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miR-21 regulates the proliferation and apoptosis of ovarian cancer cells through PTEN/PI3K/AKT

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Abstract. – OBJECTIVE: Phosphatase and tensin homologue deleted on chromosome ten (PTEN) regulates cell proliferation and apoptosis by inhibiting phosphatidylinositol-3 kinase (PI3K) and protein kinase (AKT) signaling. High expression of miR-21 was associated with ovarian cancer. This study aims to investigate whether miR-21 regulates PTEN/PI3K/AKT signaling as well as its role in the proliferation and apoptosis of ovarian cancer cells.

MATERIALS AND METHODS: Bioinformatics analysis was used to identify the binding site between miR-21 and the 3'-UTR of PTEN mRNA. A dual-luciferase reporter gene assay was performed to confirm the relationship between miR-21 and PTEN. The expression of miR-21, PTEN, and p-AKT was measured in normal ovarian cell IOSE80, ovarian cancer cell lines A2780, and SKOV3. miR-NC or miR-21 inhibitor was transfected into A2780 or SKOV3 cells followed by the analysis of the expression of miR-21, PTEN, p-AKT, cell apoptosis by flow cytometry, and proliferation by EdU assay.

RESULTS: There was a targeted relationship between miR-21 and PTEN. Compared with IOSE80 cell, levels of miR-21 and p-AKT were significantly elevated in A2780 and SKOV3 cells, with the statistical reduction of PTEN expression ($p<0.05$). The inhibition of miR-21 significantly reduced the expressions of miR-21 and p-AKT and induced PTEN level in A2780 and SKOV3 cells, which also restricted cell proliferation and promoted cell apoptosis.

CONCLUSIONS: The miR-21 expression is found elevated in ovarian cancer cells. The suppression of miR-21 increases PTEN expression, inhibits PI3K/AKT activity, promotes cell apoptosis, and reduces cell proliferation. This finding provides new leads to the future treatment of ovarian cancer.

Key Words:
miR-21, PTEN, PI3K/AKT, Ovarian cancer.

Introduction

Ovarian cancer (OC) is a kind of malignant tumors in female reproductive systems and ranked as the fifth among all malignant tumors that seriously threaten women's life[1]. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) negatively mediates phosphatidylinositol-3 kinase (PI3K) and protein kinase (AKT/PKB) signaling and plays an important role in the regulation of cell proliferation, migration, invasion, and apoptosis. The decrease of PTEN has been reported to be involved in the pathogenesis of several cancers, such as gastric cancer[2], lung cancer[3], and thyroid cancer[4]. Accumulative studies have demonstrated the association of abnormally high expression of miR-21 with the development, progression, and drug-resistance of ovarian cancer, suggesting that miR-21 might serve as a cancer suppressor gene[5-9]. This study, therefore, aims to investigate the role of miR-21 in the regulation of PTEN/PI3K/AKT signaling as well as its effect on proliferation and apoptosis of ovarian cancer cell.

Materials and Methods

Reagents

The normal human ovarian epithelial cell IOSE80 was bought from Shanghai Huiying Biotech (Shanghai, China). The human ovarian cancer cell line A2780 and SKOV3 were provided from Shanghai Yubo Biotech (Shanghai, China). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Waltham, MA, USA). Lipo 2000 was bought from Invitrogen (Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit was obtained from Qiagen (Hilden, Germany). The mouse anti-human PTEN and β-actin were acquired from Abcam (Cambridge, MA, USA). The mouse anti-human p-AKT was bought from Abnova (Pforzheim, Germany). The HRP-conjugated secondary antibody was from Shanghai Shenggong Biotech (Shanghai, China). Annexin

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V/PI was offered by Beyotime (Nanjing, Jiangsu, China). The Dual-Luciferase Reporter Assay System and pGL3 were supplied by Promega (Madison, WI, USA). MiR-NC and miR-21 inhibitor were purchased from Guangzhou RuiBo Biotech (Guangzhou, Guangdong, China). The EdU assay kit was bought from Sigma-Aldrich (Temecula, CA, USA).

Cell Culture

IOSE80, A2780, and SKOV3 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS and 1% streptomycin at 37°C with 5% CO_2. The cells were passaged at a ratio of 1:3 to 1:4 and the cells at log phase were used for experiments.

