Original Article
MicroRNA-153 regulated AKT1 expression and suppressed cell proliferation of epithelial ovarian cancer cells

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Abstract: Epithelial ovarian cancer (EOC) is the most fatal malignancies in females worldwide, with increasing incidence recently in China. MiR-153 was reported to be dysregulated in some human cancers, including EOC. In this study, we explored the roles of miR-153 and its target AKT1 in regulating growth and migration in EOC. Cell proliferation was measured with a CCK-8 assay. Real-time quantitative RT-PCR was performed to investigate expression levels of miR-153. Cell cycle features were analyzed by Flow cytometry system. The direct target gene was confirmed by dual-luciferase reporter assay. We found the expression levels of miR-153 were generally lower in the EOC tissues than in the matched normal tissues. The miR-153 mimics caused significant G0/G1 arrest in A2780 cells. Overexpression of miR-153 suppressed cell proliferation and migration in ovarian cancer. Results of dual-luciferase reporter assay suggested that AKT1 was a direct target of miR-153 in ovarian cancer cells. Overexpression of AKT1 reverses the inhibition effect of miR-153 on cell proliferation. Introduction of miR-153 into EOC cell lines leaded to inhibition of cell proliferation and migration by directly targeting AKT1. MiR-153 may have prognostic or therapeutic value for the future management of ovarian cancer patients.

Keywords: Epithelial ovarian cancer, microRNA-153, AKT1

Introduction

Ovarian cancer (OC) represents the most fatal malignancies in females worldwide, with increasing incidence recently in China [1, 2]. As the most common types of ovarian cancer, epithelial ovarian cancer (EOC), accounts for about 90% of all cases of ovarian cancer. Despite improvements in chemotherapy and surgical techniques, the relative survival rate at 5 years in advanced stage EOC patients is rarely >30% [3, 4]. However, the molecular basis of EOC is still largely unknown. Therefore, there is consequently an urgent need to define the molecular mechanisms underlying EOC and to develop more specific and more effective treatment modalities.

MicroRNAs (miRNAs) are small noncoding RNA gene products about 22 nt long that negatively regulate protein expression of target mRNA by either translational inhibition or mRNAs degradation. Growing evidence has suggested that miRNAs are associated with various biological and pathological processes, such as proliferation, differentiation, and carcinogenesis [5, 6]. Accumulating evidence has demonstrated miRNA mutations or abnormal expression in many human cancers, including ovarian cancer [7, 8]. Increasing evidences have shown that miRNAs may function as oncogenes or tumor suppressors in the occurrence of various human cancers [9, 10]. However, the mechanisms of miRNAs in tumorigenesis remains largely unclear, and further studies are needed to identify their exact roles in occurrence and development of tumors.

MiR-153 was reported to be dysregulated in some human cancers, including EOC [1].

However, the function and mechanism of miR-153 in EOC remains unknown. In this study, we observed that miR-153 is significantly down-regulated in human EOC tissue compared with adjacent-normal tissue. Next, we explored the roles of miR-153 and its target AKT1 in regulating growth and migration in EOC. Our results show that microRNA-153 functions as a tumor suppressor in epithelial ovarian cancer.

Materials and methods

Human tissue samples

The 20 EOC tissue specimens and paired non-cancerous tissues used in the present study were obtained from the Department of Obstetrics and Gynecology of the Taixing People's Hospital after surgical resection. The fresh tissue specimens were immediately placed in liquid nitrogen after surgery and stored at -80°C until the isolation of RNA. All patients recruited to this study did not receive any preoperative radiotherapy, chemotherapy, or hormonal therapy. This study was approved by The Human Ethics Committee of the Taixing People's Hospital and written informed consent was obtained from each participant.

Cell lines and cell culture

Four ovarian carcinoma cell lines (A2780, SKOV3, OVCAR3, SW626) were purchased from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). Human Ovarian Surface Epithelial Cells (HOSEpiC) was stored in our laboratory. EOC Cells were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (Sijiqing Company, Hangzhou, China), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Beyotime, Haimen, China) in a humidified atmosphere of 5% CO_2 at 37°C. The complete media were refreshed every 3 days.

MiRNA-153 mimic transfection

MiR-153 mimic or the nonspecific control (NC) was synthesized Gene Pharma (Shanghai, China). A2780 cells ($1 × 10^5$) were seeded in six-well plates and transfected with miR-153 mimic or the NC using lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The tranfected cells were subjected to further analysis as presented in the results section.

pcDNA3.1-AKT1 vectors transfection

The CDS sequence of AKT1 was synthesized by Lanjing Biotech (Zhenjiang, China) and cloned into pcDNA3.1-vector (Promega, Madison, WI, USA) between the HindIII and BamHI sites. Ovarian cancer cells A2780 were cultured at $5 × 10^5$ cells/well in 6-well plates until they reached 75% confluence. Subsequently, the cells were transiently transfected with miR-153 mimics (genepharma, Shanghai, China) and the pcDNA3.1-AKT1 vectors or empty vector using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol.

