

MiR-200b-5p inhibits proliferation of ovarian cancer cells by targeting ATAD2 and regulating PI3K/AKT signaling pathway

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Abstract. – **OBJECTIVE:** The purpose of this study was to investigate the influences of micro ribonucleic acid (miR)-200b-5p on proliferation and apoptosis of ovarian cancer (OC) cells, and to explore its correlations with the target gene ATPase family, AAA domain containing 2 (ATAD2), and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway.

MATERIALS AND METHODS: Human ovarian fibroblasts (HOFs) or human OC cell lines (A2780) were cultured in vitro, and then, A2780 cells were separately transfected with miR-200b mimics or miR-NC or cultured with ATAD2-specific inhibitor BAY-850. Thereafter, the expression levels of miR-200b and ATAD2 messenger RNA (mRNA) were measured via qRT-PCR, and the proliferative capacity of cells was detected by CCK-8 assay. Next, the cell apoptosis was determined by means of flow cytometry and one-step TUNEL assay. Finally, the targeted regulatory relationship between miR-200b and ATAD2 was examined using a Luciferase reporter assay system, and the protein expressions were detected through Western blot (WB) assay.

RESULTS: It was found that the expression level of miR-200b was remarkably lower ($p < 0.05$), while the mRNA expression level of ATAD2 was notably higher ($p < 0.05$) in A2780 cells than those in HOFs. The transfection with miR-200b mimics markedly reduced the mRNA expression level of ATAD2 ($p < 0.05$) and the proliferative capacity ($p < 0.05$) and increased the apoptosis rate ($p < 0.05$) of A2780 cells. Besides, it was detected via the Luciferase reporter assay system that miR-200b inhibited ATAD2. BAY-850 significantly decreased the expression level of ATAD2 protein ($p < 0.05$) and the proliferative capacity ($p < 0.05$) but improved the apoptosis rate ($p < 0.05$) of cells. Moreover, both miR-200b mim-

ics and BAY-850 could distinctly repress the protein expression levels of PI3K and p-Akt of the PI3K/Akt signaling pathway ($p < 0.05$) and enhance the expression of suppressor gene p53 ($p < 0.05$).

CONCLUSIONS: MiR-200b-5p can inhibit the proliferation and promote the apoptosis of OC cells through targeted inhibition of ATAD2 expression and regulation of the PI3K/Akt signaling pathway.

Key Words:

Ovarian cancer, MiR-200b-5p, ATAD2, PI3K/Akt signaling pathway, Cell proliferation, Cell apoptosis.

Introduction

Ovarian cancer (OC) accounts for 2.5% of all the malignant tumors in women, but its survival rate is fairly low (5% of cancer deaths in females) due to a lack of effective early diagnostic techniques¹. Benefiting from the improved standard of medical services over the past decades since 1970, the death rate of OC has dropped by over 30%. Nevertheless, merely less than half of the female patients can survive within 5 years after diagnosis for the absence of specific early symptoms and efficacious strategies for early detection². About 22,240 new OC cases were diagnosed in the USA in 2018, and 14,070 patients died of OC in the meantime³. Therefore, prevention improvement and early detection will be the research priorities.

Micro ribonucleic acids (miRNAs) are endogenous small single-stranded RNAs composed of

18-22 nucleotides, which can negatively regulate a majority of messenger RNA (mRNA) expressions in the cells by means of degrading mRNAs or inhibiting mRNA translation^{4,5}. MiRNAs play various critical roles in different biological processes, such as cell cycle, proliferation, apoptosis, migration, and invasion⁶⁻⁸. The miRNAs involved in tumorigenesis can be classified as tumor suppressors or oncogenes according to the expression pattern and function of miRNAs in varying types of tumors⁹. As an important aspect of OC, miRNAs may be upregulated or downregulated in malignant tumors¹⁰.

In this research, the expression of miR-200b-5p in OC cells, its correlation with the ATPase family, AAA domain containing 2 (ATAD2), and the effects of regulated signaling pathway on the proliferation and apoptosis of OC cells were investigated, so as to explore the molecular mechanism of OC development and provide potential therapeutic targets.

