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Regular paper

MiR-92a regulates PTEN/Akt signaling axis to promote paclitaxel resistance in ovarian cancer cells

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To investigate the function and possible mechanism of miR-92a in malignant behaviors such as paclitaxel resistance in ovarian cancer (OC) cells. The miR-92a and PTEN expression were detected by real-time PCR (RT-PCR). The cell viability and apoptosis were detected by MTT, colony formation and flow cytometry assay, respectively. Dual-luciferase reporter assay was adopted to verify the targeting relationship between miR-92a and PTEN. Besides, we measured the relative protein levels of PTEN and p-AKT/AKT by Western blot. MiR-92a was significantly highly expressed in OC cells, and its high expression could notably enhance paclitaxel resistance, cell proliferation and colony formation, as well as inhibit apoptosis in SKOV3-Tax cells. Further luciferase reporter assay and expression detection showed that miR-92a could target and regulate PTEN and that there was a targeted relationship between them. In addition, further exploration of the mechanism revealed that miR-92a regulated PTEN/Akt signaling pathway. MiR-92a not only promotes the proliferation, colony formation and paclitaxel resistance of SKOV3-Tax cells in OC, but also inhibits apoptosis, and it may be related to the regulation of the PTEN/Akt signaling pathway. MiR-92a serves as a potential biomarker for the malignant biological behavior of OC cells.

Keywords: MiR-92a, PTEN/Akt signaling axis, Ovarian cancer (OC), Paclitaxel resistance, Biomarkers

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Abbreviations: miRNAs, microRNAs; OC, ovarian cancer; PI, propidium iodide; Tax, paclitaxel

INTRODUCTION

Ovarian cancer (OC), a common gynecological malignancy in the world, is the fifth leading cause of cancer death (Kossai *et al.*, 2018; Funston *et al.*, 2020). Due to inconspicuous early symptoms, most patients with OC are diagnosed at an advanced stage, and thus their 5-year survival rate is approximately 30% (Sehouli Grabowski, 2019; Rooth, 2013). Chemotherapy and immunotherapy are currently the first-line clinical treatments for OC patients. However, most patients develop resistance after a period of treatment (Narod, 2016; Kurnit *et al.*, 2021; Shang *et al.*, 2019). The main treatment for OC is surgical resection of the visible tumor followed by chemotherapy adjuvant with drugs such as paclitaxel (Mueller *et al.*, 2016). Most patients with OC develop resistance to paclitaxel leading to recurrence. Nevertheless, the molecular mechanisms of paclitaxel resistance are not yet fully understood. Therefore, exploring effective clinical therapeutic targets and clarifying their mechanism will be conducive to the treatment of OC.

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 18–25 nucleotides in length. As important epigenetic regulators, miRNAs are widespread in the biological community and considered highly conservative (Filipowicz *et al.*, 2008). Much research has demonstrated that miRNAs are involved in the evolution of tumors and play a vital role. For example, miRNAs not only participate in the development of tumors by acting as tumor suppressor genes (Gai *et al.*, 2018) or proto-oncogenes (Huang *et al.*, 2019) but also regulate the proliferation, migration and invasion of tumor cells *via* serving as significant biological molecules. In addition, increasing evidence manifested that aberrantly expressed miRNAs exerted an essential effect on drug resistance or drug sensitivity in chemotherapy (Du Pertsemlidis, 2012; De Cecco *et al.*, 2017). As a known carcinogenic miRNA, miR-92a in most cases enhances the development of tumors such as lung cancer (Zhou *et al.*, 2015), cervical cancer (Lin *et al.*, 2013), and pancreatic cancer (Ohyagi-Hara *et al.*, 2013) by regulating the expression of tumor-related genes. In recent years, the biological characteristics and function of miR-92a have been preliminarily understood, but its effect and mechanism in the proliferation, apoptosis and paclitaxel resistance of OC are still unclear. Therefore, we probed into the role of miR-92a in OC by knocking down and overexpressing miR-92a in SKOV3-Tax cells, and its possible molecular regulatory mechanism by bioinformatics prediction and molecular biology experiments. And we believed that with the continuous deepening of the study of miR-92a-related downstream target genes, there would be a scientific basis and clues provided for the diagnosis, treatment and prognosis analysis of OC.

MATERIALS AND METHODS

Cell culture and transfection

Human normal ovarian epithelial cells IOSE80 and OC cell line SKOV3 were purchased from the National Collection of Authenticated Cell Cultures. All cells were cultured in RPMI-1640 medium (Gibco, USA) contain-

ing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) in an incubator at 37°C with 5% CO_2 and 95% humidity. Paclitaxel (Tax)-resistant model cells of SKOV3 (SKOV3-Tax) were constructed using induction of concentration gradient.