Real-time quantitative PCR for miR-153

The expression levels of miR-153 in 20 self-paired tissues and A2780 cells were determined by Real-time quantitative PCR. Total RNA was isolated using TRIzol® reagent (Invitrogen) from each sample according to the manufacturer's directions. For miR-153 analysis, the stem-loop RT primer was 5'-GTCGTATCCAG-TGCAGGGTCCGAGGTATTCGCACTGGATACGAC GATCAC-3' and the amplifying primers were as follows: sense, 5'-TTGCATAGTCACAAAA-3' and antisense, 5'-GTGCAGGGTCCGAGGT-3'. U6 was used as an internal control. Primers for qRT-PCR of RNU6B were: RT 5'-CGCTTCACGAAT-TTGCGTGTCAT-3'; sense, 5'-GCTTCGGCAGCAC-ATATACTAAAAT-3' and antisense, 5'-CGCTTCA-CGAATTTGCGTGTCAT-3'. The reactions of quantitative PCR were incubated in 96-well optical plates at 95°C for 5 min, followed by 35 cycles of 95°C for 15 sec and 60°C for 30 sec and were performed in a Bio-Rad CFX-96 PCR system. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. For each sample, all experiments were performed in triplicate.

Dual-luciferase reporter assay

The luciferase reporter gene construct containing 2 adjacent miR-153 targeting sites from the 3'-UTR of AKT1 mRNA was created by cloning a 41-bp fragment (5'-AACCUGGUGGCACCAGAU-GCAACCUCACUAUGGUAUGCUGG-3') into the pGL3 luciferase reporter vector (Promega) as described previously [11]. The corresponding mutant constructs were created by replacing the seed regions of the miR-153 family binding sites with 5'-UAUGC-3'. All sequences were confirmed by sequencing. The reporter constructs and the pRL-TK vector (Promega, Madison, WI, USA) were co-transfected using Lipofectamine

2000 (Invitrogen, Grand Island, NY, USA). The luciferase activities were then determined using the dual-luciferase reporter gene assay kit (Promega, USA) as described previously [12]. Each experiment was repeated three times independently.

Cell proliferation assay

Cell proliferation was measured with a CCK-8 (Beyotime; Haimen, China) assay. Twenty-four hours after transfection, cells were seeded at a density of 4×10^3/well into 96-well plates. Cells were incubated in 10% CCK-8 that was diluted in normal culture medium at 37°C. Cell viability was determined at 48 hours after transfection using an enzyme-linked immunosorbent assay reader (Multiskan FC, ThermoFisher).

Colony formation assay

Colony formation assays were performed as described by Nyhan et al. [13]. Adherent cells were trypsinised and used to generate single-cell suspensions (1×10^4 cells/mL). Aliquots of 0.2 ml of cell suspension (containing 200 viable cells) were reseeded in fresh media into a well of a six well plate, which was incubated at 37°C for 14 days. The clones were fixed with 3.7% methanol and stained with 0.1% crystal violet (Beyotime; Haimen, China) for 5 min. The cell clones contained >50 cells were counted under a microscope.

Transwell migration assay

Briefly, A2780 cells were washed and resuspended in serum-free DMEM medium at 48 h after treatment and 1×10^4 cells were added to the upper transwell chamber. Then, 500 µl DMEM containing 10% serum was added to the lower chamber and used as the chemoattractant. After 24 h at 37°C, non-migratory cells in the top chamber were gently wiped off with cotton swabs. Migrated cells at the bottom of the membrane were fixed with 95% ethanol and then stained with 0.2% crystal violet. The number of migrated cells was counted to assess the migration ability of cells.

Cell cycle assay

The 48 h after the transfection, ovarian cancer cells were harvested and washed with cold PBS and fixed with 75% ethanol at -20°C. After 24 h

fixation, the cells were washed with PBS again and stained with propidium iodide using the cell cycle analysis kit (Beyotime, Haimen, China) for 30 min. Cell cycle features were analyzed by BD FACSCanto™ II Flow cytometry system. The data were analyzed by ModFit LT software.

Western blot

Cells were washed twice with ice-cold PBS and then lysed in Lysis Reagent (Beyotime, Haimen, China). Cell lysates (approximately 30 µg of protein) were loaded on an 8% SDS-PAGE gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). Monoclonal antibodies against AKT1 were purchased from Santa Cruz Biotechnology. Antibodies to AKT1 or the endogenous control GAPDH were incubated with the PVDF membrane overnight at 4°C in the TBST buffer. Band detection via a commercial enzyme-linked chemiluminescence kit was performed according to the manufacturer's recommendation (Beyotime, Haimen, China). The protein bands were normalized to GAPDH.