Materials and Methods

Main Materials

Human ovarian fibroblasts (HOFs) and human OC cell lines (A2780) (American Type Culture Collection (ATCC; Manassas, VA, USA), SYBR Green reverse transcription (RT) Master Mix kit (TaKaRa, Tokyo, Japan), Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12), α -Minimum Eagle's Medium (α -MEM), fetal bovine serum (FBS), 2.5% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) and phosphate-buffered saline (PBS; Gibco, Rockville, MD, USA), TRIzol (Invitrogen, Carlsbad, CA, USA), BAY-850 (MCE, Monmouth Junction, NJ, USA), antibodies of ATAD2, phosphatidylinositol 3-kinase (PI3K), phosphorylated Akt (p-Akt), p53 and β -Actin (Abcam, Cambridge, MA, USA), miRNA RT kit (Applied Biosystems, Foster City, CA, USA), Luciferase reporter gene assay system (Promega, Madison, WI, USA), bioluminescent plate reader (Modulus TM), and 0.22 μ m pinhole filter (Millipore, Billerica, MA, USA). This study was approved by the Ethics Committee of Guangrao County People's Hospital.

Cell Culture

After resuscitation, HOFs and A2780 cells were seeded into a 60 mm culture dish (2×10^4 cells/mL) and then cultured in the DMEM/F12 containing 10% FBS, followed by subculture when the cell

confluence was about 90%. Later, the old medium was discarded, and the cells were washed with PBS twice and digested with a proper amount of 2.5% trypsin + 0.02% EDTA at 37°C for 3 min, which was discontinued using the DMEM/F12 containing 10% FBS. After that, the HOFs and A2780 cells were passaged at 1:2 and 1:6, respectively. Next, the cells were cultured in an incubator under 5% CO₂ at 37°C. Culture with BAY-850: the stock solution of BAY-850 was added into the DMEM/F12 containing 10% FBS to prepare a medium with a final concentration of 200 nM, which was applied to culture A2780 cells.

Cell Transfection

A2780 cells in a favorable growth were inoculated into a 24-well plate at 2×10^4 cells/mL. When the cell confluence reached approximately 90%, the cells were transfected with miR-200b mimics or miR-negative control (NC). In specific, 5 μ L of stock solution (20 μ M) of miR-200b mimics and miR-NC were added into 250 μ L of α -MEM, respectively, and mixed for incubation at room temperature for 5 min. Next, the mixed transfection solution was added into the 24-well plate, shaken well, and cultured in the incubator with 5% CO₂ at 37°C. Finally, the abundance of miR-200b in each group of cells was detected at 48 h after transfection.

Extraction of Total RNA and Quantitative RT-Polymerase Chain Reaction (qRT-PCR)

The cells cultured differently in each group were mixed with an appropriate amount of TRIzol after discarding the medium. After standing for 8 min, the cells were gently blown scattered and transferred into 1.5 mL Eppendorf (EP; Hamburg, Germany) tubes, followed by mixing with 200 μ L of chloroform and centrifugation at 4°C and 12,000 rpm for 10 min. Next, the supernatant was aspirated carefully, added with 200 μ L of isopropyl alcohol, shaken well, placed at room temperature for 10 min, and centrifuged at 4°C and 12,000 rpm for 15 min. After that, the supernatant was discarded, the precipitate was washed with freshly prepared 75% ethanol twice and dissolved in a proper amount of DEPC-treated water, and the concentration of nucleic acid was detected using a spectrophotometer. The primers were designed online by means of Premier designing tool and synthesized by Sangon Biotechnology (Shanghai, China) Co., Ltd. The primer sequences of miR-200b, ATAD2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table I.

