

miR-377 targets CUL4A and regulates metastatic capability in ovarian cancer

RUFEN YU¹, LIMEI CAI¹, YINGUI CHI¹, XIANGCUI DING¹ and XUEQING WU²

¹Department of Obstetrics and Gynecology, Ruian People's Hospital, The Third Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325200; ²Department of Obstetrics and Gynecology, The First Affiliated Hospital of Wenzhou Medical University, The First Provincial Wenzhou Hospital of Zhejiang, Wenzhou, Zhejiang 325000, P. R. China

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Abstract. The incidence and recurrence rates of ovarian cancer are still high, and once the disease metastasizes, it is nearly always fatal. Cullin 4A (CUL4A) serves a significant role in tumorigenesis and tumour progression; however, the effect and mechanisms underlying CUL4A overexpression are still unknown. The role of microRNAs (miRs) in the regulation of metastatic capability in ovarian cancer cell lines was investigated. The interaction between miR-377 and CUL4A was investigated using bioinformatics analyses and dual-luciferase reporter assays. Furthermore, miR-377 mRNA and protein levels were detected using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively and cell migration and invasion were detected using a Transwell assay. Results revealed that CUL4A expression was negatively associated with miR-377 levels in ovarian cancer tissues and cell lines. Through *in silico* analysis, the targeting effect of miR-377 on CUL4A was verified. Ectopic expression of miR-377 in SKOV3 cells downregulated the level of CUL4A, and significantly reduced the migratory ability of the cells. miR-377 overexpression led to reduced activity of the Wnt/ β -catenin signaling pathway, and regulated the expression of matrix metalloproteinase-2, and 9, and epithelial-mesenchymal transition (EMT)-associated protein. These results suggested that miR-377 is a significant negative regulator of CUL4A that controls cancer cell progression in ovarian cancer cell lines.

Introduction

According to a previous study, ovarian cancer still remains the fifth-leading cause of mortality in women, with approximately

22,280 newly diagnosed cases and 14,240 mortalities reported in 2016 worldwide (1). With such a high recurrence rate, over three quarters of ovarian cancer patients eventually relapse following primary platinum and taxane-based chemotherapy (2). Development of cytotoxic chemotherapy and novel targeted therapies have enhanced progression-free survival, however failed to have significant influence on overall survival (3,4). Malignant tumours, including ovarian cancer, are characterized by tumour metastasis from the primary site to other parts via the lymphatic system, blood vessels, or body cavity (5). To better understand the underlying molecular mechanism of carcinogenesis in order to develop more effective therapeutic strategies, more research studies need to be conducted.

Previously, the use of miRNAs as a novel effective target in cancer therapy has been extensively reported (6-10). miRNAs are small non-coding RNA molecules with a length varying from 19-25 nt. Previous studies have demonstrated that miRNAs that are generated in cells serve important roles in a variety of biological processes; in particular, they negatively regulate gene expression following transcription. A mature miRNA chain is able to combine with the 3' untranslated region (UTR) of target mRNA in an RNA-induced silencing complex; in this way, complete complementary miRNA and target mRNA result in inhibition or activation of translation of the mRNA (11-13). miRNAs take part in various physiological processes, including cell proliferation, differentiation, and apoptosis, by regulating transcription of their target genes. In neoplastic processes, aberrantly expressed miRNAs affect proliferation, migration, and invasion of cancer cells by mediating associated proteins or transcription factors so as to exert promotive or inhibitory effects on tumorigenesis and metastasis (14-16). Several lines of evidence have verified that miRNAs may directly or indirectly control oncogenes or suppressor genes to mediate protein expression of cancer-associated pathways (17,18). Based on previous studies, miR-377, a member of the large miRNA cluster on chromosome 14q32, is weakly expressed in several human malignancies, including ependymoma, osteosarcoma, neuroblastoma, gastro-intestinal stromal tumours, and gliomas (6-10). To the best of the author's knowledge, there is a lack of information about the association between miR-377 and ovarian cancer; thus, the present study investigated whether miR-377 serves

Correspondence to: Mr. Yingui Chi, Department of Obstetrics and Gynecology, Ruian People's Hospital, The Third Affiliated Hospital of Wenzhou Medical University, 108 Wansong Road, Ruian, Wenzhou, Zhejiang 325200, P.R. China
E-mail: yinguiichi@163.com

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an important role in the control of proliferation and metastasis in ovarian cancer, and in particular, if it exhibits inhibitory capabilities.

Cullin (CUL) 4A, which was demonstrated to be one of the target genes of miR-377 in the present study, belongs to the family of Cullin-Ring E3-ligases (CRLs) and serves a key role in tumorigenesis and tumor progression (19). CUL4A constitutes a helical N-terminal domain and a spherical C-terminal domain which combine with Ras like without CAAX 1 (RIT1) and damage specific DNA binding protein 1, respectively (20). CUL4A, initially reported in breast cancer, was previously demonstrated overexpressed in numerous cancers, including squamous-cell carcinoma, adrenocortical carcinoma, medulloblastoma and liver cancer (21). Overexpression of CUL4A leads to promotion of cancer cell proliferation, and is closely and positively associated with overall survival and disease-free survival of cancer patients (22). The present study focused on the role of miR-377 in the control of CUL4A expression in ovarian cancer cell lines.

Materials and methods

Tumor samples. A total of 44 ovarian cancer patients in the Third Affiliated Hospital of Wenzhou Medical University were included in the present study. During the period from February 2015 to March 2016, all cancer specimens were obtained from surgical tumor resections, and their adjacent normal ovarian tissue specimens were collected at the same time, as negative controls. The normal and cancer tissues represented matched pairs from each patient. Basic clinical and pathological data of these patients at the average age of 51.12 ± 16.35 were collected with their written informed consent forms. The present study was approved by the ethics committee of the Third Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

Cell culture and grouping. Normal ovary cell line (IOSE80) and seven ovarian cancer cells lines including CAOV3, SKOV3, A2780, OVCAR3, HO-8901, 3AO and TC-1 were all obtained from Shanghai Bogoo Biotechnology Co. Ltd. (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Thermo Fisher Scientific Inc, Waltham, MA, USA) at 37°C in 5% CO₂ incubator. Medium was replaced once every two days. Cell passage cultivation was performed when cells grew to 90% confluency.