NC mimics and miR-92a mimics, NC inhibitor and miR-92a inhibitor were purchased from GenePharma Co., Ltd. (China). SKOV3-Tax cells in the logarithmic growth phase were collected and seeded in a 6-well plate. Transfection was performed when the cells were cultured to about 80% confluence. Subsequently, the above plasmids were transferred into SKOV3-Tax cells according to the instructions of Lipofectamine 2000, and were named NC mimics group, miR-92a mimics group, NC inhibitor group and miR-92a inhibitor group, respectively. The plate was placed in the cell incubator for 48-h culture and then the cells were collected.

Real-time PCR (qRT-PCR)

Total cellular RNA was extracted with Trizol reagent (Invitrogen), and the quality and concentration of extracted RNA were determined by Nanodrop. The cDNA was synthesized by reverse transcription according to the instructions of the reverse transcription-PCR kit (Takara, Japan). The PTEN expression level and miR-92a were tested based on the instructions of the SYBR Green PCR Master Mix kit, with GAPDH and U6 as their internal control genes, respectively. The primer sequences were seen in Table 1. And the data analysis was performed by the $2^{-\Delta\Delta Ct}$ method (Zhu *et al.*, 2019).

Table 1. Primer Sequences

Genes	Primer Sequences (5' to 3')
PTEN	F ATCAACAGCCAACAAATACC
	R TTCTTATCACCGTCACCCT
miR-92a	F GCTGAGTATTGCACTTGTCCCG
	R GTGTCGTGGAGTCGGCAA
U6	F CTCGCTTCGGCAGCACA
	R AACGCTTCACGAATTGCGT
GAPDH	F GGAGCGAGATCCCTCCAAAAT
	R GGCTGTTGTCATTCTCATTCTCATGGGG

MTT

The transfected SKOV3-Tax cells were collected and seeded in a 96-well plate at 5×10^3 cells/well, and then cultured in a cell incubator until the cell adhesion was presented. Next, the cell proliferation was determined at 0 h- and 24 h- adherence as referring to the instructions of MTT kit (Beyotime, China). And then 20 μl MTT solution (5 mg/ml) was added to each well for 4-h culture. After removal of the supernatant, 150 μl DMSO was added to each well, and they were mixed well at ambient temperature for 5 min to dissolve the formazan crystals. The absorbance value at 490 nm was determined by a microplate reader. Finally, different concentrations (0, 1, 2, 3, 4, and 5 logs [Tax] nM) of paclitaxel (Tax) were added for 24-h co-culture after cell adhesion presented in the paclitaxel resistance assay, and then cell survival rate was tested by MTT.

Cell colony formation assay

The transfected SKOV3-Tax cells were collected and seeded in a 6-well plate at 800 cells/well, and then cultured in a cell incubator for 10–14 days. After they formed macroscopic clones, the culture medium was discarded. Afterwards, the cultured cells were fixed with 4% paraformaldehyde (Beyotime, China) for 15 min, and later stained with a crystal violet staining solution (Beyotime, China). The excess staining solution was washed off with PBS and eventually the cells were dried and photographed.

Flow cytometry

Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Pharmingen, USA) was applied to detect SKOV3-Tax in apoptosis. The cells were collected and washed twice with cold PBS buffer, and 500 μl of 1× Binding Buffer was adopted to prepare 1×10^6 cells/ml cell suspension. With the addition of 5 μl of Annexin V-FITC solution, the treated cells were incubated in the dark for 15 min, followed by 10 μl of PI solution at ambient temperature (20–25°C) for another incubation in the dark for 5 min. Lastly, the early and late apoptosis rates of the cells were measured by a flow cytometry system (Becton Coulter, USA) within 1 h.

Dual-luciferase reporter assay

When reaching 80~90% confluence, 293T cells were co-transfected with constructed wild-type (PTEN WT) or mutant-type (PTEN MUT) dual-luciferase reporter vectors of PTEN and miR-92a mimics or NC mimics. Subsequently, the transfected cells were incubated for 48 h and then collected to be lysed at ambient temperature for 20 min. After that, the cell centrifugation was performed, and then the supernatant was collected and stored at −20°C. Subsequently, luciferase substrate was directly added to the supernatant and the luciferase activity was measured by luminescence. The relative firefly luciferase activity was calculated with Renilla luciferase activity as an internal control (Zou *et al.*, 2019).