MiR-200b was synthesized into first-strand complementary deoxyribonucleic acid (cDNA) using the qScript microRNA cDNA kit, and the total RNA was synthesized into cDNA by virtue of the random primers from RT Master Mix kit for ATAD2 and GAPDH. Subsequently, gel electrophoresis was conducted after PCR by virtue of PCR Master Mix kit, or qRT-PCR was performed using the SYBR Green Real-Time PCR Master Mix kit and ABI 7500 sequence detection system in accordance with the manufacturer's protocol. At last, the transcription level was evaluated *via* cycle threshold (Ct) value, and the target amount normalized to internal reference was calculated through $2^{-\Delta\Delta C_t}$ method.

Cell Counting Kit-8 (CCK-8) Assay

The cells in each group were seeded into a 96-well plate at 5×10^3 cells/mL, with 6 replicate wells for each group. After attachment, the cells were treated differently and cultured continuously for 24 h or 48 h. After that, 100 μ L of complete medium containing 10 μ L of freshly prepared CCK-8 detection solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added for culture again for 4 h. Subsequently, the optical density (OD) value at the wavelength of 450 nm was measured by a microplate reader. The experiment was repeated for 3 times for data analysis.

Flow Cytometry

The cells transfected or not transfected with miR-200b mimics were digested into single-cell suspension using 0.25% trypsin, washed with PBS twice to three times, and counted, followed by cell density adjustment to 5×10^5 cells/mL. Later, the cells were incubated with Annexin V and PI at 4°C in the dark for 30 min. After washing with PBS twice to three times, the cell density was adjusted to 1×10^6 cells/mL, the cells were detected on a flow cytometer (C6), and data were analyzed using CFlow Plus software.

One-Step Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Apoptosis Assay

After discarding the medium, the cells in each group were washed in PBS twice to three times, fixed in an appropriate amount of 4% paraformaldehyde at room temperature for 10 min, and permeabilized in 0.1% Triton X-100 for 5 min after washing by PBS for 2-3 times. Later, the cells were washed again with PBS twice to three times, added with 50 μ L of TUNEL assay solution and

incubated at room temperature for 1 h. Finally, the cells were washed using PBS twice to three times, added with a proper amount of anti-fluorescence quencher and mounted, followed by observation and photography under a fluorescence microscope.

Luciferase Assay

The cells in each group were separately transfected with miR-200b mimics or miR-negative control (NC) and wild-type ATAD2 (ATAD2-WT) or mutant ATAD2 (ATAD2-MUT) for 48 h, and then, washed in PBS for 2-3 times after discarding the medium. Next, 50 μ L of freshly prepared lysis buffer was added into each group for 30 min of cell lysis. After 10 μ L of lysis buffer was obtained and added with 100 μ L of Luciferase assay reagent prepared in advance, the Luciferase activity was determined by means of the Luciferase reporter gene assay system combined with the bioluminescent plate reader. Every experiment was set with 3 parallel controls and repeated for three times.

Western Blotting (WB) Assay

The medium was removed after the cells in each group were cultured, an appropriate amount of cell lysis buffer was added, and the lysate suspension of the adherent cells was collected using a cell scraper on ice, followed by lysis at 4°C overnight, centrifugation at 13,000 rpm to extract the total protein and detection of protein concentration through bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the proteins were separated *via* 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was sealed in 5% skim milk powder and 0.1% Tris-Buffered Saline-Tween 20, and incubated with primary antibodies of ATAD2, PI3K, p-Akt, p53, and β -Actin separately by gently shaking at 4°C overnight. After that, horse radish peroxidase (HRP)-labeled secondary antibodies were added for incubation, and the proteins to be detected were subjected to exposure using enhanced chemiluminescence (ECL) reagent.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 software (IBM Corp., Armonk, NY, USA) was used for data recording and processing. The data in each group were presented as mean \pm