Tumor formation in BALB/c nude mice

Healthy, male, infertile, 18-20 g, 5-week old nude mice were purchased from Shanghai SLAC Laboratory Animals Co., Ltd. (Shanghai, China). All animal experiments were performed in accordance with the Animal Studies Ethics Committee of Jiangsu University. To establish lung cancer xenograft model, 1×10^7 A2780 cells were suspended in 200 µL phosphate-buffered saline and subcutaneously injected into the right flank of nude mice. After 7 days, the transplanted nude mice were randomly divided into two groups ($n = 10$ each). miR-153 mimics or NC mimics (Genepharma Co., Ltd, Shanghai, China) was directly injected into the implanted tumor at the dose of 1 nmol (in 20 µL phosphate-buffered saline) per mouse every 2 days for 14 times. The tumor size was monitored by measuring the length (L) and width (W) with calipers every 4 day, and the volumes were monitored by measuring the length (L) and width (W) with calipers. The volumes were calculated using the formula: $(L \times W^2)/2$.

Statistical analysis

All statistical analyses were calculated with Statistical Product and Service Solutions
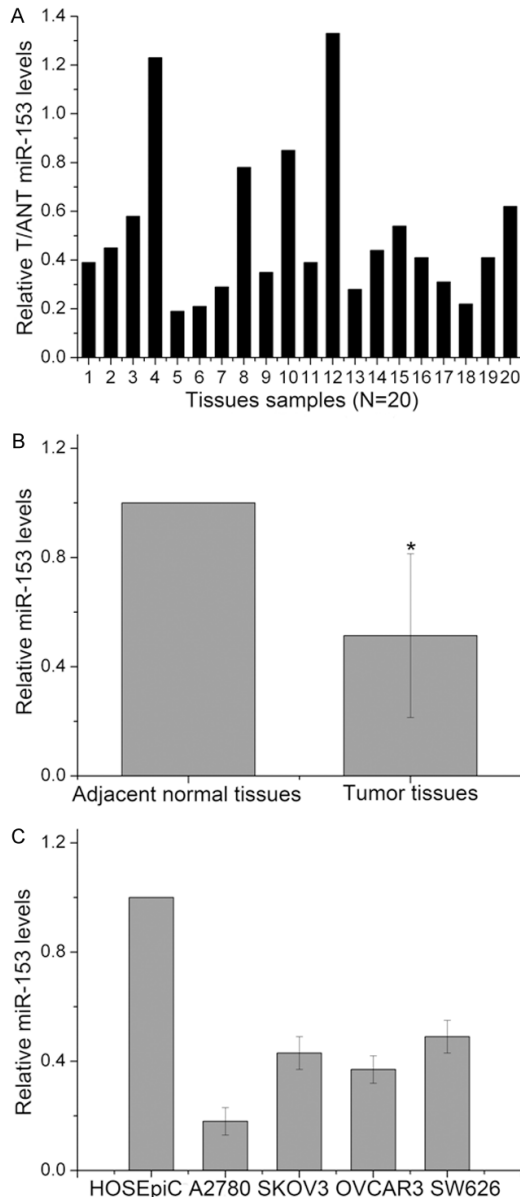
Figure 1. Identification of the expression of miR-153 in human ovarian cancer tissues and cell lines. A, B: The expression level of miR-153 in 20 pairs of ovarian cancer tissues and matched adjacent normal tissues were detected by Real-time quantitative RT-PCR. C: The expression level of miR-153 was detected in human normal epithelial ovarian cell line HOSEpiC and human ovarian carcinoma cell lines. T: tumor tissue; ANT: adjacent normal tissue. *P<0.05.

Version 12.0 (SPSS 12.0). All results were shown as the mean ± SD from three separate experiments. Differences between groups were assessed by two-tailed Student's t test. Statistical significance was considered when P<0.05.

Results

miR-153 was downregulated in ovarian cancer

In order to investigate the role of miR-153 in ovarian cancer, the expression levels of miR-153 were first determined in 20 pairs of human ovarian cancer tissues and adjacent normal tissues using Real-time quantitative PCR. The results of RT-PCR shown that miR-153 levels was decreased in the 18 patients (18/20, 90.0%) (**Figure 1A**). The results also indicated that the expression levels of miR-153 were significantly lower in the EOC tissues than in the adjacent normal tissues (**Figure 1B**). Furthermore, we detected the expression levels of miR-153 in human normal epithelial ovarian cell line HOSEpiC and human ovarian carcinoma cell lines. We found the expression level of miR-153 was significantly lower in the EOC cells than that in HOSEpiC cells (**Figure 1C**). In further experiments, the A2780 cell line was selected for the research object because the level of miR-153 in this cell line is the lowest. These findings indicate that the expression of miR-153 is down-regulated in EOC tissues and cell lines.