One cell line from six ovarian cancer cell lines was selected based on the expression of the miR-377 and CUL4A levels for the primary *in vitro* experiments of the effects of miR-377 on CUL4A in ovarian cancer in the present study. Cells were randomly allocated into three groups: Control group, mock group (cells transfected with blank plasmids) and mimics (cells transfected with miR-377 mimics). Recombinant plasmids were purchased by Nanjing Cobioer Biotechnology Co. Ltd. (Jiangsu, China). A total of 500 ng/ μ l plasmids were prepared. Transfection processes were carried out using Lipofectamine[®] 2000 reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Prior to subsequent experimentation, transfected cells were incubated at 37°C in a 5% CO₂ incubator for 48 h.

Bioinformatics analysis. To analyze the role of miRNA-377 in ovarian cancer, prediction of its target genes was carried out using the prediction software miRanda (www.microrna.org/microrna/home.do), miRDB (www.mirdb.org/), PicTar (pictar.mdc-berlin.de/) and TargetScan (www.targetscan.org/vert_71/) and the function of miRNA-377 was analyzed through the Database for Annotation, Visualization and Integrated Discovery (www.david.ncifcrf.gov/).

Luciferase reporter assay. CUL4A 3'-UTR and mutated CUL4A 3'-UTR were prepared by GeneCopoeia Inc. (Rockville, MD, USA). The 3'-UTR fragment of CUL4A with the binding site for miR-377 mimic and inhibitor was amplified via polymerase chain reaction (PCR) with the primer sequences presented in Table I and cloned into luciferase vectors (Promega Corporation, Madison, WI, USA). SKOV3 cells were seeded into 96-well plates at the density of 1×10^5 cells per well one day prior to transfection. Control luciferase reporter plasmid, CUL4A 3'-UTR and mutated CUL4A 3'-UTR were co-transfected with either miR-377 mimic or miR-377 inhibitor using Lipofectamine[®] 3000 (Thermo Fisher Scientific Inc., USA). A total of 48 h following transfection, luciferase activity was determined with Secret-Pair[™] Dual-Luciferase Reporter Assay (GeneCopoeia Inc.). The results were normalized through comparison with *Renilla* luciferase activity.

Cell Counting Kit (CCK)-8 assay. Cell viability in control, mock, and mimic groups was measured using the CCK-8 assay (Beyotime Institute of Biotechnology, Shanghai, China). Cells were seeded into a 96-well plate (100 μ l/well), and cultured at 37°C in a 5% CO₂ incubator. A total of four hours following incubation, 10 μ l CCK-8 reagent was added to each well and incubated for 1-4 h. Optical density values were read by a iMark microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm.

Transwell assay. SKOV3 cells (5×10^5 cells/ml) were seeded in the upper well of a Transwell migration system on ThinCerts[™] inserts with 8- μ m membranes (Huayue ReaCon Inc., Guangdong, China) in DMEM supplemented with 0.1% fetal bovine serum (FBS). The lower wells received the same medium supplemented with 10% FBS. Following a 24-h incubation period, the contents of the upper wells, which contained non-migrating cells were removed by cotton swabs. For invasion detection, the upper chambers were specifically coated with Matrigel. Cells that migrated or invaded through the membranes were fixated with 70% cold ethanol, stained with 0.1% crystal violet for 20 min at room temperature, and imaged using a light microscope (Olympus Corporation, Tokyo, Japan). The quantity of migrated cells was analysed from five randomly selected fields under the light microscope at a magnification of x100.

Reverse transcription-quantitative (RT-q) PCR. Quantification of miR-377 was carried out using TaqMan miRNAs assay (Thermo Fisher Scientific Inc). The PCR conditions included activating the DNA polymerase at 95°C for 15 min, followed by 40 cycles of three-step PCR (94°C for 15 sec, 55°C for 30 sec, and 70°C for 30 sec). Reverse transcription of 10 ng template RNA was performed with a TaqMan MicroRNA

Table I. Sequence of miR-377 mimics and inhibitor.

Name	Direction	Sequence (5'-3')
miR-377 mimics	Forward	TGCTGATCACACAAAGGCAACTTTTGTGTTTTGGCCACTGACT GACACAAAAGTCCTTTGTGTGAT
	Reverse	CCTGTAGTGTGTTTCCGTTGAAAACACAAAACCGGTG ACTGACTGTGTTTTCAGGAAACACACTA
miR-377 inhibitor	Forward	TGCTGGGAAGTCATACAATCCTACA TTGTTTTGGCCACTGACTGACAATGTAGGTGTATGACTTCC
	Reverse	CCTGCCTTCAGTATGTTAGGATGTAACAA AACCGGTGACTGACTGTTACATCCACATACTGAAGG
miR-377 UTR		AUCACACAAAGGCAACUUUUGU
CUL4A UTR		UGGUUUGUU-CUCGUGUGUGAU

miR, microRNA; UTR, untranslated region; CUL, cullin.

Table II. Association between CUL4A and clinical data of ovarian cancer patients.

Cancer stage	Age (<45/≥45)	CUL4A expression (low/high)
TNM		
I	6/8	9/5
II	4/8	3/9
III	7/11	4/14
P-value	0.883	0.031 ^a

^aP<0.05, Chi-square test; CUL, cullin.

Reverse Transcription kit and miRNA-specific stem-loop primers (Table I). The expression of miR-377 was normalized to U6 expression. The $2^{-\Delta\Delta C_q}$ method was performed for the quantification of gene expression data (23).