Luciferase Reporter Assay

PTEN 3'-UTR containing the binding site or mutated fragment was amplified followed by the collection of the PCR product, which was ligated into a pGL3 plasmid and transformed into DH5α cells. After that, the positive clones were selected and sequenced, which were named as pGL3-PTEN-WT and pGL3-PTEND-MUT. Using lip 2000, pGL3-PTEN-WT or pGL3-PTEND-MUT and miR-21 inhibitor or miR-NC were transfected into the human embryonic kidney (HEK) 293T cells and cultured for 48 h followed by the analysis of the relative luciferase activity using Dual-Luciferase Reporter Assay System Kit.

Cell Transfection and Grouping

100 μL serum-free Opti-MEM was used to dilute 10 μL Lip 2000, 50 nmoL miR-NC, and 50 nmoL miR-21 inhibitors followed by incubation for 5 min at room temperature. Then, Lip 2000 was mixed with miR-NC or miR-21 inhibitor and incubated for 20 min at room temperature. After that, all these reagents were added into the cell medium followed by culturing cells for 72 h.

Detection of mRNA Expression by Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent and reversely transcripted into cDNA using QuantiTect SYBR Green RT-PCR Kit. MiR-21 forward, 5'-TTGAATTCTAACACCTTCGTGGCTACAGAG-3', miR-21 reverse, 5'-TTAGATCTCATTTATCGAGGGAAGGATTG-3', PTEN forward, 5'-AGGGACGAACTGGTGTAATGA-3', PTENP reverse, 5'-CTGGTCCTTACTTCCCCATAGAA-3', β-actin forward, 5'-GAACCCTAAGGCCAAC-3', β-actin reverse, 5'-TGTCACGCACGATTTCC-3'. PCR was performed on Bio-Rad CFX96 in a total volume of 20 μL, including 10.0 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL 0.5 μm/L forward and reverse primers, 2 μg Template RNA, 0.5 μL QuantiTect RT Mix and ddH_2O. The PCR condition was designed as follows: 45°C 5 min, 94°C 30 s, 40 cycles of 95°C 5 s, 60°C 30 s, and 74°C 30 s.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysis buffer was added into tissues or cells and incubated at 4°C followed by centrifugation at 10000 × g for 15 min to obtain protein. After being quantified by bicinchoninic acid (BCA) assay, 60 μg protein was loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred into polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim milk and incubated with primary antibody against PTEN (1:2000 dilution), p-AKT (1:800) or β-actin (1:8000). After being washed with the phosphate-buffered solution (PBS) three times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution). The protein band was visualized after addition of enhanced chemiluminescence (ECL) reagent.

Analysis of Cell Apoptosis

Cultured cells were digested with trypsin and washed twice with PBS followed by the addition of 100 μL Binding Buffer, 5 μL Annexin V- Fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (PI) and incubation for 20 min under the dark. After that, 400 μL Binding Buffer was added to resuspend the cells followed by the analysis of cell apoptosis by flow cytometry.

Measurement of Cell Proliferation

The cells were collected and resuspended in RPMI-1640 complete medium containing 10% FBS followed by addition of 10 μM 5-ethynyl-2'-deoxyuridine (EdU) and incubation for 2 h at 37°C. After continuous culture for 48 h, the cells were washed twice with PBS, fixed for 15 min, permeabilized for 15 min followed by the addition of a reaction mixture. After washing, the cells were resuspended in washing buffer for analysis of cell proliferation by flow cytometry (BD, San Jose, CA, USA).

Statistical Analysis

The data were processed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and dis-

played as mean ± standard deviation (SD). The unpaired Student's *t*-test was used for the comparison of the differences between the two groups and the One-way ANOVA with Bonferroni post-hoc analysis was performed for the comparison among multiple groups. $p<0.05$ was considered as statistical significance.

Results

Targeted Relationship Between MiR-21 and PTEN

Bioinformatics analysis found a complementary binding site between miR-21 and the 3'-UTR of PTEN mRNA. The dual-luciferase assay was performed to evaluate the relationship between miR-21 and PTEN, and it showed that the transfection of miR-21 mimic significantly reduced the relative luciferase activity of HEK293T cells transfected with pGL3-PTEN-WT (Figure 1). However, miR-21 mimic or inhibitor did not affect the relative luciferase activity of HEK293T cells transfected with pGL3-PTEN-MUT (Figure 1). These data indicated a target relationship between miR-21 and PTEN.

