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MiR-506-3p suppresses the proliferation of ovarian cancer cells by negatively regulating the expression of MTMR6

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MicroRNAs have been reported to play a crucial role in ovarian cancer (OC) as the most lethal malignancy of the women. Here, we found miR-506-3p was significantly down-regulated in OC tissues compared with corresponding adjacent non-tumor tissues. Ectopic miR-506-3p expression inhibited OC cell growth and proliferation using MTT and colony formation assay. Additionally, flow cytometry analysis showed that the overexpression of miR-506-3p induced cell cycle G0/G1 phase arrest and cell apoptosis in OC cells. A luciferase reporter assay confirmed that the myotubularin-related protein 6 (MTMR6) was the target of miR-506-3p. The expression of MTMR6 was increased in OC tissues compared with adjacent tissues using immunohistochemistry. Elevated MTMR6 protein levels were confirmed in OC cells lines compared with immortalized fallopian tube epithelial cell line FTE187 using western blotting. In addition, knockdown of MTMR6 imitated the effects of miR-506-3p on cell proliferation, cell cycle progression and apoptosis in OC cells. Furthermore, rescue experiments using MTMR6 overexpression further verified that MTMR6 was a functional target of miR-506-3p. Our data indicate that miR-506-3p might serve as a tumor suppressor gene and propose a new regulatory mechanism of MTMR6 by miR-506-3p in OC.

Keywords. Apoptosis; cell proliferation; miR-506-3p; MTMR6; ovarian cancer

1. Introduction

In developed countries, ovarian cancer (OC) related mortality currently account for more than any other female genital tract tumors (McAlpine *et al.* 2014). Each year about 22,440 women will experience OC, and 14,080 deaths are expected to occur in the United States (Siegel *et al.* 2017). OC typically presents with a few months of post-menopausal bleeding, persistent pelvic, abdominal pain and distension (Brain *et al.* 2014). Despite the international standard of care for high-grade serous OC is surgery and platinum-based cytotoxic chemotherapy, whereas the overall survival of these patients have not changed significantly for several decades (Kehoe *et al.* 2015). Recent epigenetic studies have challenged the traditional view on the pathomechanism of OC, implying that microRNAs may be both cause and consequences of aberrant epigenetic events in OC (Ramassone *et al.* 2018).

MicroRNAs are an evolutionarily conserved classical of short, regulatory non-coding RNAs (~22 nucleotides) that acts to negatively regulate transcripts expression of both coding and non-coding genes (Langhe 2015). Lately, more than 2588 mature human microRNAs have been discovered and the list is growing (Strmsek and Kunej 2015). By binding predominantly to 3′UTR of target mRNAs, microRNAs play a crucial role in fundamental cellular processes, such as proliferation, differentiation, migration, apoptosis, and tissue specific functions including angiogenesis, hormone production and innate immune response (Liz and Esteller 2016; Kim *et al.* 2017). The evident connection between microRNA dysregulation and cancer was discovered in 2002 (Acunzo *et al.* 2015). From then on, numerous information regarding the role of microRNAs in cancer tumorigenesis and drug resistance has been gained (Zhao *et al.* 2015). MicroRNA-506-3p (miR-506-3p), a component of the X chromosome-linked cluster in the primate testis, was found to be involved in multiple physiological and pathological procedures in several human tumors (Huang *et al.* 2017; Wu *et al.* 2018). The pleiotropic activities and tumor-specific contributions of miR-506-3p to various cancer development and progression are achieved by binding to its specific target genes (Wen *et al.* 2015; Guo *et al.* 2017). Though miR-506-3p has recently been demonstrated to act as tumor suppressor in non-small lung cancer (Guo *et al.* 2017), retinoblastoma (Wu *et al.* 2018), cervical cancer (Wen *et al.* 2015), and osteosarcoma (Jiashi *et al.* 2018). However, the role of miR-506-3p in OC remains to be elucidated.

The myotubularin (MTM)-related protein family in humans consists of 14 members with specificity for phosphatidylinositol 3-phosphate (PtdIns3P) and PtdIns (3,5) (Clague and Lorenzo 2005). Members of this family encode a central protein tyrosine phosphatase domain (PTP), pleckstrin homology/GRAM (PH/G) and coiled-coil (CC) domains (Choudhury *et al.* 2006). Several studies have been published in recent years that the malignant biological behaviors are regulated by MTMs (Yoo *et al.* 2004). For instance, myotubularin-related protein 3 (MTMR3) is responsible for miR-99a-mediated regulation of oral cancer cells proliferation, migration and invasion (Kuo *et al.* 2014). By using new software for cell tracking, Oppelt *et al.* (2014) observed that MTMR3 deficiency caused severe reduction in velocity in three cancer cell lines. In colorectal cancer, MTMR7 overexpression resulted in the inactivation of insulin-mediated AKT-ERK signaling and proliferation (Weidner *et al.* 2016). MTMR6 could negatively regulate $Ca2^{+}$-activated K^{+} channel $K_{Ca}3.1$ which implicate in the proliferative activity of some cancer cells (Srivastava *et al.* 2005). A recent study revealed that MTMR6 has an unexpected role in preventing cell death (Clague and Lorenzo 2005). Notably, MTMR6 was identified as miR-190b-regulated factor that involved in the pathophysiologic mechanism of SIV infection in the intestinal mucosa (Mohan *et al.* 2014).

In the present study, the crosstalk between miR-506-3p and MTMR6 as a cause for OC cancer cells proliferation, cell cycle progression and apoptosis, as well as the mechanism underline this crosstalk were discovered. Our findings have the potential for broadening our understanding of epigenetics of caner etiology.