To determine mRNA levels of CUL4A, β -catenin, Wnt3a, cyclin D1, matrix metalloproteinases (MMP)-2 and -9, metastasis-associated protein (MTA)-1, and metalloproteinase inhibitor (TIMP), total RNA was firstly reverse transcribed using the Takara PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). Quantification of mRNA was carried out by TaqMan Gene Expression Assay (Thermo Fisher Scientific Inc, USA). PCR was carried out by activating the DNA polymerase at 95°C for 10 min, followed by 40 cycles of two-step PCR (95°C for 15 sec and 60°C for 45 sec). Target gene expression was normalized to GAPDH expression. Primer sequences were listed as follows: CUL4A forward, 5'CAAGAAGCTTCCGAGACAGACC3' and reverse, 5'TGCTTGATAGACATTGGGGA3'; β -catenin forward, 5'TATAAGAGCTCCTTG TCGGC3' and reverse, 5'CTGAAGCTGCTCCTCAGACC3'; Wnt3a forward, 5'CTGGAGCTAGTGTCTCTCTCT3' and reverse, 5'GGAAGAAGCCTCATCCACCA3'; cyclin D1 forward, 5'CAATGACCCCGCACGATTTTC3' and reverse, 5'AAGTTGTTGGGGCTCCTCAG3'; MMP-2 forward, 5'TGT GTTGTCCAGAGGCAATG3' and reverse, 5'ATCACTAGG

CCAGCTGGTTG3'; MMP-9 forward, 5'TTTGAGTCCGGTGGACGATG3' and reverse, 5'GCTCCTCAAAGACCGAGTCC3'; MTA1 forward, 5'AAACTGCCCTGAGTGTGGT3' and reverse, 5'AAATATGTTGACCCAGCTCATCT3'; TIMP1 forward, 5'GCCTGACGGTCATATGGTAGA3' and reverse, 5'GAATGCGCCAAAAACCCCAT3' and GAPDH forward, 5'GAATGGGCAGCCGTTAGGAA3' and reverse, 5'AAAAGCATCACCCGGAGGAG3'. The $2^{-\Delta\Delta C_q}$ method was performed for the quantification of gene expression data (23).

Western blot analysis. Total protein of cells in each group was extracted using the ProteoPrep® Total Extraction Sample kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cell lysates were prepared with the cell lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation (12,000 x g) at 4°C for 10 min, the supernatant was collected. Concentration of the protein was determined by Bradford assay (Bio-Rad Laboratories, Inc.). A total of 30 mg of each protein sample were separated on 10-15% SDS-PAGE (Merck KGaA, Darmstadt, Germany). The membranes were blocked using 5% fat-free milk in TBST at room temperature for 1 h. Membranes were incubated with primary antibodies at 4°C for 6 h and then at room temperature for 4 h. The primary specific antibodies used were anti-CUL4A (catalog no. ab72548; 1:1,000; Abcam, Cambridge, MA, USA), anti- β -catenin (catalog no. ab32572; 1:5,000; Abcam), anti-Wnt3a (catalog no. ab19925; 1:1,000; Abcam), anti-cyclin D1 (catalog no. ab134175; 1:10,000; Abcam), anti-MMP2 (catalog no. ab92536; 1:1,000; Abcam), anti-MMP9 (catalog no. ab38898; 1:1,000; Abcam), anti-MTA1 (catalog no. ab71153; 1:2,000; Abcam) and anti-GAPDH (catalog no. ab8245; 1:2,000; Abcam). The membranes were incubated with the following secondary antibodies at room temperature for 1 h: Goat anti-mouse IgG H&L (catalog no. ab6789; 1:2,000; Abcam), goat anti-rabbit IgG H&L (catalog no. ab6721; 1:2,000; Abcam) and donkey anti-goat IgG H&L (catalog no. ab6885; 1:2,000; Abcam). Blots were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). Densitometry of the bands was performed using Quantity One software, version 4.6.9 (Bio-Rad Laboratories, Inc.).