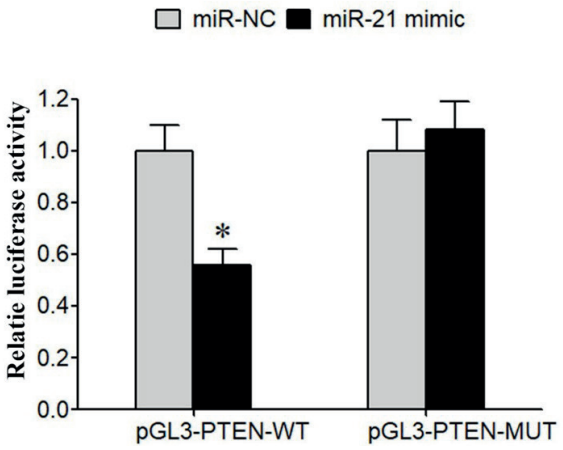

Figure 1. Dual-luciferase assay analysis of the relationship between miR-21 and PTEN. Compared with miR-NC, *$p<0.05$.

Increased MiR-21 and Reduced PTEN Expression in OC Cells

Compared with the normal human ovarian epithelial cell IOSE80, in human ovarian cancer cell line A2780 and SKO3 cell miR-21 level was significantly increased and PTEN mRNA expression was statistically reduced ($p<0.05$) (Figure 2A). In accordance with the expression profile of PTEN mRNA, compared with IOSE80 cell, a declined expression of PTEN protein was found in A2780 and SKOV3 cell (Figure 2B).

Transfection of MiR-21 Inhibitor Upregulates PTEN Expression in OC Cell

Compared with miR-NC group, the inhibition of miR-21 significantly reduced miR-21 expression in A2780 (Figure 3A) and SKOV 3 cell (Figure 3B) with the up-regulation of PTEN mRNA. Meanwhile, the expression of PTEN protein was also increased and p-AKT expression was reduced after the transfection of miR-21 inhibitor (Figure 3C).

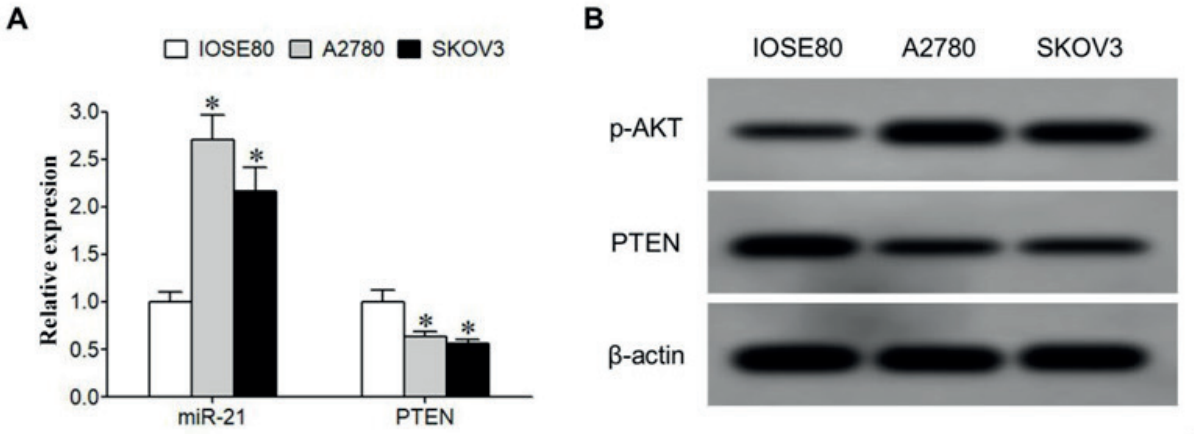

Figure 2. Expression of miR-21 and PTEN in OC cells. Total RNA and protein was isolated from IOSE80, A2078 or SKOV3 cells followed by measuring miR-21 and PTEN mRNA by qRT-PCR ***(A)***, or PTEN protein expression by Western blot ***(B)***. Compared with IOSE80, * $p<0.05$.