2. Materials and methods

2.1 *Clinical tissue samples*

A total of 20 pairs of tumor tissues and adjacent non-tumor tissues were collected from OC patients who underwent surgical resection in the Department of Gynaecology, Yan'an University Affiliated Hospital (Shanxi, China) between January 2016 and December 2017. Before surgical resection, all patients were confirmed not to receive chemotherapy or radiotherapy and signed the written informed content. The basic clinicopathological characteristics of all enrolled patients were summarized in table 1. The stage for every patient was retrospectively assessed based on a modified International Federation of Gynecology and Obstetrics (FIGO) staging system (Javadi *et al.* 2016). The collected tissues were immediately snap-frozen and stored in liquid nitrogen. The present study was approved by the Ethics Committee of Yan'an University Affiliated Hospital (Shanxi, China).

2.2 *Cell lines and culture condition*

Human OC cell lines (ES-2, SKOV-3, HO-8910 and OVCAR3) and the immortalized fallopian tube epithelial cell line FTE187 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The OC cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Sigma). FTE187 cells were kept in ovarian epithelial cell medium (GIBCO, Carlsbad, CA, USA) with 10% FBS. All cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Table 1. Clinicopathological features of ovarian cancer patients (n = 20)

Clinical pathologic parameters	Cases (n = 20)
Age	
<50	8
≥50	12
Tumor size (cm)	
<4	15
≥4	5
FIGO stage	
I/II	14
III/IV	6
Histological grade	
Low	9
Moderate	6
High	5

FIGO federation of gynecology and obstetrics

2.3 *Cell transfection*

The miR-506-3p mimics (miR-506-3p) and negative control (miR-NC) were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). Two different small interfering RNAs for MTMR6 (si-MTMR6-1 and si-MTMR6-2) and si-NC were purchased from RiboBio Co., Ltd (Guangzhou, China). The pcDNA3.1-MTMR6 (MTMR6) and pcDNA3.1 empty vector were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For cell transfection, OVCAR3 and SKOV-3 cells were seeded in six-well plates at a density of 3×10^5 cells per well and transfected with miR-506-3p, miR-NC, si-MTMR6 or si-NC. In the rescue experiment, OVCAR3 cells were co-transfected with miR-506-3p and MTMR6 plasmids. All cell transfections were performed using Lipofectamine^{TM} 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

2.4 *Bioinformatics analysis*

To predict the potential target genes of miR-506-3p, several software programs, including PicTar 5 (*https://pictar.mdc-berlin.de/*), TargetScan version 7.1 (*http://www.targetscan.org*) and miRBase release 22 (*http://www.mirbase.org/*) were used.

2.5 *Dual luciferase reporter assay*

The 3′-untranslated regions (3′UTRs) of human MTMR6 cDNA with the potential target sites for miR-506-3p were amplified and inserted into the psiCHECK2 plasmid (Promega Corporation, Madison, WI, USA), named as MTMR6 wild type (WT). The corresponding site-directed mutagenesis of miR-506-3p in the 3′UTR of MTMR6 was performed using Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific), called as MTMR6 mutant type (MUT). Then, OVCAR3 and SKOV-3 cells were seeded in 24-well plates at a density of 2×10^6 cells per well and co-transfected with 50 pmol of miR-506-3p or miR-NC and 20 ng of MTMR6 WT or MTMR6 MUT reporter plasmid using Lipofectamine™ 2000 (Invitrogen, USA). Luciferase activities were quantified 48 h post-transfection using Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions.

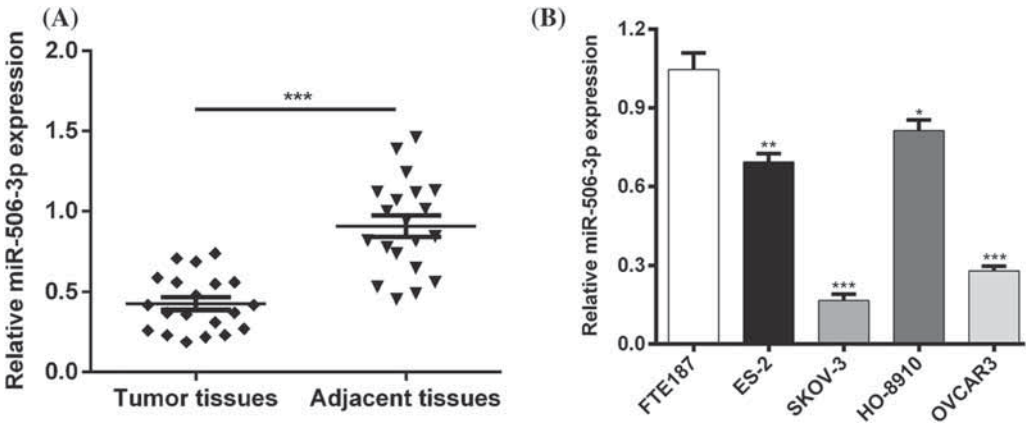

Figure 1. MiR-506-3p was significantly down-regulated in ovarian cancer tissues and cell lines. Quantitative PCR analysis of miR-506-3p expression in (**A**) tumorous tissues and adjacent non-tumor tissues of 20 patients with ovarian cancer selected for the study, as well as in (**B**) ovarian cancer cell lines and FTE187 cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