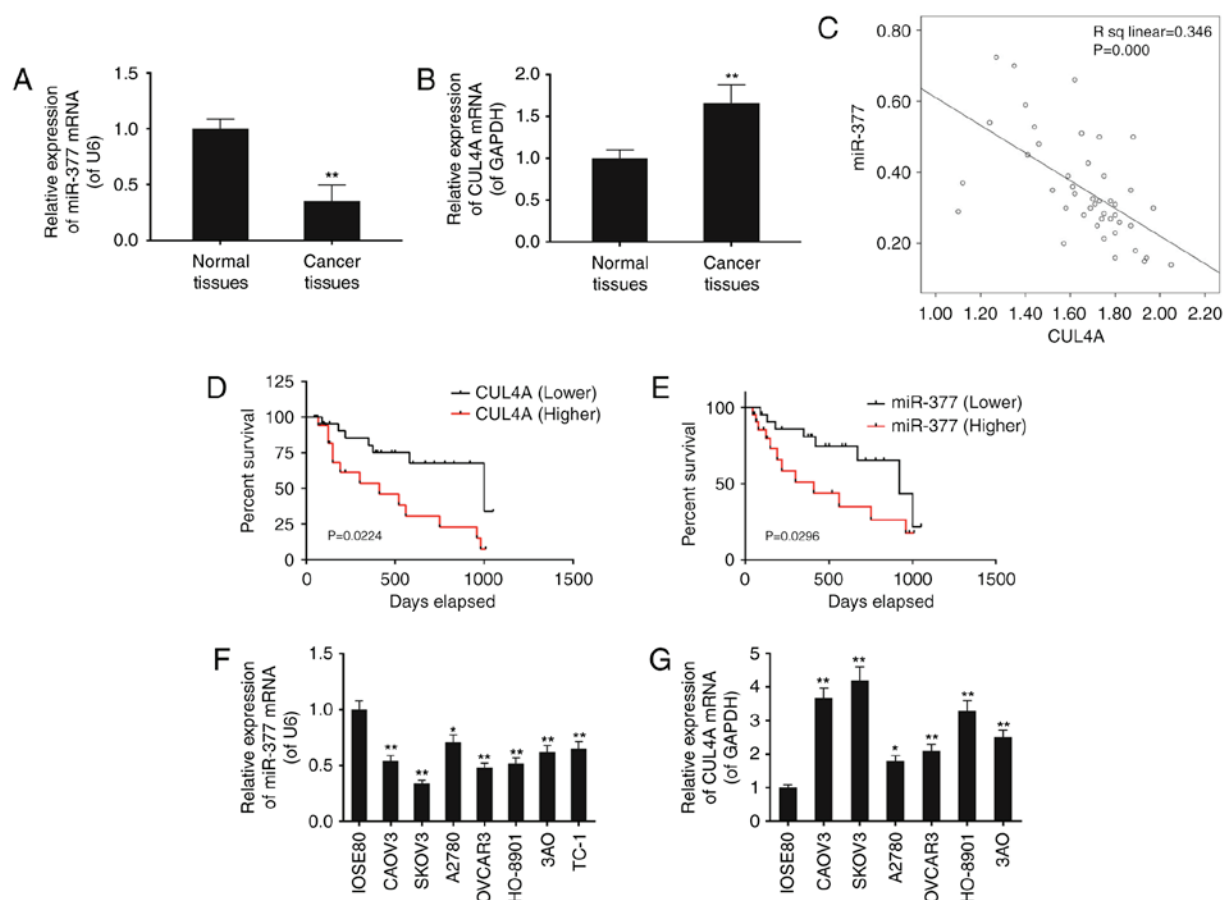


Figure 1. The expression levels of miR-377 and CUL4A in ovarian cancer tissues and cell lines (CAOV3, SKOV3, A2780, OVCAR3, HO-8901 and 3AO) were assessed. (A) miR-377 levels were decreased in cancer tissues compared with their adjacent normal tissues, whereas (B) mRNA levels of CUL4A were elevated. (C) Linear correlation of mRNA levels between miR-377 and CUL4A were assessed. Survival analysis demonstrated that ovarian cancer patients with (D) increased level of CUL4A and (E) decreased level of miR-377, lived shorter than the patients with decreased CUL4A and increased miR-377 expression level, respectively. (F) The miR-377 level was significantly lower in ovarian cancer cells compared with normal cells (IOSE80), whereas (G) mRNA levels of CUL4A were greater in ovarian cancer cells compared with normal cells (IOSE80). Data are expressed as the mean \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. IOSE80 normal cells. CUL4, cullin 4A; miR, microRNA.

Statistical analysis. Statistical analysis was performed using SPSS software, version 22.0 (IBM Corp., Armonk, NY, USA). Each experiment was repeated three times, and data are presented as the mean \pm standard deviation. Student's t-test and one-way analysis of variance, followed by Tukey and Bonferroni post-hoc tests were used in either two or multiple group comparisons, for statistical significance. Spearman's nonparametric correlation test was used to analyze the correlations between the miR-377 and CUL4A mRNA levels in tissue samples. The Chi squared test was used for analysis of the significance of CUL4A to clinicopathological characteristics. The method of survival analysis was used for testing divided phases. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-377 and CUL4A in ovarian cancer tissues and cell lines. The present study included a total of 44 patients at the average age of 51.12 ± 16.35 (Table II). The expression of miR-377 and CUL4A in surgically resected ovarian cancer specimens and adjacent normal ovarian tissues was assessed using RT-qPCR. Compared with the normal ovarian tissues, the levels of miR-377 in ovarian cancer specimens

was decreased, and the expression of CUL4A was increased, and the correlation analysis indicated a negative correlation between miR-377 and CUL4A expression (Fig. 1A-C). It was also detected that the survival rate of ovarian cancer patients with decreased levels of CUL4A and increased miR-377 was greater compared with those that demonstrated the opposing trends ($P < 0.05$; Fig. 1D and E).

Decreased miR-377 expression along with increased CUL4A expression was also detected in seven ovarian cancer cell lines including CAOV3, SKOV3, A2780, OVCAR3, HO-8901, 3AO and TC-1, compared with in the normal cell line IOSE80, however this difference was most evident in SKOV3 cells ($P < 0.05$; Fig. 1F and G).

Verification of CUL4A as the direct target of miR-377. *In silico* analysis of human miR-377 began with surveys of its target-prediction. A total of four prediction software programs; miRanda, miRDB, PicTar, and TargetScan, detected 3,812, 767, 185, and 4,920 target genes, respectively. Based on the result of the Venn diagram, a total of 52 reliable target genes of miR-377 were discovered through intersection calculation of predicted target genes from the four online programs (Fig. 2A). Subsequent gene ontology (GO) analysis of gene