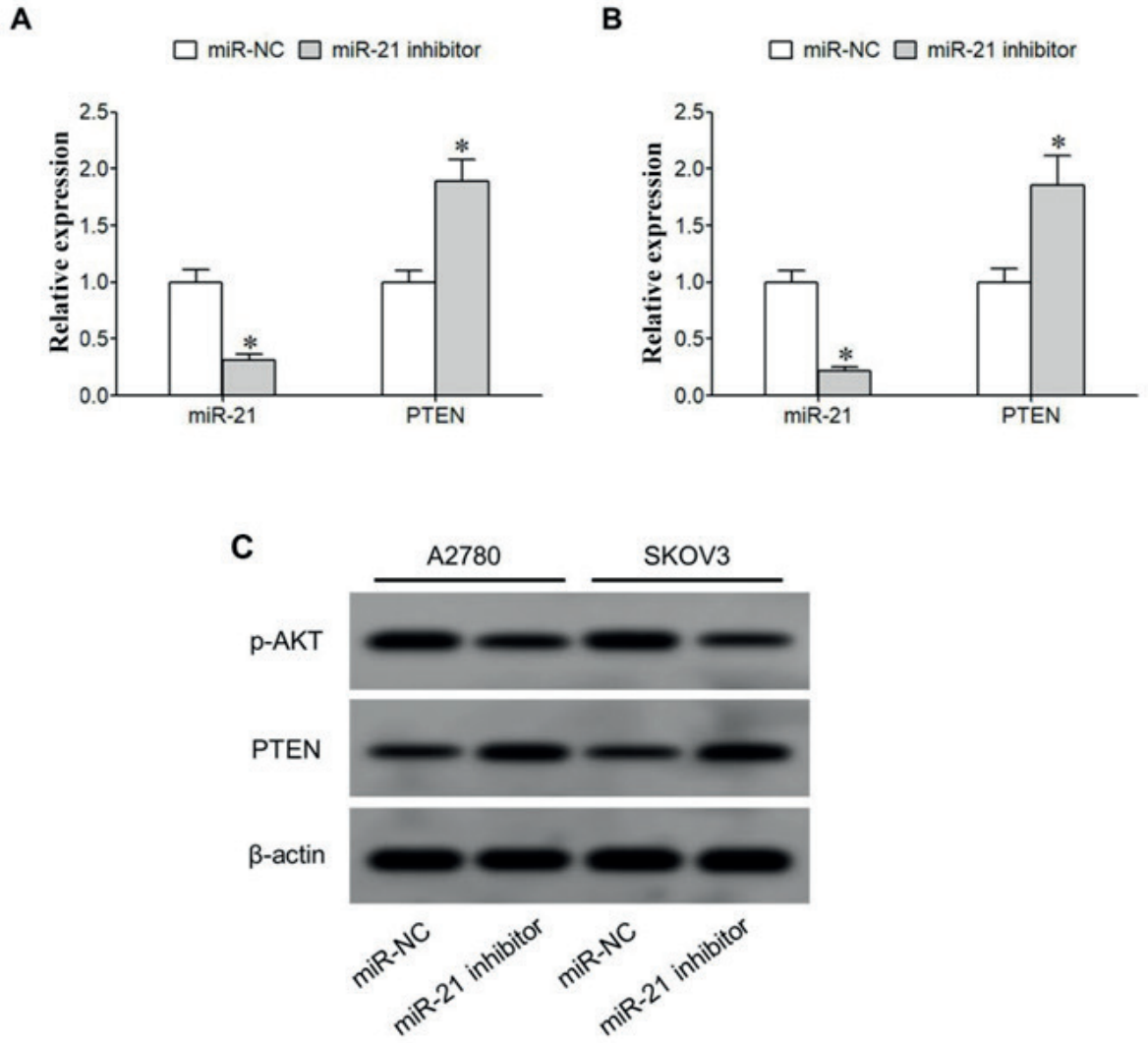

Figure 3. Expression of miR-21, PTEN, and p-AKT in OC cell transfected with miR-21 inhibitor. MiR-21 inhibitor was transfected into the OC cells followed by measuring the level of miR-21 and PTEN mRNA by qRT-PCR ***(A, B)***, protein expression of PTEN and p-AKT by western blot ***(C)***. Compared with miR-NC, * $p<0.05$.

Transfection of MiR-21 Inhibitor Promotes OC Cell Apoptosis

Flow cytometry analysis showed that the apoptosis of A2780 (Figure 4A) and SKOV3 (Figure 4B) was significantly increased after the transfection of miR-21 inhibitor compared with cells transfected with miR-NC.