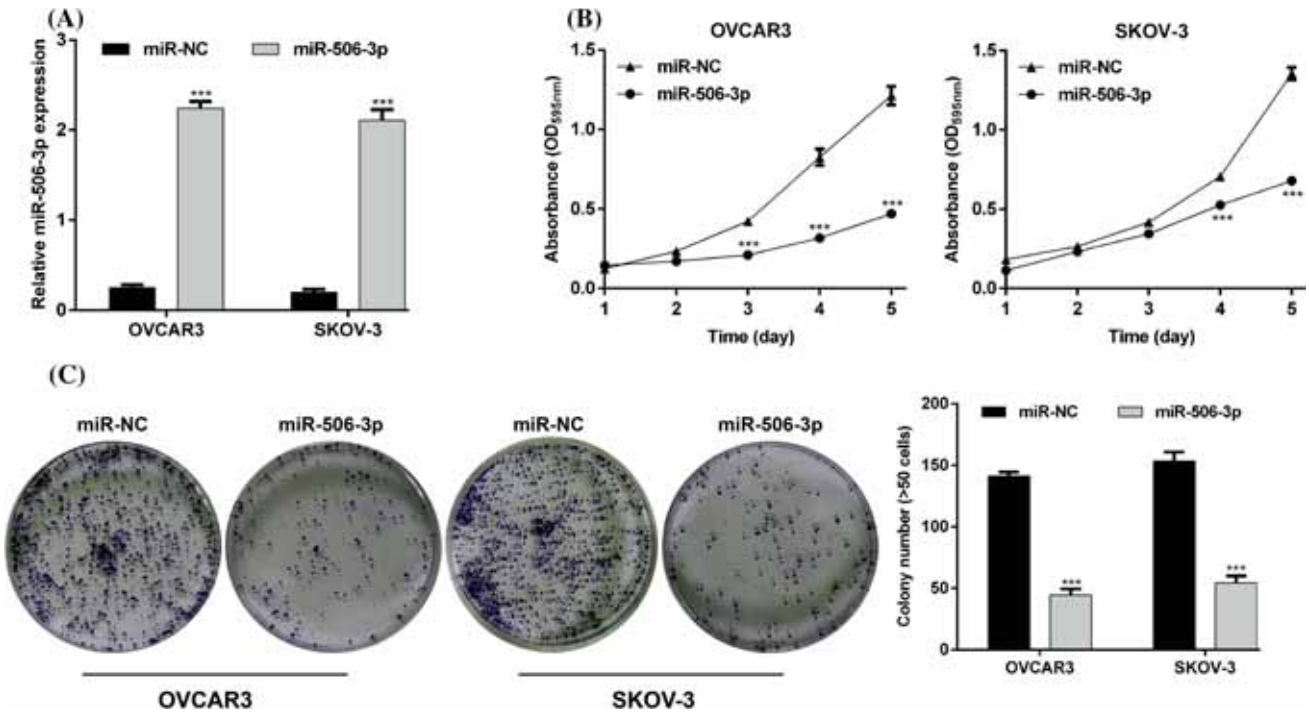

Figure 2. The effects of miR-506-3p expression on the proliferation of ovarian cancer cells. OVCAR3 and SKOV-3 cells were transfected with the miR-506-3p or miR-NC, respectively. (**A**) Quantitative real time PCR analysis of miR-506-3p expression in OVCAR3 and SKOV-3 cells after 48 h transfection. (**B**) MTT assay was used to analyze cell proliferation. (**C**) The proliferation capacity of OVCAR3 and SKOV-3 cells was determined by colony formation assay. $***p < 0.001$.

2.6 *Immunohistochemical (IHC) staining*

Some of tissue samples were selected for IHC staining. In brief, tissues samples were fixed in formalin and embedded with paraffin. Paraffin blocks were sliced into 5-μm-thick sections and hydrated with gradient alcohol, pretreated with microwaves for antigen repairmen. After blocked with sheep serum, the slides were incubated with primary antibody against MTMR6 (1:1000, ab241290, Abcam) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated immunoglobulin G for 30 min, and developed with 3,3′-diaminobenzidine buffer (DAB) as the substrate. PBS was used for negative control in place of the primary antibodies.

2.7 *Quantitative real time PCR*

Total RNA was isolated from OC tissues and cell lines using TRIpure RNA isolation kit (BioTeke, Beijing, China). For miR-506-3p detection, cDNA was synthesized with M-MLV reverse transcriptase (BioTeke) using primer sequences (forward: 5′-ACACTCATAAGGCACCCTTC-3′ and reverse: 5′-TCTACTCAGAAGGGGAGTAC-3′). To detect the MTMR6 mRNA level, the RNA was reversely transcribed with Oligo (dT) and random primers, and used for real-time PCR with MTMR6 primer sequences (forward: 5′-CAGCAGCCTGGCAGATAATCGTT-3′ and reverse: 5′-TAAGCTGACCACAGCAGGTTCTGA-3′). The PCR amplification was carried out in the ABI 7900 HT Fast PCR System (Life Technologies). The relative expression of miR-506-3p or MTMR6 was determined by comparing its threshold cycle (Ct) to that of U6 or GAPDH, respectively using the $2^{-\Delta\Delta CT}$ method.

2.8 *MTT assay*

Cell proliferation was evaluated using MTT assay. In brief, OVCAR3 or SKOV3 cells were seeded into 96-well plates with 3×10^3 per well. At indicated culturing time points (1, 2, 3, 4 and 5 day), 10 μL of MTT solution (Beyotime, China) at a final concentration of 5 μg/mL was added to each well and the cells were incubated for another 2 h. Then the formazan was completely dissolved in 100 μL DMSO and the absorbance values at 595 nm using a microplate reader (TECAN, Salzburg, Switzerland).

2.9 *Colony formation assay*

After transfected with miR-506-3p or miR-NC, OVCAR3 or SKOV3 cells were trypsinized and seeded at a density of 500 cells per well into six-well plates. Following seven days' culture, the naturally formed colonies were fixed with 4% paraformaldehyde for 20 min and then stained with 0.5% crystal violet (Biosharp, Jiangsu, China) for 15 min. After washing with PBS three times, the number of colonies containing more than 50 cells was counted for each group.

2.10 *Flow cytometry analysis*

Flow cytometry analysis was performed to determined cell cycle distribution and apoptosis in OVCAR3 or SKOV3 cells. Briefly, transfected cells were reseeded in 6-cm dishes at a density of 2×10^5 cells per dish. After 48 h, cells were digested with 0.25% trypsin, centrifuged, collected and fixed in precooling 70% ethanol overnight. For cell cycle analysis, immobilized cells were incubated in 500 μl PBS containing 50 μg/ml PI for 30 min and analyzed using BD FACSCalibur Flow Cytometer (BD Biosciences, CA, USA). For apoptosis analysis, immobilized cells were incubated with 5 μl Annexin V-FITC and 5 μl PI (Beyotime, Haimen, China) for 20 min in the dark at 25°C, then analyzed using BD FACSCalibur Flow Cytometer (BD Biosciences, CA, USA).