Overexpression of miR-153 attenuates the proliferative capacity of ovarian cancer cells

To further confirm the effect of miR-153 on the EOC cell proliferation, we used the miR-153 mimics to infect A2780 cells. The results of RT-PCR confirmed that miR-153 expression level was significantly increased in A2780 cells transfected with miR-153 mimic compared to cells transfected with NC (**Figure 2A**). Further, the CCK-8 and colony formation assays showed that the overexpression of miR-153 significantly attenuates the proliferative capacity of A2780 cells, compared with cells transfected with NC (**Figure 2B-D**). In the transwell assay, we observed that the overexpression of miR-153 led to a decrease in the number of cells that had migrated (**Figure 2E**, **2F**). The cell cycle assay showed that the A2780 cells transfected with the miR-153 mimics had a significant increase in the G1/G0 phase cell population at 72 h post-infection, compared with negative control transfected cells (**Figure 2G**, **2H**). Meanwhile, a concomitant decrease in the S phase cell population at 72 h post-infection in A2780 cells also occurred. These results sug-
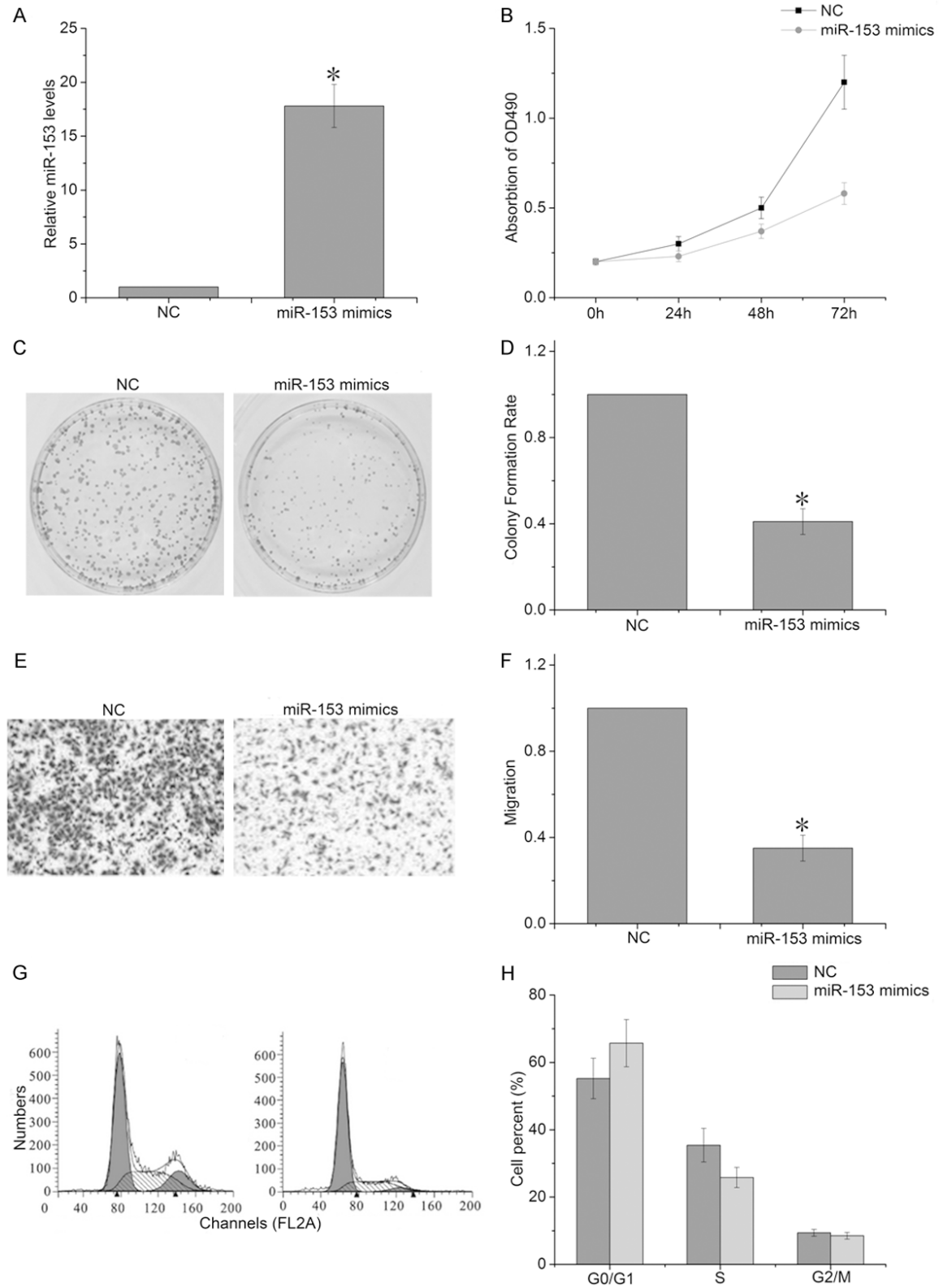
Figure 2. MiR-153 suppresses ovarian cancer cell growth and induces cell cycle arrest. A: MiR-153 expression levels were examined to validate the effect of transfection on A2780 cells; B: CCK-8 assay was performed every 24 h until 72 h after transfection, and the proliferation curves of A2780 cells were plotted. C, D: Ectopic miR-153 expression significantly inhibited A2780 cell proliferation by colony formation assay; E, F: Representative images and bar graphs revealing the migration ability of A2780 cells 72 h after transfection. G, H: PI staining and flow cytometry were used to analyze the cell cycle distribution of A2780 cells. *P<0.05.
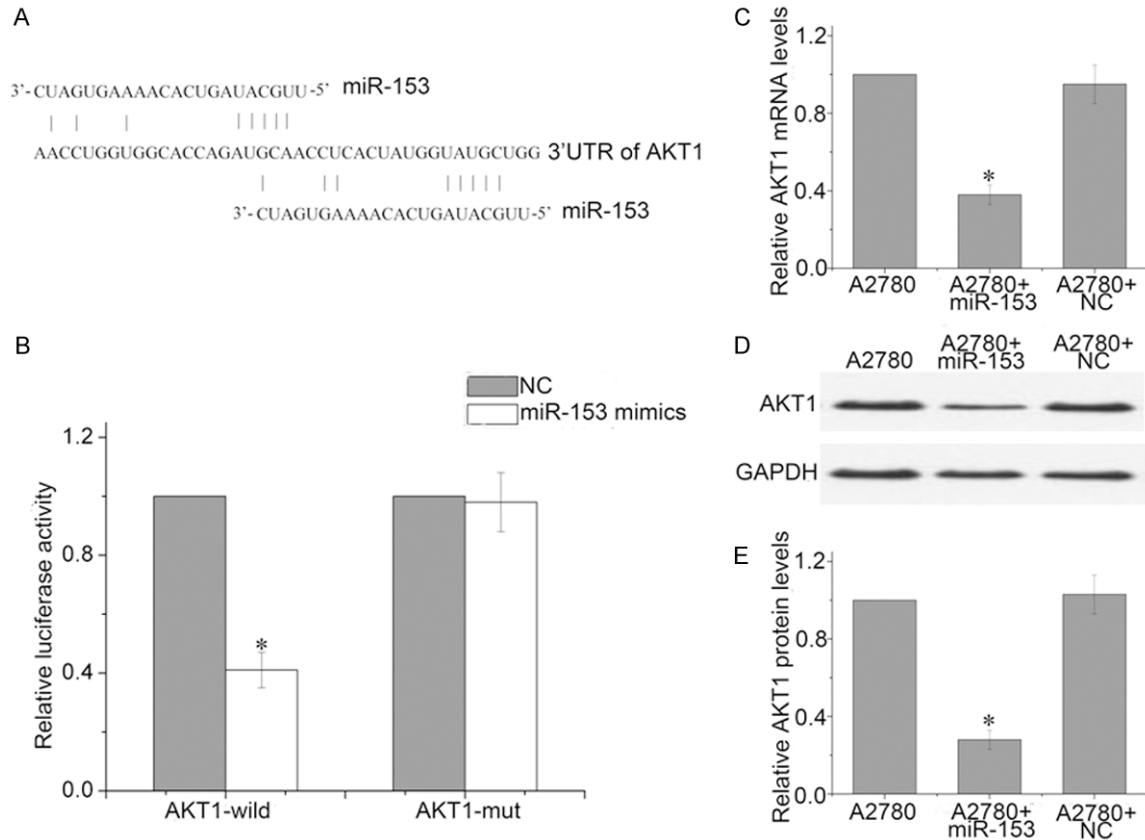
A