Transfection of MiR-21 Inhibitor Inhibits Proliferation of OC Cell

EdU assay further revealed that the proliferation of A2780 (Figure 5A) and SKOV3 (Figure 5B) cell was significantly reduced after the transfection of miR-21 inhibitor.

Discussion

Approximately 225,000 individuals are diagnosed with ovarian cancer each year in the world, around 145,000 patients die because of that each year, and it seriously threatens women's health and life quality[10]. Therefore, the investigations of the pathogenic mechanism of ovarian cancer, as well as abnormally expressed signaling molecules, have great clinical significance in the diagnosis and treatment of patients with ovarian cancer. Once activated, PI3K will be recruited into the plasma membrane, where it catalyzes phosphatidylinositol 4,5-trisphosphate (PIP2)

into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits AKT to plasma membrane from cytoplasm, leading to phosphorylation of AKT at Ser473 and Thr308 by phosphoinositide-dependent protein kinase (PDK). Activated AKT plays an important role in the regulation of cell proliferation, cell cycle, and cell apoptosis[11-13]. PTEN can dephosphorylate PIP3 and antagonize PI3K-mediated phosphorylation of PIP2, leading to the inhibition of PI3K and AKT signaling transduction[14-17]. The down-regulation of PTEN has been reported to participate in the pathogenesis of several cancers, such as gastric cancer[2], lung cancer[3] or thyroid cancer[4]. MicroRNA is a non-coding sing-strand RNA with a length of 22-25 nucleotides and can downregulate several genes by degrading target mRNA or inhibiting the translation of target mRNA, thus participating in the regulation of tissue or organ development, cell proliferation, apoptosis or differentiation[18]. Abnormal expression of miR-21 has been shown to be associated with the development, the pathogenesis of several cancers, such as gastric cancer[19], bladder cancer[20], and esophageal cancer[21], indicating a potential tumor-suppressing function. Based on the result of dual-luciferase assay indicating that the transfection of miR-21 mimic significantly reduced the relative luciferase activity in HEK293T cells transfected with pGL3-PTEN-WT, we found a targeted relationship between miR-21 and PTEN mRNA. In addition, a significant increase of miR-21 and p-AKT levels with reduced PTEN expression was found in ovarian cancer cell line A2780 and SKOV3 cell compared with that in the normal human ovarian epithelial cell, suggesting that miR-21 might be involved in the pathogenesis of ovarian cancer. Consistent with our findings, the low miR-21 expression was demonstrated in ovarian cancer tissues compared with normal cancer-adjacent tissues[5]. Meanwhile, the level miR-21was associated with TNM stage and clinical stage[6,22,23]. Notably, the ovarian cancer patients with higher miR-21 expression are characterized with short survival and poor prognosis compared with patients with lower miR-21 level.