2.11 *Western blotting*

The cellular protein was extracted with RIPA lysis buffer (Beyotime, Jiangsu, China). Approximately 30 μg protein samples was separated by 10% polyacrylamide gel, and then transferred onto a PVDF membrane (Millipore, MA, USA). After blocking with 5% nonfat dry milk, the membrane was incubated with antibodies against MTMR6 (1:1000, ab241290, Abcam) and GAPDH (1:50000, 10494-1-AP, Proteintech Group, Inc., IL, USA), followed by incubation with horseradish peroxidase-labeled secondary antibody (1:5000, sc-2054, Santa Cruz Biotechnology Inc., USA). These protein signals were visualized with an ECL kit (Beyotime).

2.12 *Statistical analysis*

All quantitative data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with the SPSS software package (version 18.0, SPSS). Analyses of two groups were compared using Student's t test (two-tailed) and multiple groups were assessed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was accepted when a p value less than 0.05.

3. Results

3.1 *MiR-506-3p was down-regulated in OC tissues and cell lines*

To explore whether miR-506-3p plays important roles in the progression of OC, the expression of miR-506-3p was

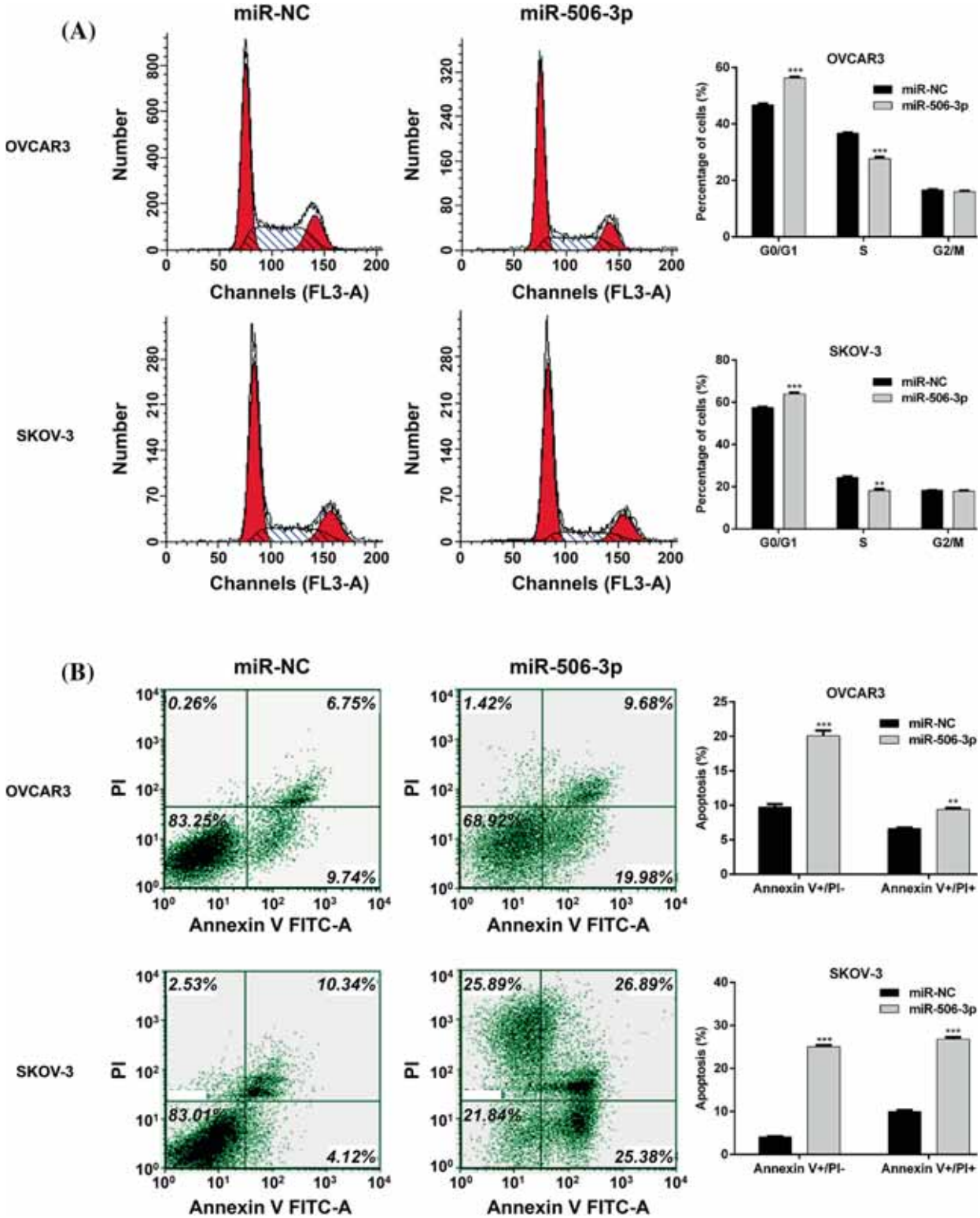

Figure 3. The effects of miR-506-3p expression on the cell cycle and apoptosis of ovarian cancer cells. OVCAR3 and SKOV-3 cells were transfected with the miR-506-3p or miR-NC, respectively. (**A**) OVCAR3 and SKOV-3 cells were stained with PI and subjected to flow cytometry analysis (left panel). The percentage of cells in G0/G1 phase, S phase and G2/M phase was the average value of three repeated experiments (right panel). (**B**) OVCAR3 and SKOV-3 cells were stained with Annexin V/PI and subjected to flow cytometry analysis (left panel). The percentage cells in early apoptosis (Annexin V+/PI −) and late apoptosis (Annexin V+/PI +) was the average value of three repeated experiments (right panel). $^{**}p < 0.01$, $^{***}p < 0.001$.

determined in 20 pairs of tumor tissues and adjacent tissues using quantitative real time PCR. As shown in figure 1A, the expression level of miR-506-3p in OC tissues was significantly lower than that in adjacent normal tissues ($p < 0.001$). In addition, we found miR-506-3p expression was significantly down-regulated in OC cell lines, ES-2 ($p < 0.01$), SKOV-3 ($p < 0.001$), HO-8910 ($p < 0.05$) and OVCAR3 ($p < 0.001$), compared to immortalized fallopian tube epithelial cell line FTE187 (figure 1B). The results demonstrated that the decreased expression of miR-506-3p might be closely associated with the progression of OC.