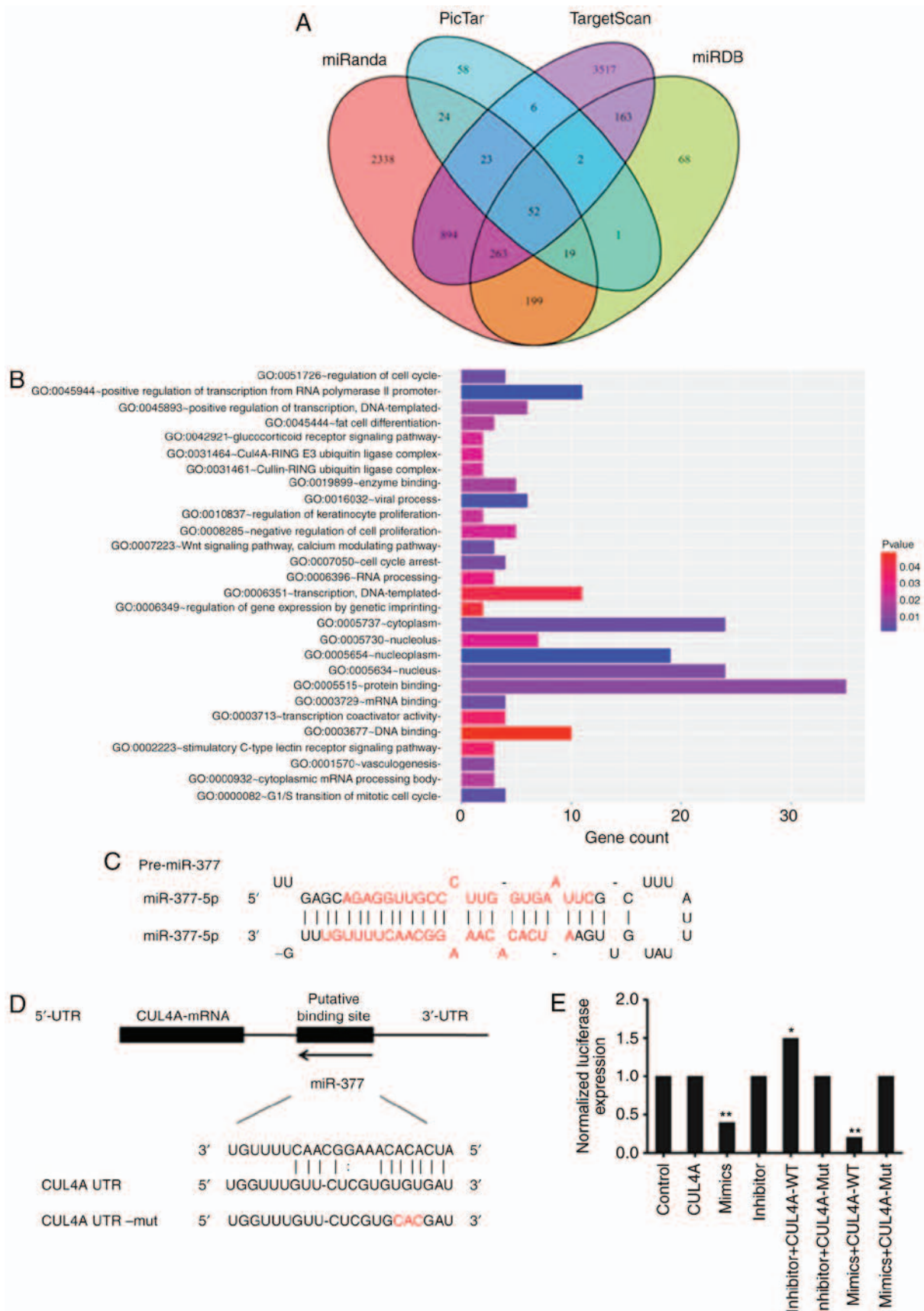


Figure 2. Target genes of miR-377 identified through bioinformatic and luciferase reporter analysis. (A) A total of 52 reliable target genes of miR-377 were discovered through intersection calculation of predicted target genes from miRanda, miRDB, PicTar and TargetScan. (B) Gene ontology analysis of gene function revealed 28 annotations of miR-377-associated biological processes, genes were enriched in processes of regulation of gene expression, cell proliferation and signal transduction. (C) Cervical-loop structures of pre-miR-377. (D) Mature hsa-miR-377 (miR-377) was predicted to interact with the CUL4-3' UTR from positions 319 to 349 via a 7-mer seed match interaction. (E) Relative luciferase activity demonstrated that miR-377 reduced luciferase reporter activity. Data are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control. CUL4, cullin 4A; miR, microRNA; mut, mutant; WT, wild type; UTR, untranslated region.

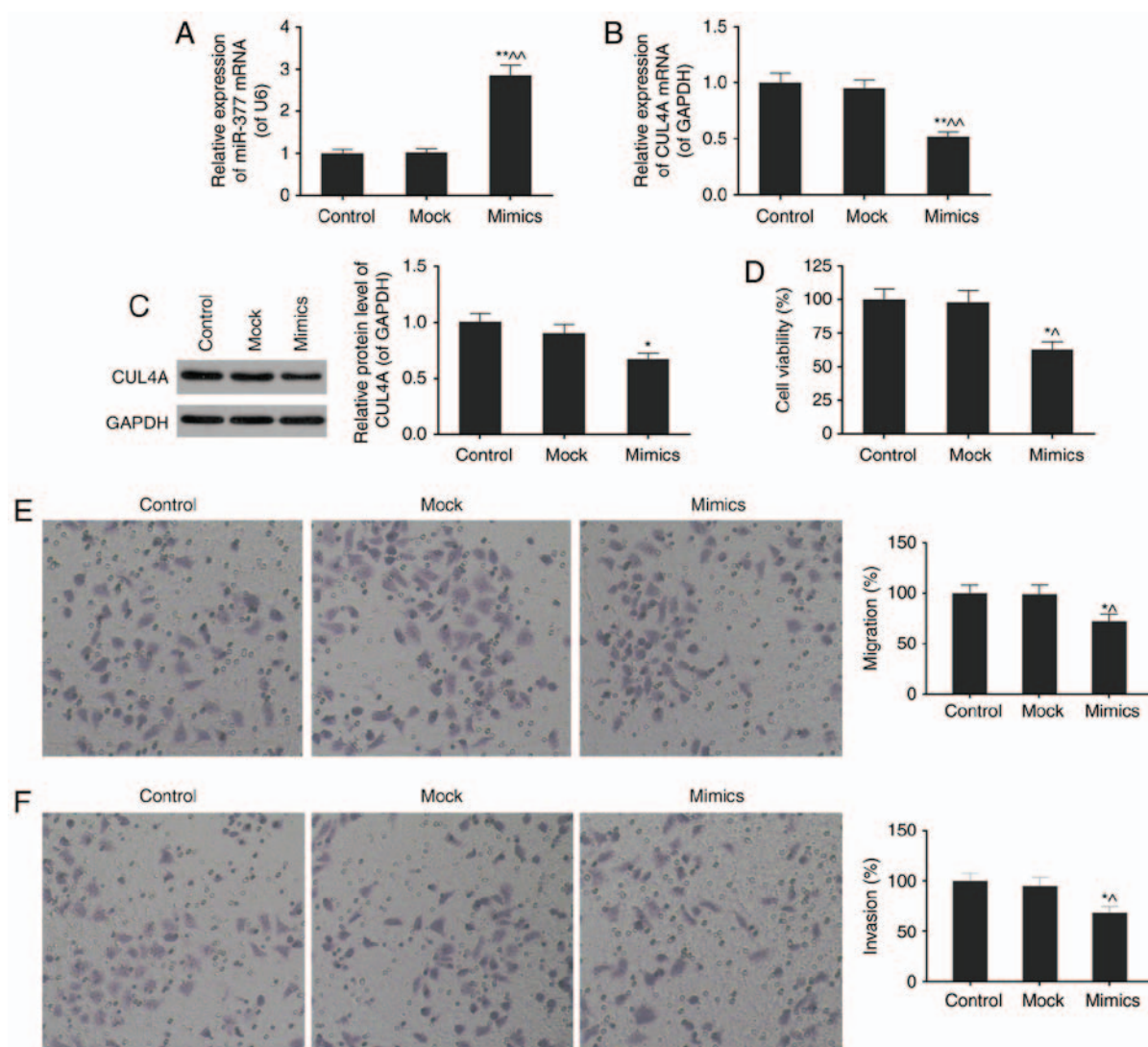


Figure 3. miR-377 overexpression affects cell viability, migration and invasion. (A) Ectopic miR-377 expression decreased the (B) CUL4A mRNA level. (C) The expression of CUL4A protein was inhibited by miR-377 mimics. (D) miR-377 overexpression decreased the viability of SKOV3 cells. miR-377 attenuated (E) cell migration and (F) cell invasion rates in SKOV3 cells (magnification, x100). Data are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control, ^ $P < 0.05$ and ^^ $P < 0.01$ vs. mock. CUL4, cullin 4A; miR, microRNA.