3'-CUAGUGAAAACACUGAUACGUU-5' miR-153

AACCUGGUGGCACCAGAUGCAACCUCACUAUGGUAUGCUGG 3'UTR of AKT1

3'-CUAGUGAAAACACUGAUACGUU-5' miR-153

C

D

B

E

Figure 3. AKT1 is a direct target of miR-153 in ovarian cancer cells. A: Seed sequences of miR-153 in the 3'-UTR of AKT1. B: Luciferase reporter assay data identified that cotransfection of the A2780 cells with miR-153 and WT AKT1 3'-UTR produced a significant decrease in luciferase activity, whereas cotransfection with MUT AKT1 3'-UTR and miR-153 mimics demonstrated no difference with the control group. C: Reverse transcription-quantitative polymerase chain reaction was performed to examine the AKT1 mRNA levels in the A2780 cells transfected with miR-153 mimics or NC, respectively. D, E: Western blot analysis was performed to examine the protein level of AKT1 in the A2780 cells transfected with miR-153 mimics or NC, respectively. $*P<0.05$.

gest that the overexpression of miR-153 inhibited proliferation and induced cell cycle arrest of EOC cells.

AKT1 is a direct target of miR-153 in ovarian cancer cells

To determine the molecular mechanism by which miR-153 induces cell growth arrest and senescence, we used an open-target prediction program (TargetScanHuman 6.2, http://www.targetscan.org/) to predict the targets of miR-153. We found AKT1 was a potential miR-153 target (**Figure 3A**). To determine whether AKT1 is the direct target gene for miR-153, a dual-luciferase reporter assay was performed. The results indicated that the luciferase activity of the reporter containing the AKT1 gene's wide-type 3'-UTR decreased (52%) following

treatment with miR-153 mimics. By contrast, the inhibitory effect of the miR-153 mimics was abolished in the mutated construct (**Figure 3B**). In addition, qRT-PCR and western blot analysis revealed that the expression of AKT1 mRNA and protein was inhibited by treatment with miR-153 mimics in A2780 cells (**Figure 3C-E**). Taken together, these data suggest that miR-153 reduces AKT1 expression by inhibiting translation and/or causing mRNA instability.

Overexpression of AKT1 reverses the inhibition effect of miR-153 on cell proliferation