3.2 *MiR-506-3p overexpression inhibited OC cell proliferation*

Since miR-506-3p was lower expressed in OC cell lines, OVCAR3 and SKOV-3 cells with the lowest miR-506-3p expression were selected for gain-of-function assay by transfection with miR-506-3p or miR-NC. As shown in figure 2A, quantitative real time PCR confirmed the expression of miR-506-3p was significantly increased in both OVCAR3 and SKOV-3 cells after miR-506-3p transfection ($p < 0.001$). Next, MTT assay was used to assess cell proliferation and the results showed the cell proliferative rate of OVCAR3 and SKOV-3 cells was significantly repressed after transfected with miR-506-3p, compared to miR-NC (figure 2B, $p < 0.001$). Subsequently, the proliferative capacity of OC cells was further determined by colony formation assay and the statistical data indicated that miR-506-3p overexpression remarkably decreased the number of colonies from 141.3 ± 3.1 to 44.3 ± 5.0 in OVCAR3 cells and from 153.3 ± 7.4 to 54.3 ± 5.7 in SKOV-3 cells (figure 2C, $p < 0.001$). All of these results demonstrated that miR-506-3p might a negative regulator in OC cell proliferation.

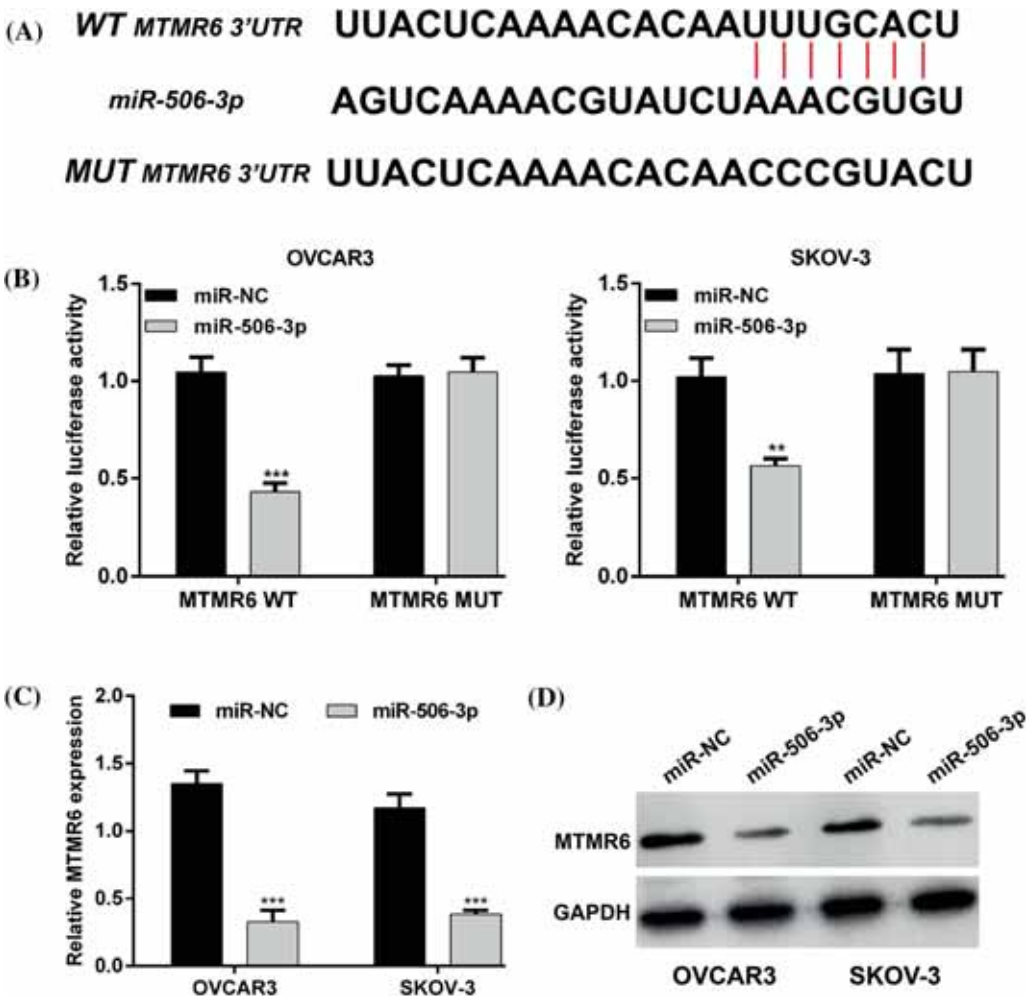

Figure 4. MTMR6 was identified as a target gene of miR-506-3p in ovarian cancer cells. (**A**) Putative binding region between miR-506-3p and MTMR6 mRNA. (**B**) A dual-luciferase reporter assay was performed on OVCAR3 and SKOV-3 cells to detect the relative luciferase activities of wild type and mutant MTMR6 reporters. (**C**) Quantitative real time PCR and (**D**) Western blot analysis of MTMR6 expression in OVCAR3 and SKOV-3 cells following miR-506-3p or miR-NC transfection. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3 *MiR-506-3p induced OC cell cycle arrest and apoptosis*