function revealed 28 annotations of biological processes which were associated with miR-377 ($P < 0.05$). The target genes of miR-377 were primarily enriched during processes regulating gene expression, cell proliferation, and signal transduction (Fig. 2B).

Based on literature review, of the 52 predicted target genes, CUL4A was selected as it has been previously demonstrated to highly implicated in tumor progression and patient survival (22,24). To investigate whether miR-377 is involved in the regulation of CUL4A protein expression, the alignment of miR-377/CUL4A was analysed (Fig. 2C). miR-377 is located in a miRNA cluster on chromosome 14q32.2. The stem-loop for miR-377 includes two different mature miRNA sequences, miR-377 (MIMAT0000730) from positions 45-66 and miR-377* (MIMAT0004689) from positions 7-28 (25). miR-377 was predicted to interact with a 7-mer seed match with the CUL4A-3' UTR from position 319-349 (Fig. 2D).

miR-377 interacts with CUL4A-3' UTR. A luciferase reporter involving the human CUL4A-3' UTR was applied to identify

if miR-377 interacted directly with the CUL4A-3' UTR. Luciferase activity was tested 24 h following transfection of SKOV3 cells with the reporter miR-377 overexpression and inhibition constructs. In the assay system, a reduction in luciferase expression revealed a specific miR-377-3' UTR interaction. It was demonstrated that luciferase activity was significantly decreased in miR-377 overexpressed cells; in particular, in the miR-377 mimics + CUL4A-WT group, the luciferase expression was reduced to one fifth of the level in the control group ($P < 0.01$; Fig. 2E). Mutation of the predicted binding sites in CUL4A-3' UTR eliminated this decrease in luciferase reporter activity. This result suggested that miR-377 is involved in the control of CUL4A expression.

miR-377 downregulates CUL4A mRNA and protein expression levels in SKOV3 cells. As presented in Fig. 1D and E, the differential expression of miR-377 and CUL4A in SKOV3 cell line compared with normal cells was the most significant of the six ovarian cancer cell lines. In the present study, SKOV3 was selected for subsequent experimentation to probe into

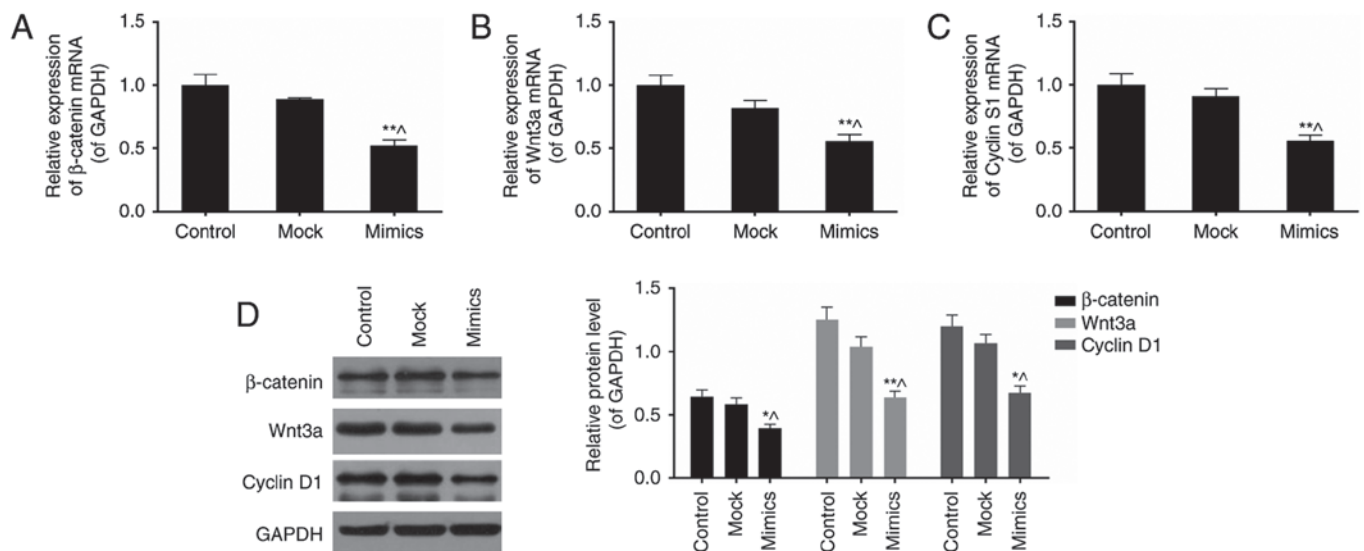


Figure 4. miR-377 overexpression inhibits Wnt/β-catenin signaling pathway. Ectopic miR-377 expression inhibited the mRNA levels of (A) β-catenin, (B) Wnt3a and (C) cyclin D1 and (D) protein levels of β-catenin, Wnt3a and cyclin D1 were reduced in miR-377 overexpression cells. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 vs. control, ^P<0.05 vs. mock. CUL4, cullin 4A; miR, microRNA.

the effects of miR-377 on ovarian cancer *in vitro*. The effect of expression of the miR-377 mimics on the expression level of CUL4A was subsequently assessed using RT-qPCR and western blotting (Fig. 3A-C). The miR-377 expression levels in the mimics group exhibited an almost three-fold increase compared with normal SKOV3 cells (P<0.05; Fig. 3A). Overexpression of miR-377 downregulated CUL4A mRNA and protein expression levels. As presented in Fig. 3C, the expression of miR-377 mimics resulted in an approximately 20% decrease in CUL4A protein expression compared with expression of the mock.