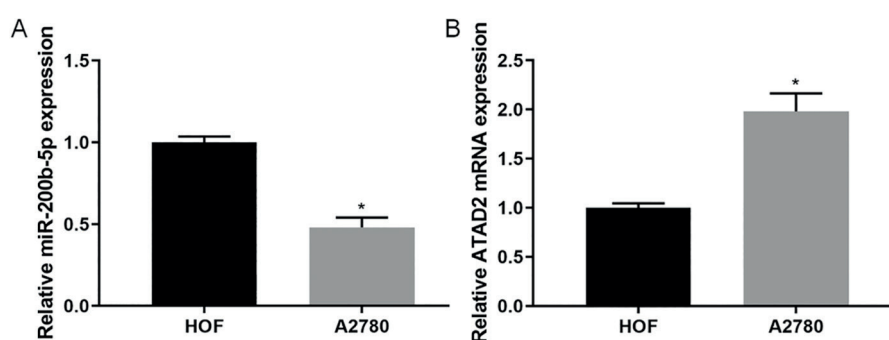


Figure 1. Expressions of miR-200b-5p and ATAD2 mRNA in ovarian fibroblasts and OC cells. **A**, Expression level of miR-200b-5p detected via qRT-PCR. **B**, Expression level of ATAD2 mRNA determined via qRT-PCR. *a significant difference ($p < 0.05$).

standard deviation ($\bar{x} \pm s$), and independent-sample t -test was adopted for comparison between groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

Expression Levels of MiR-200b-5p and ATAD2 mRNA in Cells

The detection results of expressions of miR-200b-5p and ATAD2 mRNA in ovarian fibroblasts and OC cells *via* qRT-PCR indicated that OC cells had a remarkably lower expression level of miR-200b-5p ($p < 0.05$) (Figure 1A) but a notably higher expression level of ATAD2 mRNA than ovarian fibroblasts ($p < 0.05$) (Figure 1B).

MiR Transfection of OC Cells

According to the expressions of miR-200b-5p and ATAD2 mRNA measured after OC cell transfection measured through qRT-PCR, the expression level of miR-200b-5p was markedly upregulated ($p < 0.05$) (Figure 2A), while that of ATAD2 mRNA was evidently downregulated ($p < 0.05$) (Figure 2B) after transfection with miR-200b mimics.

Impacts of MiR-200b on Proliferation and Apoptosis of OC Cells

Based on the results of CCK-8 assay, miR-200b remarkably attenuated the proliferative capacity of OC cells ($p < 0.05$) (Figure 3A). It was manifested in flow cytometry that the apoptosis rate was increased prominently from $(0.2 \pm 0.1)\%$ to $(10.7 \pm 1.4)\%$ after transfection with miR-200b mimics compared with that of OC cells not transfected with miR-200b mimics (Control group) ($p < 0.05$) (Figure 3B).

Relationship Between MiR-200b and ATAD2 Expressions

The qRT-PCR results for the influence of miR-200b mimics transfection on the expression of ATAD2 mRNA in OC cells revealed that the expression level of ATAD2 mRNA declined distinctly in OC cells transfected with miR-200b mimics in comparison with that in OC cells not transfected with miR-200b mimics ($p < 0.05$) (Figure 4).

Targeting Relationship Between MiR-200b and ATAD2

According to the online prediction of RNA-central, ATAD2 might be a target gene of miR-

Table I. Primer sequences for miR-200b-5p, ATAD2 and GAPDH detection.

| Name | Sequence |
|---------------|------------------------------|
| MiR-200b-5p F | 5'-CATCTTACTGGGCAGCATTGGA-3' |
| MiR-200b-5p R | 5'-CAGTGCGTGTCTGGAGT-3' |
| ATAD2 F | 5'-CTCAGTCTGGAGCACATCGG-3' |
| ATAD2 R | 5'-TCTTAAAGCACGTGTCCGGT-3' |
| GAPDH F | 5'-GTCAAGGCTGAGAACGGGAA-3' |
| GAPDH R | 5'-AAATGAGCCCCAGCCTTCTC-3' |