Numerous reports suggest uncontrolled cellular proliferation is strongly linked with cell cycle deregulation. Thus, we further investigated the effects of miR-506-3p overexpression on cell cycle distribution in OC cells. As shown in figure 3A, the percentage of cells in G0/G1 phase was significantly increased from miR-NC group to miR-506-3p group in OVCAR3 cells (46.71% ± 0.48% vs. 56.27% ± 0.45%, $p < 0.001$) and in SKOV-3 cells (57.41% ± 0.41% vs. 63.93% ± 0.64%, $p < 0.001$) in SKOV-3 cells. Accordingly, miR-506-3p overexpression significantly decreased the percentage of cells in S phase in both OVCAR3 ($p < 0.001$) and SKOV-3 cells ($p < 0.01$). These results showed miR-506-3p overexpression induced cell cycle G0/G1 phase arrest in OC cells. Furthermore, flow cytoemtry analysis was performed to analyze cell apoptosis. As shown in figure 3B, the early apoptotic and late apoptotic rate were remarkably elevated from miR-NC group to miR-506-3p group in both OVCAR3 ($p < 0.01$, $p < 0.001$) and SKOV-3 cells ($p < 0.001$), which further demonstrated that miR-506-3p overexpression could promote OC cell apoptosis.

3.4 *MTMR6 is a direct target of miR-506-3p in OC*

To further explore the molecular mechanism underlying miR-506-3p regulating OC cell proliferation, cell cycle and apoptosis, we performed bioinformatics analyses to identify the target genes of miR-506-3p. As shown in figure 4A, the 3′-UTR of MTMR6 was identified to contain a putative target sequence for miR-506-3p, which might be implicated in the pathogenesis of OC. A luciferase assay was then carried out to verify whether MTMR6 is a direct target of miR-506-3p. As illustrated in figure 4B, the miR-506-3p transfection significantly reduced the luciferase activity of the MTMR6 WT compared with the miR-NC-transfected OVCAR3 ($p < 0.001$) and SKOV-3 cells ($p < 0.01$). However, no significant differences were observed between OC cells transfected with miR-NC and miR-506-3p when co-transfected with the MTMR6 MUT. Moreover, miR-506-3p overexpression significantly down-regulated the expression of MTMR6 at mRNA and protein levels in both OVCAR3 and SKOV-3 cells, as determined by quantitative real time PCR (figure 4C, $p < 0.001$) and western blot analysis (figure 4D). The results further suggested that miR-506-3p directly bound to the 3′UTR of MTMR6 in OC cells.

3.5 *Knockdown of MTMR6 imitated the effects of miR-506-3p on OC cells*

To explore the biological function of MTMR6 in OC, the expression levels of MTMR6 in clinical OC tissue and paired adjacent tissues were analyzed using IHC staining. As depicted in figure 5A, Representative images of MTMR6 staining showed the MTMR6 expression were obviously higher in OC tissues than that in adjacent tissues. In addition, we found the protein expression of MTMR6 was elevated in four OC cell lines, compared with FTE187 cells (figure 5B). Subsequently, we performed loss-of-functional assays in OVCAR3 cells with relative higher MTMR6 expression levels by transfecting with si-MTMR6-1 or si-MTMR6-2. Since si-MTMR6-1 could efficiently suppress the expression of MTMR6 compared with si-MTMR6-2, we thus chose it for the subsequent analysis, as determined by western blotting (figure 6A). MTT assay and flow cytometry analysis further demonstrated that MTMR6 knockdown significantly inhibited cell proliferation (figure 6B), induced cell cycle G0/G1 phase arrest (figure 6C) and apoptosis (figure 6D) in OVCAR3 cells.

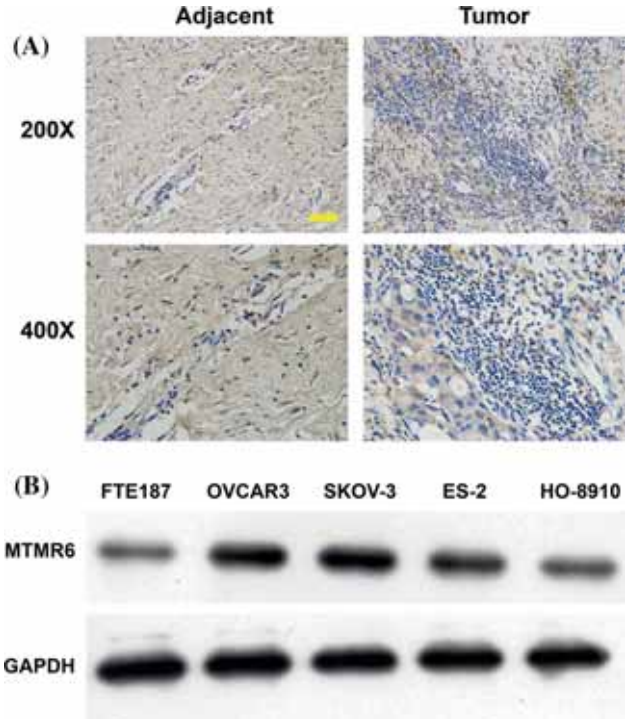

Figure 5. MTMR6 expression was up-regulated in ovarian cancer tissues and cell lines. (**A**) Representative immunohistochemical images are presented about MTMR6 expression in OC and paired paracarcinoma tissues. (**B**) Western blotting analysis was performed to analyze the protein expression of MTMR6 in ovarian cancer cell lines and FTE187 cells.