Western blot

RIPA lysate was added to the cells in each group and cells were lysed on ice for 20 min and centrifugated at 10000 rpm, 4°C for 20 min to obtain the protein supernatant. Afterwards, the protein concentration was detected by the BCA kit (Solarbio, China). There was 20 μl of total protein separated using SDS PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Next, the protein was transferred to PVDF membranes, blocked with 3% BSA blocking solution for 1 h at ambient temperature, and then incubated overnight at 4°C in diluted primary antibodies (anti-PTEN, ab32199, Abcam; anti-p-Akt, #9271, CST; anti-Akt, #9272, CST; anti-GAPDH, ab8245, Abcam; USA). Later, the membranes were washed twice with TBST, and then a diluted secondary antibody (ZSGB-Bio Co., Ltd., China) was added for 1-h incubation at ambient temperature. After that, the membranes were washed three times with TBST, and then ECL luminescence solution was added to the membranes. And then, the membranes were exposed to

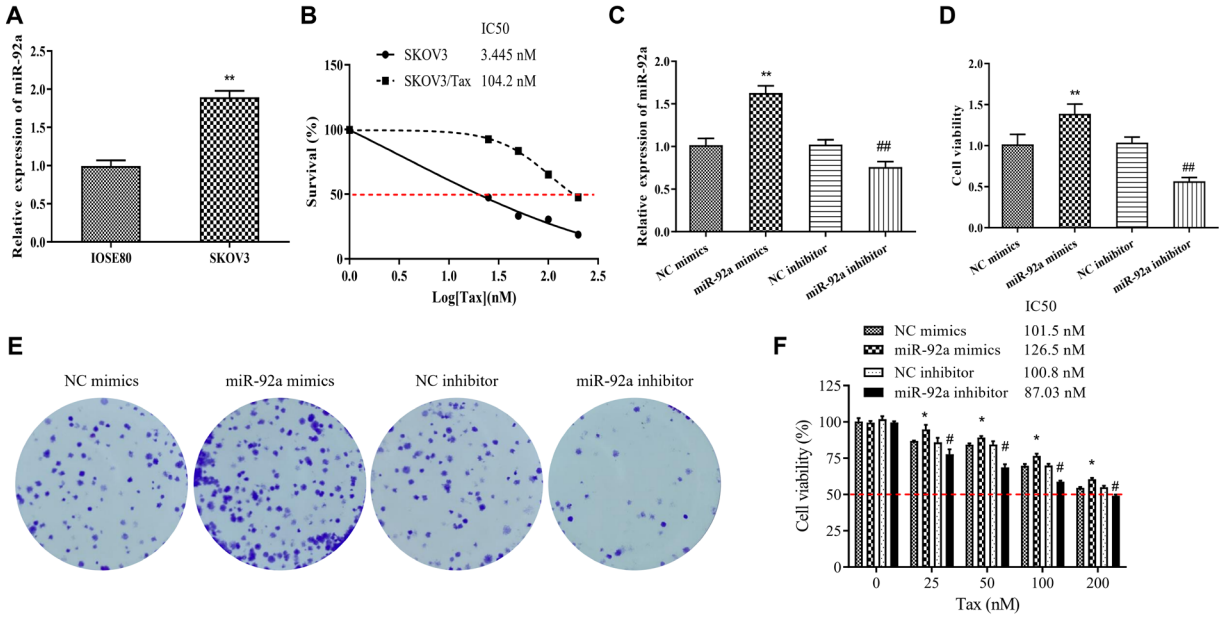

Figure 1. MiR-92a promotes SKOV3 cell proliferation, colony formation and improves paclitaxel resistance of the cells.
(**A**) qRT-PCR to detect the miR-92a expression level in normal ovarian epithelial cells IOSE80 and ovarian cancer (OC) cells SKOV3, ***P*<0.01 *vs*., IOSE80; (**B**) MTT to test the effect of paclitaxel (Tax) on the activity of SKOV3 and SKOV3-Tax; (**C**) qRT-PCR to determine the miR-92a expression level in SKOV3-Tax cells of each group; (**D/E**) MTT and colony formation assay to assess the activity (**D**) and colony formation ability (**E**) of SKOV3-Tax cells in each group, respectively; (**F**) MTT to detect the effect of different concentrations of Tax on the cell activity of SKOV3-Tax cells in each group, **P*<0.05 and ***P*<0.01 *vs*., NC mimics, #*P*<0.05 and ##*P*<0.01 *vs*., NC inhibitor.

the gel imaging system for photography. Finally, the gray values of the protein bands were analyzed by Image pro plus software, and then the relative expression level of the proteins was analyzed with GAPDH as an internal control.