miR-377 mimics attenuate viability, migration and invasion of SKOV3 cells. The effects of miR-377 mimics on biological processes in SKOV3 cells were next investigated, including cell viability, migration, and invasion (Fig. 3D-F). In the miR-377 mimic group, cell viability was markedly inhibited by the high level of miR-377, compared with normal cell group (P<0.05; Fig. 3D). Furthermore, with miR-377 overexpression, migration and invasion rates were attenuated (P<0.01; Fig. 3E and F). These results implied the important role of miR-377 in ovarian cancer.

miR-377 mimics inhibit Wnt/β-catenin signaling pathway in SKOV3 cells. β-catenin, Wnt3a, and cyclin D1 are three key components in the Wnt/β-catenin signaling pathway (26). To investigate the effects of miR-377 on this signalling pathway, expression levels of these three genes were assessed in normal and miR-377 mimics conditions (Fig. 4A-D). As a result, overexpression of miR-377 downregulated the expression of β-catenin, Wnt3a, and cyclin D1 (P<0.05).

miR-377 mimics affect the expression of MMP-2, MMP-9, MTA1 and TIMP2 in SKOV3 cells. Subsequently, the influence of miR-377 overexpression on tumor metastasis-associated genes including MMP-2 and 9, MTA1 and TIMP2 was assessed. MMPs and TIMPs serve important roles in extracellular matrix turnover (27). MTA1, a member of MTAs family, is an

integral component of the nucleosome remodeling and histone deacetylation complexes (28). It has been reported to be highly expressed in metastatic cells compared with non-metastatic ones (29). Expression levels of MMP-2, MMP-9, MTA1 and TIMP2 were influenced by miR-377 overexpression (Fig. 5A-E). Cells transfected with the miR-377 overexpression construct expressed lower levels of MMP-2, MMP-9, and MTA1, however increased levels of TIMP2 (P<0.05).

Discussion

The expression of miR-377 is silenced in a number of human malignancies, which has been verified in previous studies (6-10). In fact, the large miRNA cluster on chromosome 14q32 including miR-377 is termed as 'the largest miRNA tumour suppressor cluster' (9). The present analysis identified a total of 52 target genes of miR-377, and demonstrated that these genes were enriched in primary functions of regulation of gene expression, cell proliferation, and signal transduction. Aberrant expression of miR-377 in the ovarian cell line SKOV3 reduced cell viability, and resulted in inhibition of migration and invasion of the cancer cells, which suggested that miR-377 is likely to function as a suppressor in ovarian cancer.

There is a general lack of information regarding target genes of miR-377. A previous study demonstrated that miR-377 targets frizzled class receptor 4, which is required for epithelial-mesenchymal transition (EMT) and metastasis in prostate cancer cells (30). miR-377 has additionally been demonstrated to target cyclin dependant kinase 6 and E2F transcription factor 3 (31). However, previously it was unknown if miR-377 targeted CUL4A.

It was demonstrated that EMT is an essential process for the metastasis of cancer cells (32-34). Through EMT, cancer cells obtain stronger capability to move and migrate into the circulatory system via the extracellular matrix and basement membrane of blood vessels, and form secondary metastases as a consequence (35,36). A key factor which