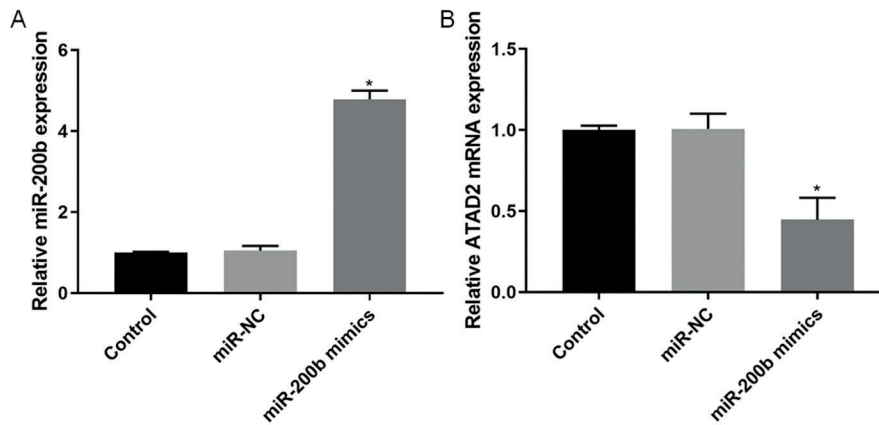


Figure 2. Expressions of miR-200b-5p and ATAD2 mRNA after transfection of OC cells. **A**, Expression level of miR-200b-5p measured by qRT-PCR. **B**, Expression level of ATAD2 mRNA determined through qRT-PCR. *a significant difference ($p < 0.05$).

200b-5p, so the Luciferase reporter gene vector carrying ATAD2, the predicted target of miR-200b, was constructed correspondingly. The comparison of miR-200b and ATAD2 target sequences are shown in Figure 5A. Then, the cells were co-transfected with ATAD2-WT plasmid and ATAD2-MUT plasmid, as well as miR-200b mimics and miR-NC, respectively, and it was discovered that the Luciferase activity of ATAD2-WT could be inhibited by miR-200b mimics ($p < 0.05$), but there was no apparent influence on the Luciferase activity of ATAD2-MUT ($p > 0.05$) (Figure 5B).

Impacts of ATAD2 Expression on Proliferation and Apoptosis of OC Cells

In order to verify the impacts of ATAD2 expression on the proliferation and apoptosis of OC

cells, OC cells were cultured with ATAD2-specific inhibitor BAY-850. It was indicated in WB assay for ATAD2 protein expression that BAY-850 remarkably repressed the protein expression level of ATAD2 in the cells ($p < 0.05$) (Figure 6A). Besides, the results of CCK-8 assay manifested that BAY-850 weakened the cell proliferation evidently ($p < 0.05$) (Figure 6B). However, the cell apoptosis was enhanced notably by BAY-850 according to the one-step TUNEL assay results ($p < 0.05$) (Figure 6C and D).

Correlations of MiR-200b and ATAD2 with the PI3K/Akt Signaling Pathway

After OC cells were treated with miR-200b mimics or BAY-850, the WB assay results displayed that both miR-200b mimics and BAY-850

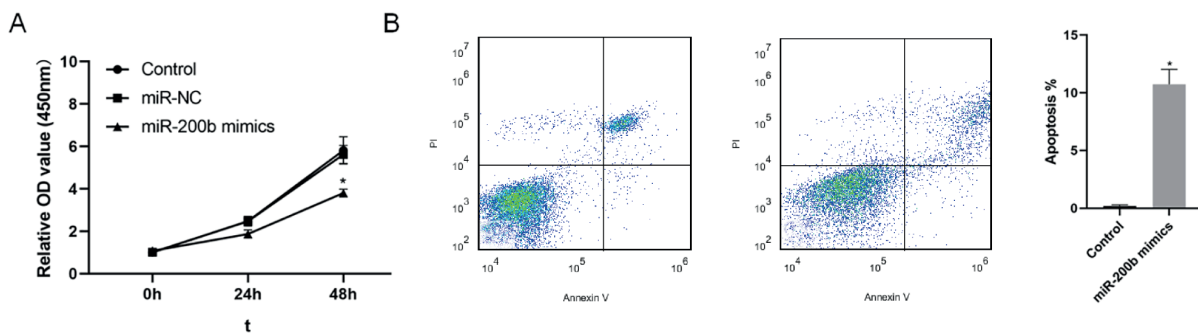


Figure 3. Impacts of miR-200b on proliferation and apoptosis of OC cells. **A**, Cell apoptosis detected via CCK-8. **B**, Apoptosis rate examined using flow cytometry. * $p < 0.05$ vs. other groups, with significant differences.

