or vimentin expression by the miR-361-5p mimic. Accordingly, the transwell invasive assay verified that reintroduction of c-Met reinforced the cell migratory and invasiveness ability of EOC cells, which were inhibited by the miR-361-5p mimic (**Figure 4F**, **4G**). Moreover, the analysis of correlation of c-Met and miR-361-5p in human EOC samples showed the inverse correlation between c-Met and miR-361-5p (**Figure 4H**). Together, these results demonstrated that miR-361-5p suppresses EMT by targeting the c-Met/Akt/mTOR pathway in EOC cells.

Discussion

Growing evidence has indicated that deregulation of miRNAs contributes to the pathogenesis of most human malignancies including ovarian cancer [8]. miR-361-5p was identified to be downregulated in diverse cancers, including prostate cancer, cutaneous squamous cell carcinoma (SCC), hepatocellular carcinoma, colorectal cancer and gastric cancer [10-13]. However, very little was known about its role and molecular mechanism in EOC. In the present study, we showed that miR-361-5p was downregulated in ovarian cancer cell lines and tumor tissues compared with normal ovarian surface epithelial cells and normal ovarian tissues. In addition, we demonstrated that miR-361-5p suppressed EOC cell migration and invasion, suggesting a fundamental role of miR-361-5p as a tumor suppressor in ovarian cancer. To investigate the underlying mechanisms, we found that miR-361-5p repressed EOC cell EMT progression by directly targeting the RPL22L1 and c-Met/Akt/mTOR signaling pathways.

Epithelial-mesenchymal transition (EMT), one of the important mechanisms that induces invasion and metastasis of tumors, is a process by which epithelial cells lose their polarity and are converted to a mesenchymal phenotype [19]. Because the major effect of miR-361-5p was considered to function on cell migration and gene expression regulation, we detected the change of EMT markers in SKOV3 and OVCAR3 cells transfected with miR-361-5p and miR-361-5p-in. Over-expression of miR-361-5p increased the expression of E-cadherin and reduced the expression of vimentin and fibronectin in SKOV3 and OVCAR3 cells, while knockdown of miR-361-5p had the opposite effect. The results supported the conclusion that miR-361-5p may reverse the EMT process to inhibit cell migration.

A recent study showed that RPL22L1 played a critical role in EOC progression by enhancing cell invasion and metastasis via inducing EMT [16]. In the present study, RPL22L1 was predicted as the direct target of miR-361-5p by Targetscan and miRanda. In addition, our results demonstrated that upregulating RPL22L1 reverses the miR-361-5p-induced EMT inhibition, verifying that the EMT induced by miR-361-5p deficiency in EOC requires RPL22L1 participation. We first provided evidence that RPL22L1 is direct target of miR-361-5p.

c-Met is a well-characterized oncogene and is frequently overexpressed in a variety of human cancers [20]. In this study, we also observed that c-Met is directly inhibited by the binding of miR-361-5p to its 3'UTR, leading to the suppression of EMT. Taken together with previous studies, it seems that miR-361-5p serves as a pivotal mediator in the regulation of ovarian cancer metastasis.

In summary, we found, for the first time, that miR-361-5p directly regulated RPL22L1 and c-Met expression in EOC. Our data provide evidence that miR-361-5p suppresses the migration and invasion of EOC cells and inhibits the EMT progress. The c-Met/Akt/mTOR pathway is also involved in miR-361-5p-mediated EMT regulation. Our study suggests previously unidentified therapeutic strategies for EOC by targeting miR-361-5p. These findings provide a better understanding of the development and progression of EOC and may be an important implication for future therapy of the EOC.

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Disclosure of conflict of interest

None.

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