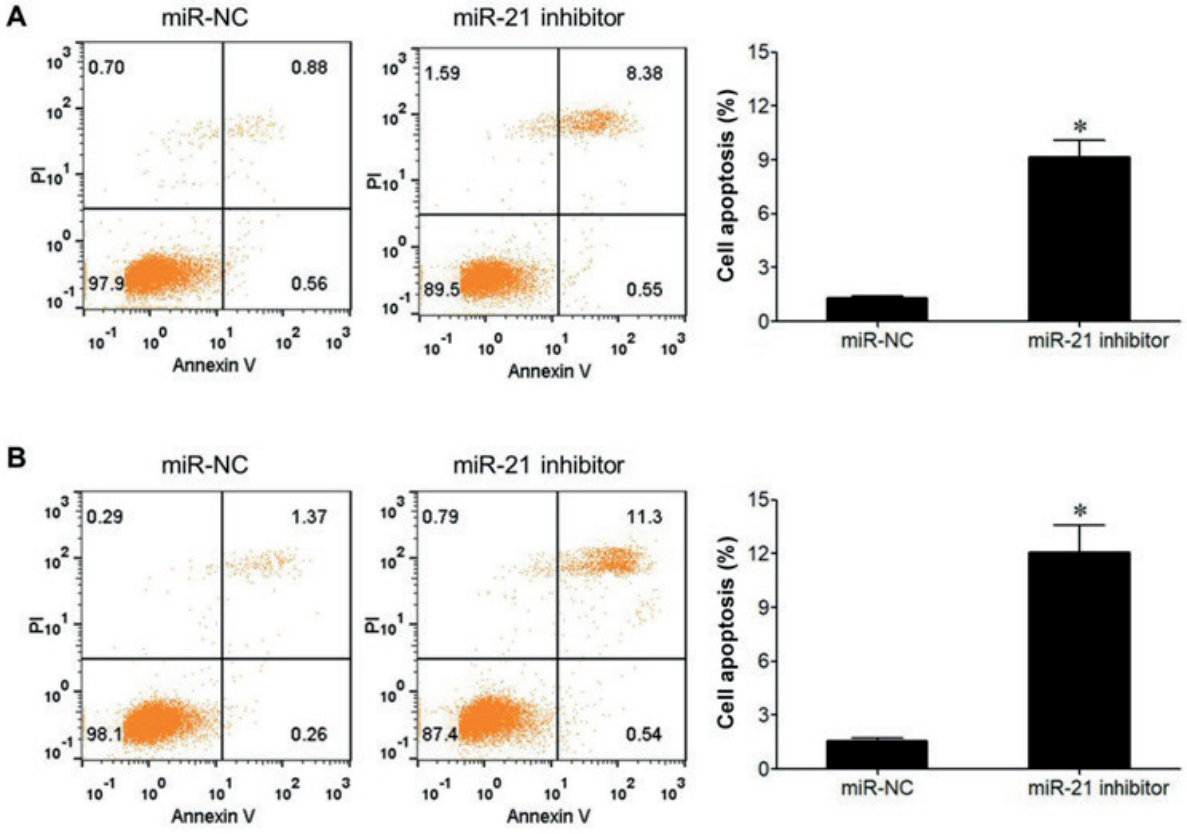

Figure 4. Cell apoptosis after transfection of miR-21 inhibitor. MiR-21 inhibitor was transfected into the OC cell line A2078 ***(A)*** or SKOV3 cell ***(B)*** and cell apoptosis was detected by flow cytometry. Compared with miR-NC, * $p<0.05$.