3.6 *Inhibition effects of miR-506-3p on OC cells could be partially reversed by enhanced expression of MTMR6*

As MTMR6 was confirmed as a target gene of miR-506-3p and negatively regulated by miR-506-3p overexpression in OC cells. We thus speculated that MTMR6 might mediate the inhibitory effects of miR-506-3p on cell proliferation, cell cycle progression and apoptosis. To validate our hypothesis, rescue experiments were performed in OVCAR3

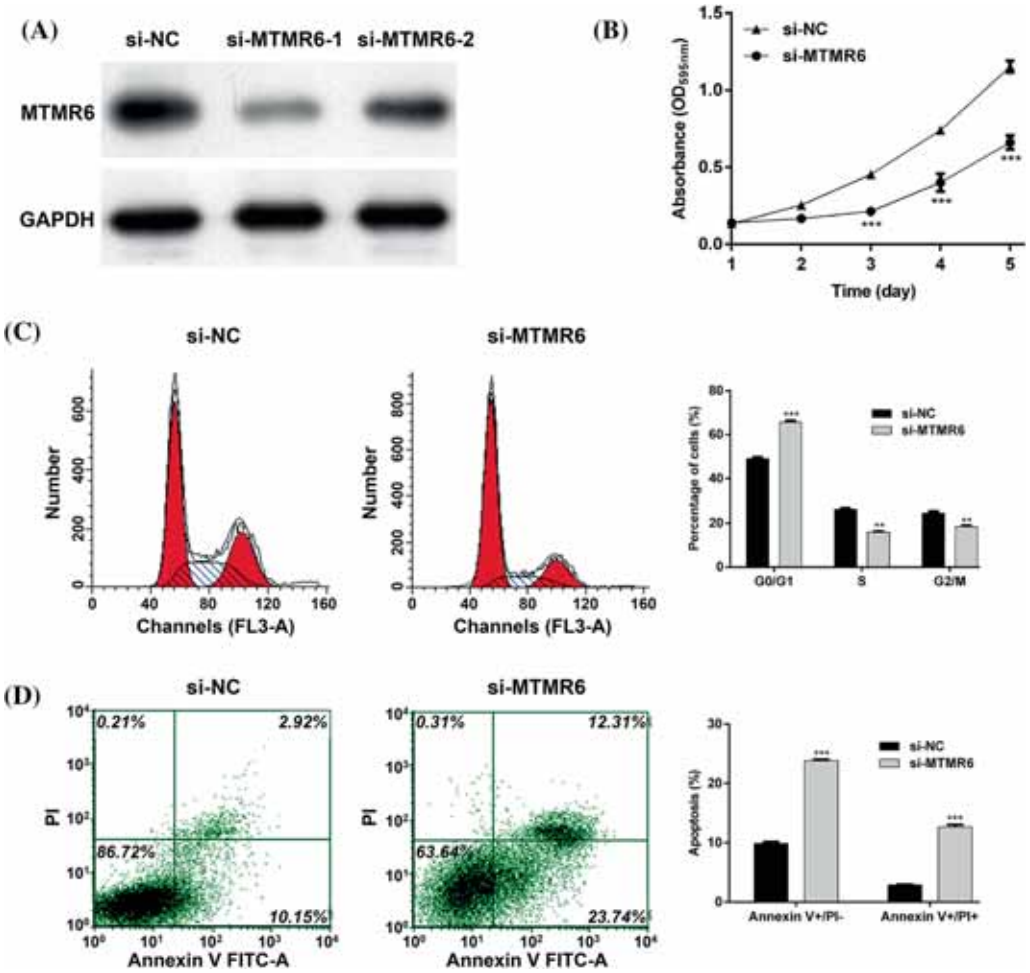

Figure 6. Knockdown of MTMR6 affected cell proliferation, cell cycle distribution and apoptosis in ovarian cancer cells. (**A**) OVCAR3 cells were transfected with si-MTMR6-1 or si-MTMR6-2, followed by detection of MTMR6 protein using western blotting. (**B**) MTT assay was used to evaluate cell proliferation ability. (**C**) Cell cycle distribution and (**D**) apoptosis rate were determined by Flow cytometry analysis. $^{**}p < 0.01$, $^{***}p < 0.001$.

cells by co-transfected with miR-506-3p and MTMR6. Firstly, quantitative real time PCR confirmed up-regulated expression of MTMR6 after co-transfected with miR-506-3p and MTMR6 compared with solely miR-506-3p transfection, but slightly down-regulation of MTMR6 compared with solely MTMR6 transfection (figure 7A). As expected, overexpression of MTMR6 partially rescued the impaired cell proliferation (figure 7B, $p < 0.001$) and cell cycle G0/G1 phase arrest (figure 7C, $p < 0.001$) and apoptosis (figure 7D, $p < 0.001$) in OVCAR3 cells. These data demonstrated that miR-506-3p suppressed cell proliferation and induced G0/G1 phase arrest and apoptosis possibly through targeting MTMR6 in OC cells.

4. Discussion

OC is considered to be the most lethal gynecological malignancy (Coward *et al.* 2015). Reasons for high lethality are mainly due to complex mechanisms of pathogenesis, difficulties for the early detection, and lack of effective treatment strategies (Kinose *et al.* 2014). Recent discoveries of miRNAs unraveled a novel mechanism of gene modulation and supply new clues about the underlying etiology of OC (Katz *et al.* 2015). Accumulating evidence suggests that numerous miRNAs display aberrant expression in a variety of tumor types, including OC (Kinose *et al.* 2014). Although low expression of miR-506-3p has been observed in cervical cancer (Wen *et al.* 2015), retinoblastoma (Wu *et al.* 2018), osteosarcoma (Jiashi *et al.* 2018), and in general associated with a less aggressive tumor phenotype, the current state of knowledge concerning the role of miR-506-3p in OC remains limited. Consistent with these reports, the present study also demonstrated a downregulation of miR-506-3p in OC tissues and established OC cell lines. Gain-of-function experiments revealed that enforced expression of miR-506-3p in OVCAR3 and SKOV-3 cells with low endogenous levels significantly depressed cell proliferation, induced G0/G1 phase arrest and apoptosis.