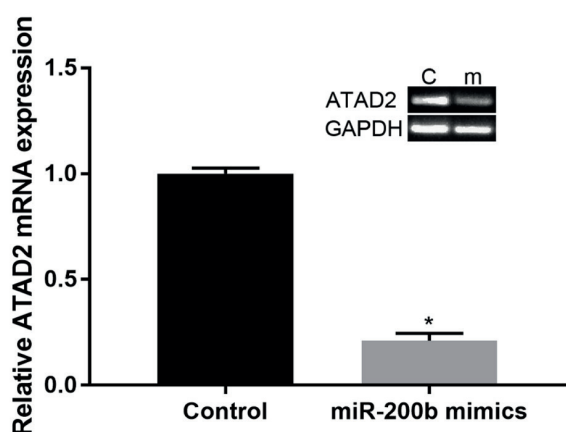


Figure 4. Expression of ATAD2 mRNA in OC cells after transfection with miR-200b mimics detected through RT-PCR. Control and C represent OC cells not transfected with miR-200b mimics, and m stands for OC cells transfected with miR-200b mimics. * a significant difference ($p < 0.05$).

could distinctly repress the protein expression levels of PI3K and p-Akt of the PI3K/Akt signaling pathway ($p < 0.05$) and prominently elevate the protein expression of p53 ($p < 0.05$) (Figure 7).

Discussion

MiR-200a, miR-200b, miR-200c, miR-141, and miR-429 are the members of miRNA cluster significantly associated with epithelial-mesenchymal transition, of which miR-200b and miR-200c are considered as essential regulatory factors for tumor invasion, metastasis, and sensitivity¹¹. Therefore, the miR-200 family members, especially miR-200b and miR-200c, control critical cellular processes, such as motility and stemness, and

their regulators also play vital roles in these processes. Liu et al¹² measured the expression levels of miR-200b and miR-200c in 93 cases of ovarian tumor tissues and 32 cases of normal ovarian tissues using *in situ* hybridization, and they found that both miR-200b and miR-200c were down-regulated in ovarian tumor compared with those in normal tissues. In this research, the expression level of miR-200b in OC cells and ovarian fibroblasts was analyzed, and it was manifested that OC cells had an evidently lower expression level of miR-200b than normal cells. Besides, the overexpression of miR-200b in cancer cells could effectively repress the cell proliferation and promote the cell apoptosis.

The expression level of ATAD2 and cell proliferation and apoptosis were studied, so as to further testify the relationship between miR-200b and target gene ATAD2. ATAD2 protein, a member of the AAA ATPase family marked by a structurally conserved ATPase domain, is responsible for the binding and hydrolyzation of ATP and involved in various cellular processes, including cell cycle regulation, protein folding and degradation, organelle biogenesis and intracellular trafficking^{13,14}. As an emerging oncogene, ATAD2 protein has close correlations with the etiology of diverse advanced human cancers and are highly expressed in many human tumor cell lines¹⁵, so it is an efficacious therapeutic target of multiple human cancers^{16,17}. Wan et al¹⁸ illustrated that highly expressed ATAD2 probably exists in ovarian tumor tissues, which may be related to the tumor stage, greater omentum metastasis, ascites, and CA-125. Increased level of ATAD2 may be a crucial player in tumor metastasis and migration. In particular, ATAD2 can serve as a prognostic marker and therapeutic target of OC. Moreover, the follow-up of the patients manifested that the