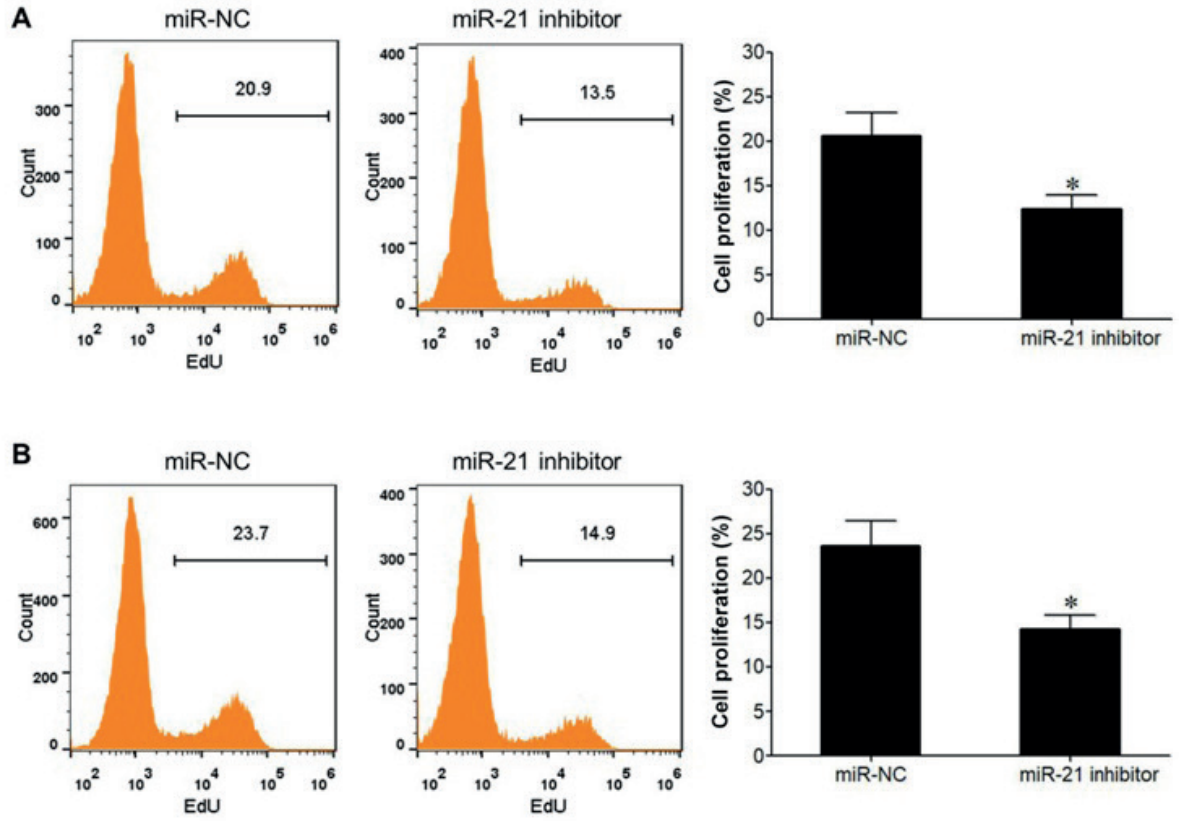

Figure 5. Cell proliferation after transfection of miR-21 inhibitor. MiR-21 inhibitor was transfected into OC cell line A2078 ***(A)*** or SKOV3 cell ***(B)*** and cell proliferation was detected by flow cytometry. Compared with miR-NC, * $p<0.05$.

To further investigate whether miR-21 affects ovarian cancer by regulating PTEN/PI3K/AKT, miR-21 inhibitor was transfected into the ovarian cancer cell, and we found that miR-21 inhibitor significantly reduced the expressions of miR-21 and AKT, while increased the PTEN expression. A previous study showed that the transfection of miR-21 inhibitor to ovarian cancer cell significantly upregulated the expression of its target gene RBPMS, while inhibited cell proliferation and invasion[5]. In addition, other evidence indicated that the inhibition of miR-21 in ovarian cancer cell significantly increased PDCD4 expression, promoted cell apoptosis, and reduced drug-resistance[6,7]. In line with these previous studies, in the present study, we detected that the suppression of miR-21 inhibited ovarian cancer cell proliferation and promoted cell apoptosis. However, whether miR-21 exerts similar effects in ovarian cancer patients remains unclear and requires further *in vivo* investigations.

Conclusions

We showed the aberrant elevation of miR-21 in ovarian cancer cells. The inhibition of miR-21 promotes ovarian cancer cell apoptosis by targeting PTEN/PI3K/AKT signaling, which offers a theoretical basis for further investigation of the anti-ovarian cancer therapy.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Siegel RL, Fedewa SA, Miller KD, Goding-Sauer A, Pinheiro PS, Martinez-Tyson D, Jemal A. Cancer statistics for Hispanics/Latinos, 2015. CA Cancer J Clin 2015; 65: 457-480.
2) Wang P, Guan Q, Zhou D, Yu Z, Song Y, Qiu W. MiR-21 inhibitors modulate biological functions of

gastric cancer cells via PTEN/PI3K/mTOR pathway. DNA Cell Biol 2018; 37: 38-45.