Statistical analysis

All results were expressed as mean ± standard deviation (S.D.) and GraphPad Prism 9.0 was utilized to plot. Statistical analysis was performed with GraphPad Prism 9.0 and SPSS 24.0 software. *T*-test was adopted to compare the two groups, and a one-way analysis of variance was employed for comparison among multiple groups. $P<0.05$ was considered a statistically significant difference.

RESULTS

MiR-92a promotes SKOV3-Tax cell proliferation, colony formation and improves paclitaxel resistance of cells

Examination of miR-92a expression in normal ovarian epithelial cells IOSE80 and OC cells SKOV3 revealed that miR-92a expression was notably higher in SKOV3 than in IOSE80 ($P<0.01$, Fig. 1A). To clarify the role

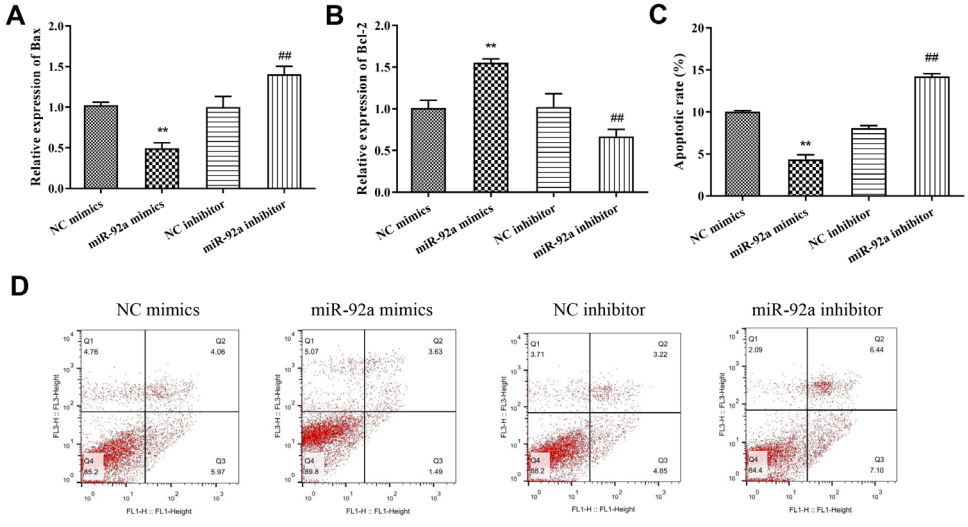

Figure 2. MiR-92a inhibits apoptosis of SKOV3-Tax cells.
(**A/B**) qRT-PCR to detect the expression level of pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 in SKOV3-Tax cells of each group; (**C/D**) flow cytometry to test the apoptosis rate of SKOV3-Tax cells in each transfection group. ***P*<0.01 *vs*., NC mimics, ##*P*<0.01 *vs*., NC inhibitor.

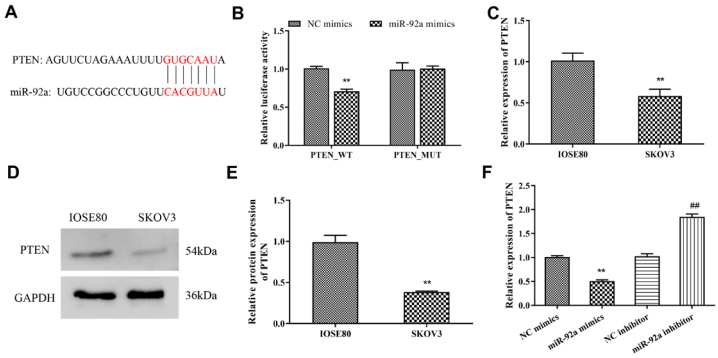

Figure 3. MiR-92a targets PTEN.
(**A**) TargetScan to predict the targeting sequences between miR-92a and PTEN; (**B**) dual-luciferase reporter assay to validate the targeting relationship between miR-92a and PTEN; C, qRT-PCR to detect the PTEN expression level in IOSE80 and SKOV3 cells, ***P*<0.01 *vs*., IOSE80; (**D/E**) Western blot to detect PTEN protein expression level in IOSE80 and SKOV3 cells, ***P*<0.01 *vs*., IOSE80; F, qRT-PCR to detect PTEN expression level in SKOV3-inhibitor cells after knockdown or overexpression of miR-92a, ***P*<0.01 *vs*., NC mimics, ##*P*<0.01 *vs*., NC inhibitor.