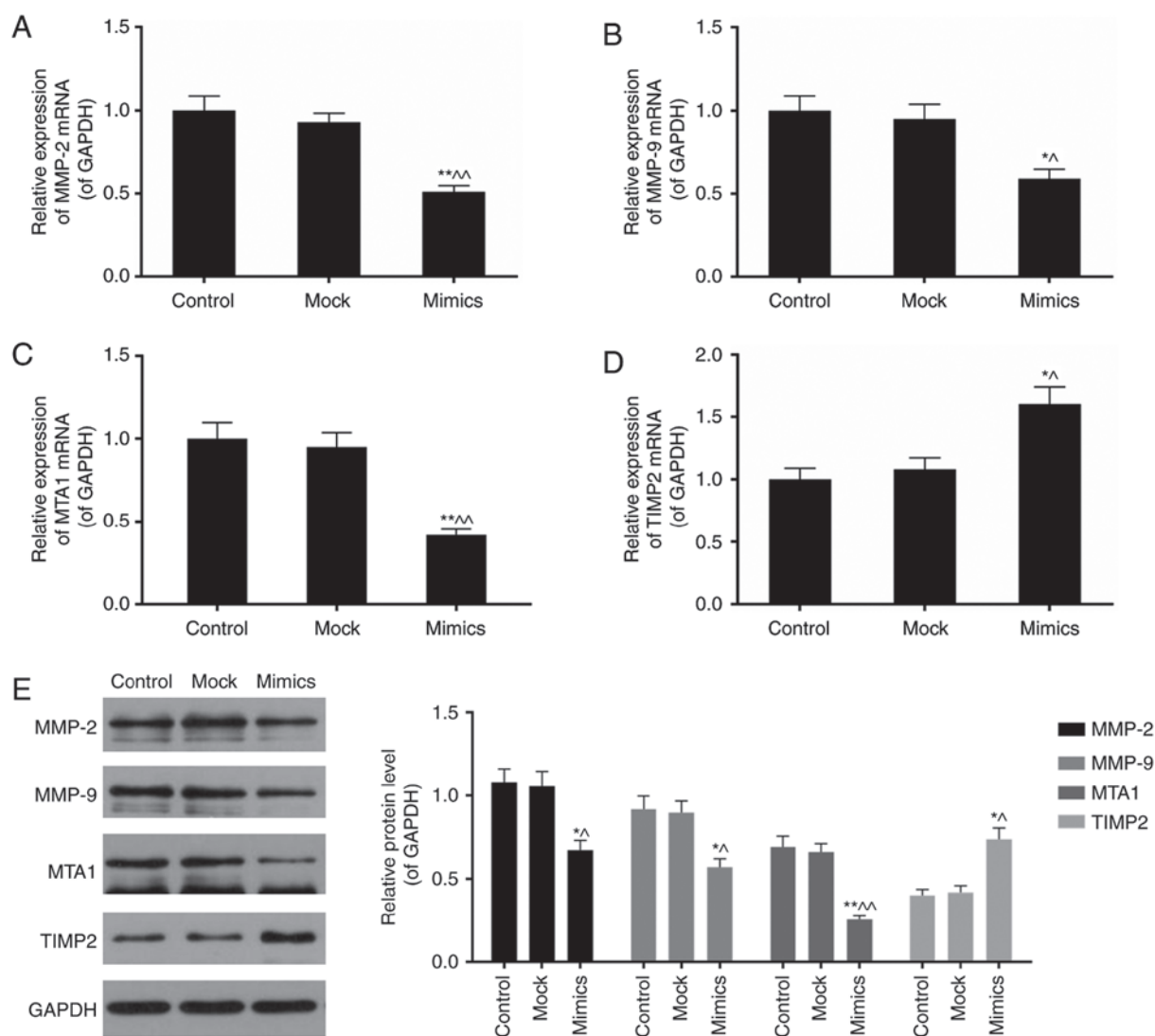


Figure 5. miR-377 overexpression influences the expression of MMP-2, MMP-9, MTA1 and TIMP2. Aberrant miR-377 level downregulated the expression of (A) MMP-2, (B) MMP-9 and (C) MTA1 mRNA, however upregulated (D) TIMP2 mRNA expression levels. (E) MMP-2, MMP-9 and MTA1 protein expression levels were decreased whereas TIMP2 protein expression was elevated in the miR-377 mimics group. Data are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control, ^ $P < 0.05$ and ^^ $P < 0.01$ vs. mock. MTA, metastasis-associated protein; miR, microRNA; MMP, matrix metalloproteinase; TIMP, metalloproteinase inhibitor.

regulates the occurrence of EMT in ovarian cancer may be not only an important biomarker of prognostic prediction of ovarian cancer, however also an important target in further studies for intervention treatments to more effectively inhibit or interdict metastasis of ovarian cancer. Ubiquitin ligase E3, which is classified into two categories, termed, ring-finger E3 ubiquitin ligase family and homologous to E6AP C-terminus (HECT) ubiquitin ligase family, according to the difference in transfer methods of ubiquitin, serves a role in ubiquitination-mediated degradation of proteins (19,37,38). Belonging to the cullin-ringbased E3-ligases (CRLs), a principal part of the ring-finger E3 ubiquitin ligase family, CUL4A was first identified in breast cancer and its amplification is strongly associated with tumorigenesis and tumour migration of several cancers (39,40). CUL4A, which was demonstrated to be overexpressed in ovarian cancer tissues and cell lines in the present study, is a direct target of miR-377, and its activity is regulated by miR-377. The present study demonstrated that the decrease in CUL4A protein levels, which were detected

following overexpression of miR-377 resulted in inhibition of viability, migration, and invasion of SKOV3 cells.

A number of signal transduction pathways, including transforming growth factor- β , mitogen activated protein kinase, Notch and Wnt, participate in the process of EMT of tumour cells (26). The Wnt signaling pathway transmits signals from the cell surface into the cell nucleus by secreting Wnt signalling proteins, transmembrane receptor proteins, and other intracellular proteins (41). The canonical Wnt signaling pathway, also named Wnt/ β -catenin signaling pathway, serves a vital role in proliferation, differentiation, movement, and morphology of numerous cells (42-44). In the presence of Wnt signalling, Wnt proteins combine with the cell surface receptor Frizzled and members of the low-density lipoprotein receptor-related protein (LRP) family, LRP-5, -6 to activate intracellular dishevelled protein, inhibit phosphorylation of β -catenin, and protect intracytoplasmic unphosphorylated β -catenin from degradation via the ubiquitin-proteasome system. β -catenins in the cytoplasm gather and shift toward

the cell nucleus so as to interact with the lymphoid enhancer factor/T cell factor to activate the transcription of target genes, including cyclin D1 and c-myc. The protein complex of β -catenin/E-cadherin combines with the actin cytoskeleton to reduce cell-cell adhesion and induce the EMT process of tumours, which strengthens the migration and invasion abilities of tumour cells (45-49).

In the Wnt/ β -catenin signaling pathway, Wnt3, a secretory protein of the pathway, is the promoter, and β -catenin, an important regulatory factor that conducts signals from the cell membrane or cytoplasm to the cell nucleus, is the executor of the regulatory function on its downstream targets. Cyclin D1, one of its targets, is a key intranuclear transcription factor that regulates the cell cycle (50). The present study demonstrated that aberrant miR-377 expression, attenuated the mRNA and protein levels of β -catenin, Wnt3a, and cyclin D1, which indicated the reduced activation of the Wnt/ β -catenin signaling pathway by miR-377 mimics. The results of the present study suggested that miR-377 reduces the migratory ability of SKOV3 cells, potentially by inhibiting the EMT process via the Wnt/ β -catenin signalling pathway.

MMP2, together with MMP9, is able to degrade type IV collagen, and degradation of the basement membrane allows cancer cells to migrate out of the tumour, resulting in metastasis (51). MTA1 suppresses the expression of numerous tumour suppressor genes contributing to cell migration and invasion (52). In the present study, it was demonstrated that the expression levels of MMP-2, MMP-9, MTA1, and TIMP2 were significantly influenced by miR-377 overexpression at the mRNA and protein levels. Cells transfected with the miR-377 overexpression construct expressed lower levels of MMP-2, MMP-9, and MTA1, however increased levels of TIMP2, which suggested that miR-377 is necessary for attenuating migration-associated protein expression.

In conclusion, the results of the present study suggested that miR-377 acted as a negative regulator of CUL4A, which resulted in reduced cancer cell viability and migration in an ovarian cancer cell line. It was then hypothesized that downregulation of miR-377 in ovarian cancer may promote tumorigenesis and metastasis through activation of CUL4A expression, however further *in vitro* and *in vivo* research is required to verify this.

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Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

RY and YC conceived and designed the study. XD analyzed and interpreted the patient data regarding the relationship of

miR-377 and CUL4A expression in ovarian cancer tissues. LC and XW performed the cell experiments to investigate the role of miR-377 in metastatic capability regulation. RY was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the Third Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Written informed consent was obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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