3) Yang WB, Zhang WP, Shi JL, Wang JW. MiR-4299 suppresses non-small cell lung cancer cell proliferation, migration and invasion through modulating PTEN/AKT/PI3K pathway. Eur Rev Med Pharmacol Sci 2018; 22: 3408-3414.
4) Ramírez-Moya J, Wert-Lamas L, Santisteban P. MicroRNA-146b promotes PI3K/AKT pathway hyperactivation and thyroid cancer progression by targeting PTEN. Oncogene 2018; 37: 3369-3383.
5) Baez-Vega PM, Echevarria Vargas IM, Valiyeva F, Encarnacion-Rosado J, Roman A, Flores J, Marcos-Martinez MJ, Vivas-Mejia PE. Targeting miR-21-3p inhibits proliferation and invasion of ovarian cancer cells. Oncotarget 2016; 7: 36321-36337.
6) Chan JK, Blansit K, Kiet T, Sherman A, Wong G, Earle C, Bourguignon LY. The inhibition of miR-21 promotes apoptosis and chemosensitivity in ovarian cancer. Gynecol Oncol 2014; 132: 739-744.
7) Echevarría-Vargas IM, Valiyeva F, Vivas-Mejía PE. Upregulation of miR-21 in cisplatin resistant ovarian cancer via JNK-1/c-Jun pathway. PLoS One 2014; 9: e97094.
8) Liu S, Fang Y, Shen H, Xu W, Li H. Berberine sensitizes ovarian cancer cells to cisplatin through miR-21/PDCD4 axis. Acta Biochim Biophys Sin (Shanghai) 2013; 45: 756-762.
9) Xie Z, Cao L, Zhang J. MiR-21 modulates paclitaxel sensitivity and hypoxia-inducible factor-1α expression in human ovarian cancer cells. Oncol Lett 2013; 6: 795-800.
10) La Vecchia C. Ovarian cancer: epidemiology and risk factors. Eur J Cancer Prev 2017; 26: 55-62.
11) Lei Y, Wang YH, Wang XF, Bai J. LINC00657 promotes the development of colon cancer by activating PI3K/AKT pathway. Eur Rev Med Pharmacol Sci 2018; 22: 6315-6323.
12) Yan R, Li K, Yuan DW, Wang HN, Zhang Y, Dang CX, Zhu K. Downregulation of microRNA-4295 enhances cisplatin-induced gastric cancer cell apoptosis through the EGFR/PI3K/Akt signaling pathway by targeting LRIG1. Int J Oncol 2018; 53: 2566-2578.
13) Zhang Y, Fang J, Zhao H, Yu Y, Cao X, Zhang B. Downregulation of microRNA-1469 promotes the development of breast cancer via targeting HOXA1 and activating PTEN/PI3K/AKT and Wnt/β-catenin pathways. J Cell Biochem 2019; 120: 5097-5107.
14) Jiang Y, Chang H, Chen G. Effects of microRNA-20a on the proliferation, migration and apoptosis of multiple myeloma via the PTEN/PI3K/AKT signaling pathway. Oncol Lett 2018; 15: 10001-10007.
15) Lin H, Huang ZP, Liu J, Qiu Y, Tao YP, Wang MC, Yao H, Hou KZ, Gu FM, Xu XF. MiR-494-3p promotes PI3K/AKT pathway hyperactivation and human hepatocellular carcinoma progression by targeting PTEN. Sci Rep 2018; 8: 10461.
16) Yao H, Su S, Xia D, Wang M, Li Z, Chen W, Ren L, Xu L. F-box and leucine-rich repeat protein 5 promotes colon cancer progression by modulating PTEN/PI3K/AKT signaling pathway. Biomed Pharmacother 2018; 107: 1712-1719.
17) Long ZW, Wu JH, Cai H, Wang YN, Zhou Y. MiR-374b promotes proliferation and inhibits apoptosis of human GIST cells by inhibiting PTEN through activation of the PI3K/Akt pathway. Mol Cells 2018; 41: 532-544.
18) Huang YH, Lin KH, Chen HC, Chang ML, Hsu CW, Lai MW, Chen TC, Lee WC, Tseng YH, Yeh CT. Identification of postoperative prognostic microRNA predictors in hepatocellular carcinoma. PLoS One 2012; 7: e37188.
19) Larki P, Ahadi A, Zare A, Tarighi S, Zaheri M, Souri M, Zali MR, Ghaedi H, Omrani MD. Up-regulation of miR-21, miR-25, miR-93, and miR-106b in gastric cancer. Iran Biomed J 2018; 22: 367-373.
20) Ghorbanmehr N, Gharbi S, Korsching E, Tavallaei M, Einollahi B, Mowla SJ. MiR-21-5p, miR-141-3p, and miR-205-5p levels in urine-promising biomarkers for the identification of prostate and bladder cancer. Prostate 2019; 79: 88-95.
21) Xia Y, Wang Y, Wang Q, Ghaffar M, Wang Y, Sheng W, Zhang F. Increased miR-203-3p and reduced miR-21-5p synergistically inhibit proliferation, migration, and invasion in esophageal cancer cells. Anticancer Drugs 2019; 30: 38-45.
22) Chen Y, Chen Q, Liu Q, Gao F. Human epididymis protein 4 expression positively correlated with miR-21 and served as a prognostic indicator in ovarian cancer. Tumour Biol 2016; 37: 8359-8365.
23) Mahmoud EH, Fawzy A, A Elshimy RA. Serum MicroRNA-21 negatively relates to expression of programmed cell death-4 in patients with epithelial ovarian cancer. Asian Pac J Cancer Prev 2018; 19: 33-38.