To determine whether miR-153 inhibits EOC cells proliferation through AKT1, a rescue experiment was performed to further confirm the role of AKT1 in ovarian cancer. An overexpression vector, pcDNA3.1-AKT1, was con-
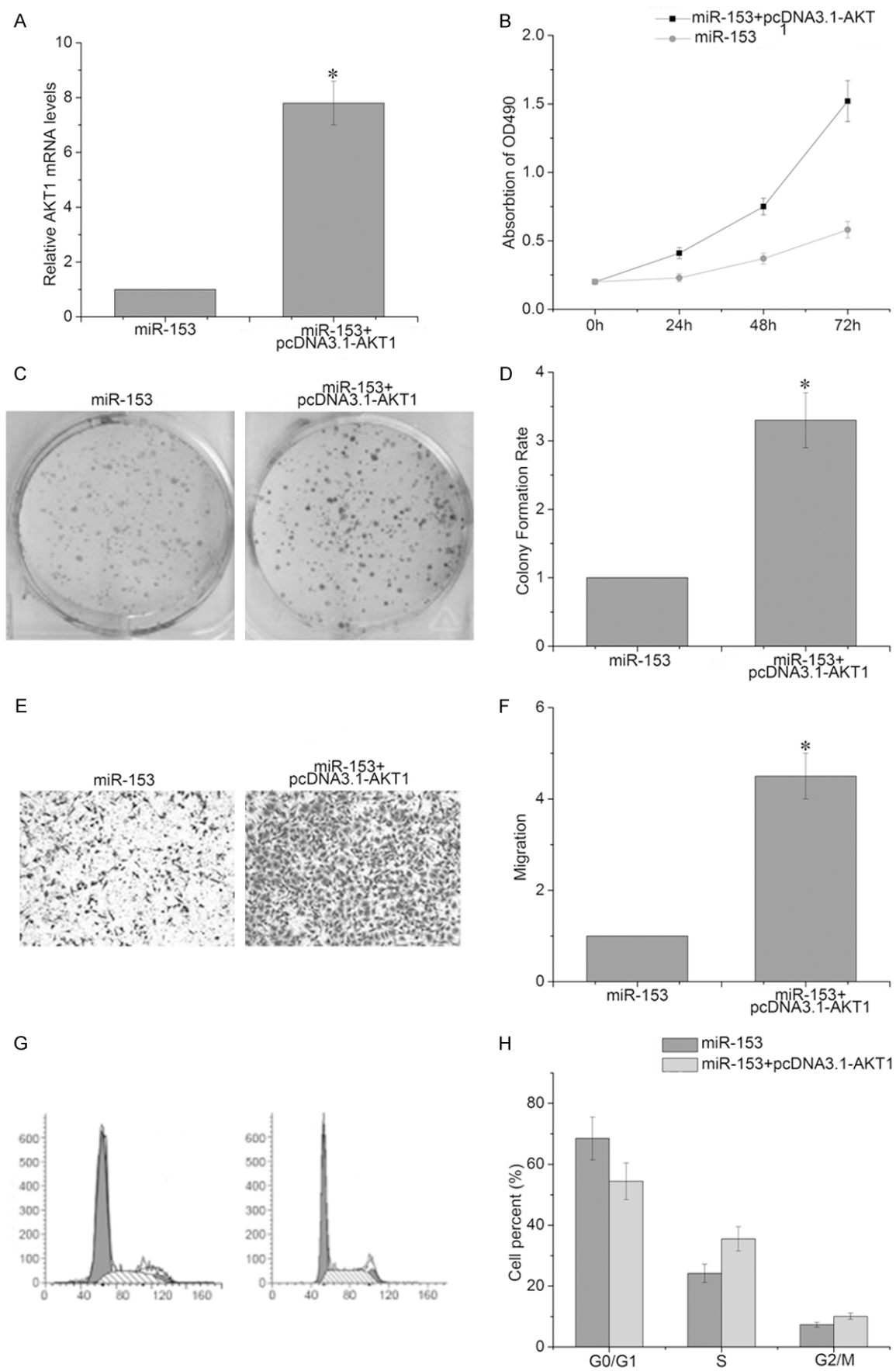
Figure 4. Overexpression of AKT1 reverses the Inhibition Effect of miR-153 on cell proliferation. A: The mRNA levels were detected by RT-PCR in A2780 cells cotransfected with pcDNA3.1-AKT1 and miR-153 mimics. B: The CCK-8 assay was performed every 24 h until 72 h after co-transfection with miR-153 mimics and pcDNA3.1-AKT1 or with miR-153 and control vector. C, D: Representative micrographs and quantifications of crystal violet stained colonies formed by the A2780 cells co-transfected with miR-153 mimics and pcDNA3.1-AKT1 or with miR-153 and control vector. E, F: Cell migration of A2780 cells that were treated as indicated in a transwell cell migration assay, as shown by the quantification and representative images. G, H: PI staining and flow cytometry were used to analyze the cell cycle distribution of A2780 cells that were treated as indicated. *P<0.05.
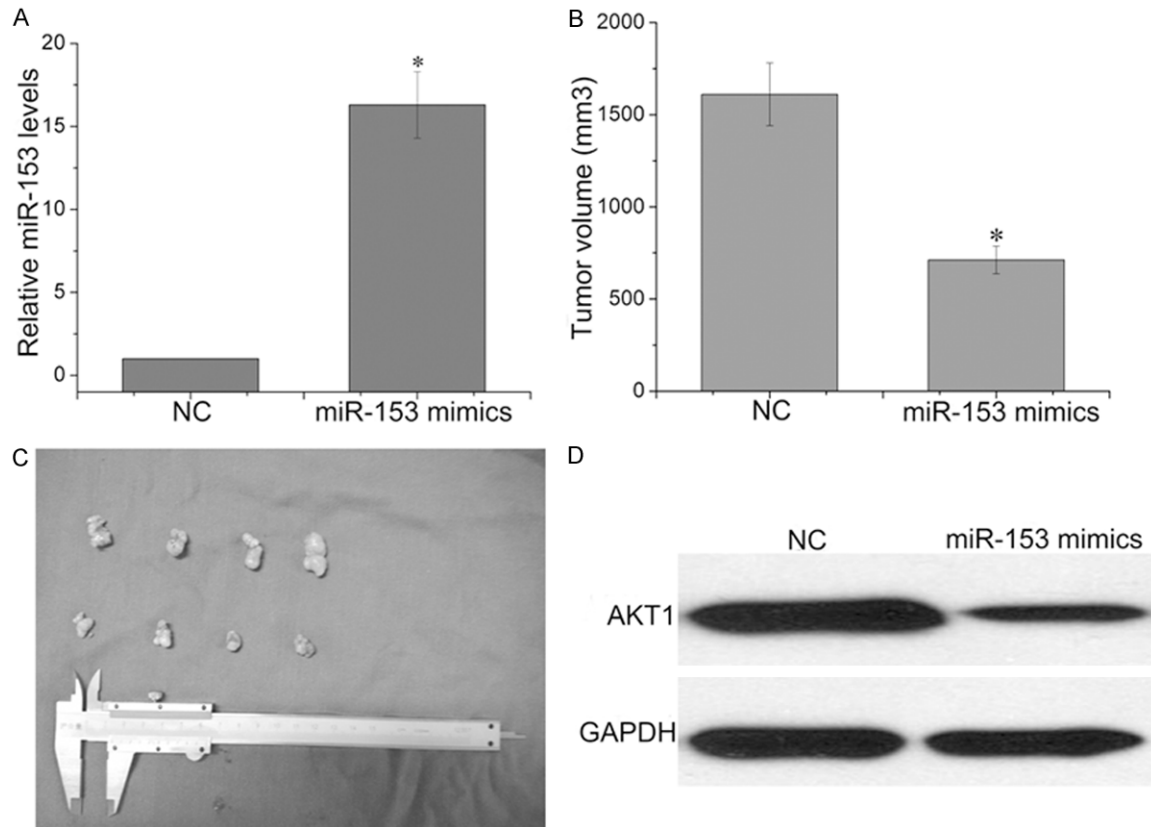

Figure 5. miR-153 suppresses ovarian cancer growth in vivo. A: A2780 cells were transfected with miR-153 mimics or NC. Real-time RT-PCR was conducted to examine the expression levels of miR-153. B: At 28 days after implantation, all mice were sacrificed. The tumor xenograft was obtained. C: The volume of the tumor xenograft was examined. D: Western blot assay was conducted to examine the protein expression of AKT1 in xenografted tumor tissues. *P<0.05.