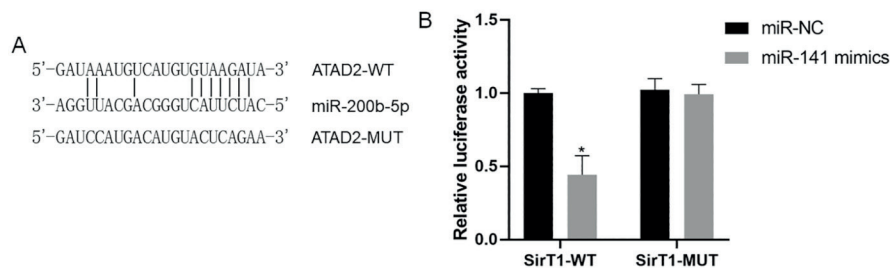


Figure 5. Interrelation between ATAD2-WT and ATAD2-MUT under the actions of miR-200b mimics and miR-NC verified via luciferase reporter gene assay system. **A**, Comparison chart sequences of miR-200b and target gene ATAD2. **B**, Relative luciferase activity. *a significant difference between two groups ($p < 0.05$).

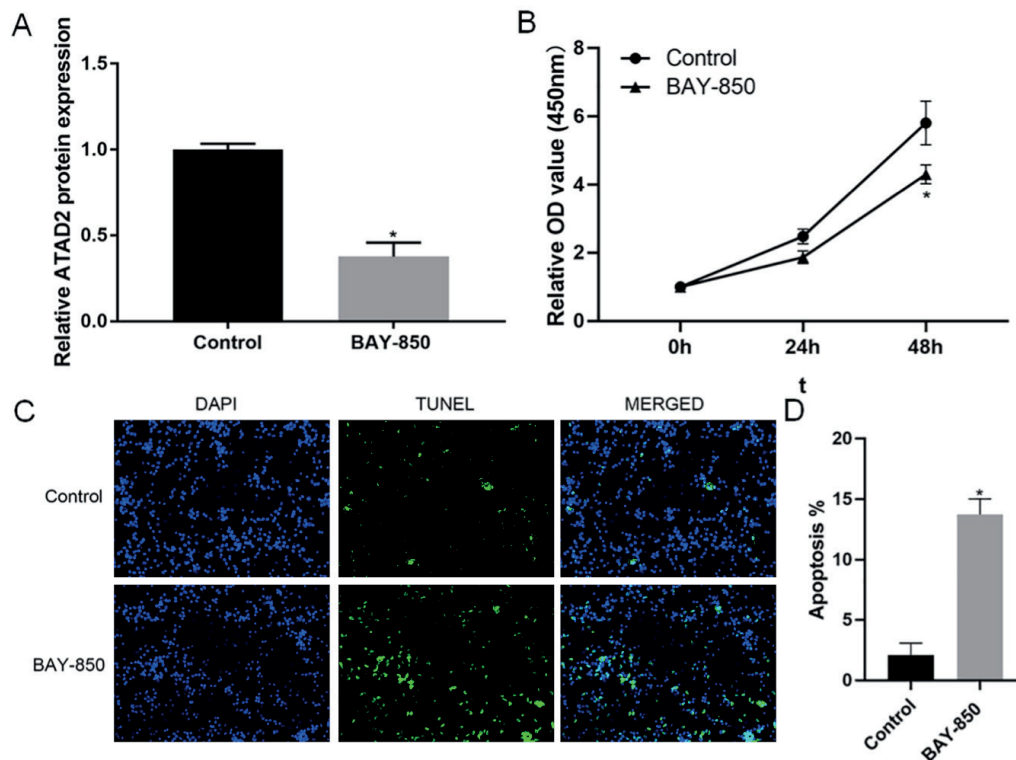


Figure 6. Impacts of ATAD2 expression on proliferation and apoptosis of OC cells. **A**, ATAD2 protein expression measured by WB assay. **B**, Cell proliferation examined through CCK-8. **C**, Fluorescence images for cell apoptosis by means of one-step TUNEL assay, (magnification: 400 \times). **D**, Apoptosis rate. Control stands for OC cells cultured routinely, and BAY-850 or B represents OC cells cultured with ATAD2-specific inhibitor BAY-850. Scale bar: 100 μ m, * a significant difference between groups ($p < 0.05$).

5-year survival rate of patients with high expression of ATAD2 is relatively low¹⁸. It was observed and analyzed in this research that ATAD2 gene and protein were highly expressed in OC cells, but the proliferative capacity of OC cells was reduced distinctly, and the apoptosis was increased after inhibiting the expression.