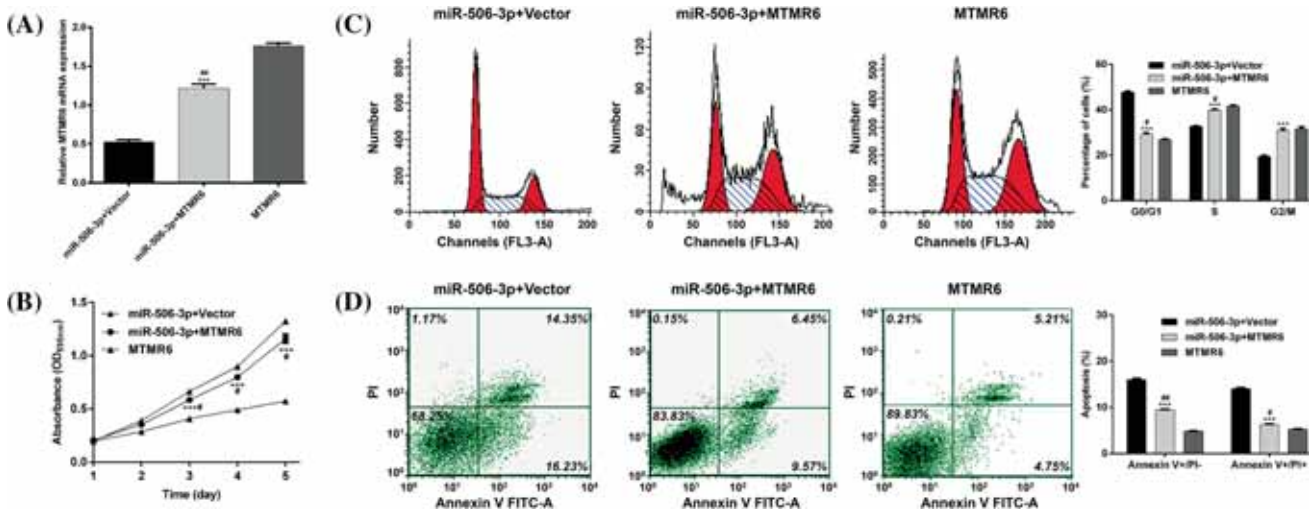

Figure 7. Overexpression of MTMR6 partially rescues the inhibitory effects of miR-506-3p on ovarian cancer cells. OVCAR3 cells were transfected with MTMR6 solely, or miR-506-3p together with plasmid expressing MTMR6 or empty vector. (**A**) Quantitative real time PCR was used to detect the MTMR6 expression levels. (**B**) MTT assay was conducted to determine the proliferation rate of OVCAR3 cells. Flow cytometry analysis was performed to assess (**C**) cell cycle distribution and (**D**) apoptosis in OVCAR3 cells. ***$p < 0.001$, compared with miR-506-3p + Vector; #$p < 0.05$, ##$p < 0.01$, compared with MTMR6.

Findings from the current study are quite interesting as we identified MTMR6 is a direct target of miR-506-3p. Further study revealed that miR-506-3p up-regulation-mediated knockdown of MTMR6 is a critical event in preventing growth, triggering cell cycle distribution and apoptosis in OC cells. Multiple lines of evidence show that MTMR6 specially interacts with KCa3.1, a Ca2+-activated K channel, through CC domains that exist in both domains (Srivastava *et al.* 2005). The CC and phosphatase domains of MTMR6 are required for inhibition of KCa3.1 channel activity (Mohan *et al.* 2014). Specially, dephosphorylation of PI(3)P occurs at the 3′ position by the MTMR6 is an important mechanism for abrogation of KCa3.1 channel activity (Srivastava *et al.* 2005). Moreover, KCa3.1 has well-established roles in the promotion of proliferation by several cancer cell lines (Faouzi *et al.* 2016), fibroblasts (Xie *et al.* 2018), T cells (Chiang *et al.* 2017), bronchial smooth muscle cells (Faouzi *et al.* 2016), and monocytes/macrophages (Zhang *et al.* 2015). Treatment with KCa3.1 inhibitors have been shown to arrest cell cycle progression at G0/G1 phase, including human endometrial cancer cells and breast cancer cells (Ouadid-Ahidouch *et al.* 2004; Wang *et al.* 2007). Here, OC cells proliferation was inhibited and G0/G1 phase arrest was induced by overexpression of miR-506-3p, we hypothesize that the KCa3.1 channels were partially deactivated in these cells. Though treatment of OC cells with miR-506-3p mimics significantly decreased the levels of MTMR6, but the negative regulation of KCa3.1 by MTMR6 may disturbed by the interaction between miR-506-3p 3′UTR and MTMR6 5′UTR or other unknown mechanisms. Further studies will be necessary to reveal the potential mechanisms mentioned above.

Oncogenetic transformation is often associated with an anti-apoptotic gene expression program in cancer cells (Diaz *et al.* 2015). Emerging evidence suggests that MTM-related phosphatase is also involved in the regulation of apoptosis (Zou *et al.* 2012). MTMR6, MTMR7, and MTMR8 have been suggested as anti-apoptotic molecules (Zou *et al.* 2012). In HeLa cells and primary human skeletal muscle myotubes, depletion of MTMs expression induced caspase-dependent apoptosis signaling through inhibiting growth factor triggered Akt phosphorylation (Razidlo *et al.* 2011). Besides, silencing of MTMs also blocks Akt-dependent signaling via targeting rapamycin complex 1 (Razidlo *et al.* 2011). It has been shown that inhibition of KCa3.1 potassium channel by its specific blocker TRAM-34 results in induced apoptosis and depressed proliferation in human hepatocellular carcinoma cells (Liu *et al.* 2015). In the current study, our results suggest that miR-506-3p-induced apoptosis in OC cells may not all but at least partially depend on the direct inhibition of MTMR6. However, the exact mechanism through which MTMR6 mediates downstream effects needs further investigation.

In conclusion, blockage of MTMR6 expression by miR-506-3p has many biological activities, including anti-proliferative, disturbance in cell cycle progression, and pro-apoptosis. The present study may provide new insight into the underlying key factors regarding the tumorigenesis and development of OC.

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