structed to increase endogenous AKT1 expression (**Figure 4A**). The results of the CCK-8 and colony formation assays shown that the proliferation suppressing effect of miR-153 was reversed by AKT1 in A2780 cells (**Figure 4B-D**). Additionally, the transwell assay indicated that AKT1 significantly reversed the effect of miR-153 on the migration capability of A2780 cells (**Figure 4E**, **4F**). Furthermore, cell cycle analysis demonstrated that BTG1 decreased the proportion of cells in G0/G1 phase, and inhibited the effect of miR-153 on cell cycle progression (**Figure 4G** and **4H**). These findings suggested

that the overexpression of AKT1 could reverse the effect of miR-153 on the proliferation and cycle progression of ovarian cancer cells.

miR-153 suppresses ovarian cancer growth in vivo

To confirm the tumor suppressor role of miR-153, we established a BALB/c nude mouse xenograft model using A2780 cells. The A2780 cells were pre-treated with miR-153 or NC and injected into BALB/c athymic nude mice. RT-PCR analyses demonstrated the increased

expression of miR-153 in tumors developed from miR-153-treated cells relative to NC-treated cells (**Figure 5A**). The tumor volume was measured every 4 days until day 28. The tumor volume of the A2780 cells treated with miR-153 mimics was significantly reduced relative to NC (**Figure 5B**, **5C**). We also performed Western blot assay to determine the expression of AKT1 in xenografted tumor tissues (**Figure 5D**). We found that overexpression of miR-153 significantly inhibited the expression of AKT1. These results indicated that overexpression of miR-153 significantly inhibits the tumorigenicity of A2780 cells in vivo.

Discussion

Although several miRNAs have been reported to play a role in the carcinogenesis of EOC, the function of miR-153 in EOC remains unclear. In this study, we observed that the levels of miR-153 were remarkably decreased in EOC tissues or EOC cell lines in comparison with paired adjacent normal tissues and normal epithelial ovarian cells. Furthermore, we found that overexpression of miR-153 can inhibit A2780 cells proliferation and induce cell cycle arrest. We also confirmed that AKT1 is a direct target of miR-153. Our findings suggested that miR-153 may attenuate the proliferative capacity of ovarian cancer cells.

MicroRNAs (miRNAs) are a class of endogenous small RNA molecules of approximately 18-24 nucleotides that modulate the post-transcriptional level of target gene mRNAs by binding to 3'-UTR directly [14, 15]. Recently, growing evidences indicated that miRNAs are involved in biological and pathological process regulation, such as cell proliferation, migration and apoptosis [16]. Some of the miRNAs have been reported that they act as oncogenes genes or tumor suppressor in ovarian cancer. For example, Liu et al. reported that MiR-383 was overexpressed in both human EOC tumors and EOC cell lines [17]. Xiaohong Z. et al. confirmed the oncogenic effects of miR-203 on the proliferation of ovarian cancer cells [18]. Recently several miRNAs have been proven to inhibit the growth and to enhance the drug sensitivity of EOC cells [19, 20]. An increasing body of evidence indicated that miR-153 expression was downregulated in multiple malignancies [21, 22]. However, to our knowledge the biological role and underlying molecular mechanism of miR-153 in EOC remains unclear. Here, our findings demonstrated that ectopic expression of miR-153 inhibited proliferation, colony formation, and migration by repressing AKT1, suggesting that miR-153 might act as tumor suppressor in EOC.

AKT, a serine/threonine-protein kinase, also known as protein kinase B (PKB), is involved in various signaling pathways, such as those of cell proliferation, survival, metabolism [23, 24]. Overactivation of AKT can affect many downstream effectors and mediate multiple pathways including cell proliferation and survival and thus favor tumorigenesis. Lee et al. reported that overexpression of phosphorylated Akt (pAkt) in histologic specimens from EOC patients was shown to be associated with poor prognostic [25]. Yin et al. found Wip1 modulate ovarian cancer metastasis through negative regulation of p-Akt [26]. Blocking of PI3K/AKT pathway has been reported to be negatively correlated with EOC tumor growth [27]. Consistently, we found that in the present study p-AKT levels were increased in the EOC tissues compared to paired noncancerous tissues. Moreover, overexpression of AKT1 reverses the inhibition effect of miR-153 on EOC cell proliferation. We also confirmed that AKT1 is the direct target of miR-153 in EOC cells. Our findings indicated that microRNA-153 suppressed EOC cell proliferation via regulating AKT1 expression.

In summary, we have found that miR-153 is mostly downregulated in ovarian cancer tissues. Transfection of miR-153 into EOC cell lines leads to inhibition of cell proliferation and induces cell cycle arrest by directly targeting AKT1. Hence, our data suggest that miR-153 and AKT1 may become promising therapeutic candidates and targets for EOC treatment.

Disclosure of conflict of interest

None.

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