The PI3K/Akt signaling pathway plays an important role in modulating cell cycle, metabolism, survival, motility, chemoresistance, angiogenesis, and genomic stability¹⁹. In addition, it exerts vital regulatory effects in the progression of a variety of human cancers^{20,21}. Wee et al²² have discovered that the PI3K/Akt/mTOR signaling pathway overactivated by EGFR aberration and its activation induced by radiation are key players in cancer development²³. Researchers found that Akt2 small interfering RNA (siRNA) has a significant apoptosis-inducing effect on mutant OC OVCAR-8 cells with increased copy number of AKT2. Moreover, Akt2 siRNA manifests

strong inhibitory effect on growth of OC A2008 (PIK3CA mutation) and UPN251 (increased copy number of PIK3CA) cells, while PIK3CA siRNA exerts effective inhibitory effect on growth of OC A2008 and UPN251 cells²⁴. The results of this research suggested that the PI3K/Akt signaling pathway has potent correlations with specific genetic aberrations of OC cells and targeted components of siRNA, prominently affecting the proliferation, apoptosis, migration, and invasion of OC cells. It was also shown in this research that the PI3K/Akt signaling pathway was highly activated in OC cells, and overexpressing miR-200b or suppressing ATAD2 protein could downregulate the expression level of the pathway, repress the cell proliferation, and enhance the apoptosis. Besides, it was investigated that the downregulation of ATAD2 is associated with the reduced expressions of regulatory factors for cell proliferation (e.g., cyclin-E1, cyclin-B1, cyclin-A2, E2F1, EZH2 and B-Myb) and survival pathway-related proteins (such as IRS2, SGK1, VEGF α , Akt, and

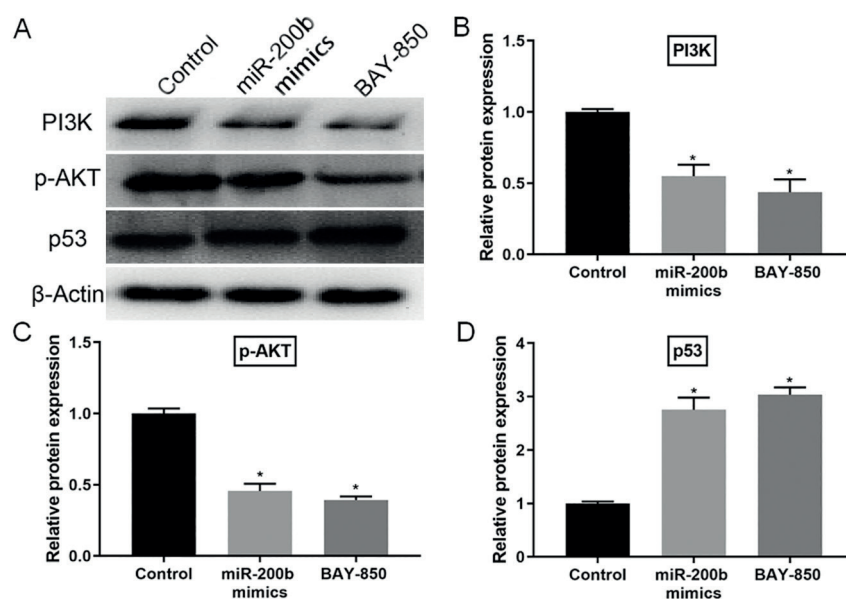


Figure 7. Influences of miR-200b and ATAD2 on the PI3K/Akt signaling pathway determined via WB assay. **A**, WB assay results. **B**, Relative protein expression of PI3K. **C**, Relative protein expression of p-Akt. **D**, Relative protein expression of p53. * $p < 0.05$ vs. Control group, with significant differences.

p-Akt), demonstrating that the cell proliferation and survival are indeed controlled by ATAD2²⁵.

Conclusions

Briefly, miR-200b-5p can inhibit the proliferation and promote the apoptosis of OC cells through targeted inhibition of ATAD2 expression and regulation of the PI3K/Akt signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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