of miR-92a in OC, we induced SKOV3-Tax, a cell line with apparent resistance to paclitaxel, by concentration gradient (Fig. 1B). Then miR-92a was knocked down and overexpressed respectively in SKOV3-Tax cells by transfection, and the efficiencies of knockdown and overexpression were shown in Fig. 1C. Compared with NC mimics, miR-92a mimics remarkably increased miR-92a expression in SKOV3-Tax cells; compared with NC inhibitor, miR-92a inhibitor markedly inhibited miR-92a expression in SKOV3-Tax cells (*P*<0.01). Meanwhile, the results of MTT and colony formation assays revealed a noticeable climb in proliferation, colony formation and cell activity under different concentrations of Tax treatment of SKOV3-Tax cells in the miR-92a mimics group, compared with the NC mimics group; while in the miR-92a inhibitor group, the above items exhibited an evident reduction compared with the NC inhibitor group (Fig. 1D/E). These results suggested that miR-92a promoted SKOV3-Tax cell proliferation, colony formation as well as resistance to Tax.

MiR-92a inhibits apoptosis of SKOV3-Tax cells

Subsequently, the effect of miR-92a on the apoptosis of SKOV3-Tax cells was further explored. As results shown, compared with the NC mimics group, the apoptosis rate of SKOV3-Tax cells in the miR-92a mimics group was greatly reduced, and the expression level of pro-apoptotic gene Bax in the cells was considerably lowered, while the level of anti-apoptotic gene Bcl-2 was evidently increased (*P*<0.01). By contrast, the opposite results were shown after inhibiting miR-92a in SKOV3-Tax cells by miR-92a inhibitor: The apoptosis level of the cells and the Bax expression level were significantly increased, whereas the Bcl-2 expression was markedly decreased (*P*<0.01) (Fig. 2A–D), indicating that overexpression of miR-92a inhibited apoptosis of SKOV3-Tax cells, while knockdown of miR-92a enhanced apoptosis of SKOV3-Tax cells.

MiR-92a targets PTEN

MicroRNAs mediate the expression of target genes mainly by directly targeting the 3 -untranslated region (3-UTR) of mRNAs, thereby regulating the behavior of a variety of malignancies (Shi *et al*., 2021). And we predicted the downstream target gene of miR-92a on the online website TargetScan (https://www.targetscan.org/vert_72/). The outcomes reported that miR-92a was able to target and regulate PTEN and their targeting sequences were displayed in Fig. 3A. The results of subsequent dual-luciferase reporter assay also manifested that co-transfection of miR-92a mimics notably suppressed the luciferase activity of cells in the PTEN-WT group, but the luciferase activity of the PTEN-MUT group was not obviously changed (Fig. 3B). And the above confirmed that there was a targeting relationship between PTEN and miR-92a. The mRNA and protein expression level of PTEN in SKOV3 cells was significantly lower than those in IOSE80 cells (*P*<0.01, Fig. 3C–E). In addition, the PTEN expression in the cells was remarkably declined after the overexpression of miR-92a in SKOV3-Tax cells, while the opposite was true after the knockdown of miR-92a (Fig. 3F). From the above, it was suggested that miR-92a could target and regulate PTEN expression.

MiR-92a regulates PTEN/Akt signaling pathway in SKOV3-Tax cells

Several studies have demonstrated that through the PTEN/Akt signaling pathway, miR-92a promotes the malignant behavior of tumors such as prostate cancer, nasopharyngeal carcinoma as well as osteosarcoma (Yanshen *et al*., 2021; Zhang *et al*., 2016; Xiao *et al*., 2017). To clarify miR-92a function in OC to promote proliferation, inhibit apoptosis and improve chemoresistance to Tax whether, through the PTEN/Akt signaling pathway, we analyzed the expression of PTEN/Akt signaling pathway by Western blot. And a noticeable decrease was revealed by the results in the PTEN expression level (*P*<0.01), while a marked climb in both the phosphorylation level of Akt and the ratio of p-Akt/Akt (*P*<0.01) in SKOV3-Tax cells in the miR-92a mimics group when compared with the NC mimics group. Besides, in SKOV3-Tax cells in the miR-92a inhibitor group, the PTEN expression level remarkably rose (*P*<0.01), while the phosphorylation level of Akt and the ratio of p-Akt/Akt notably reduced (*P*<0.01) compared with the NC inhibitor group (*P*<0.01) (Fig. 4A/B). All these indicated that miR-92a was able to regulate the PTEN/Akt signaling pathway in SKOV3-Tax cells.

DISCUSSION

Despite the progress at the social medical level, the incidence of malignant tumors is still growing year by year,

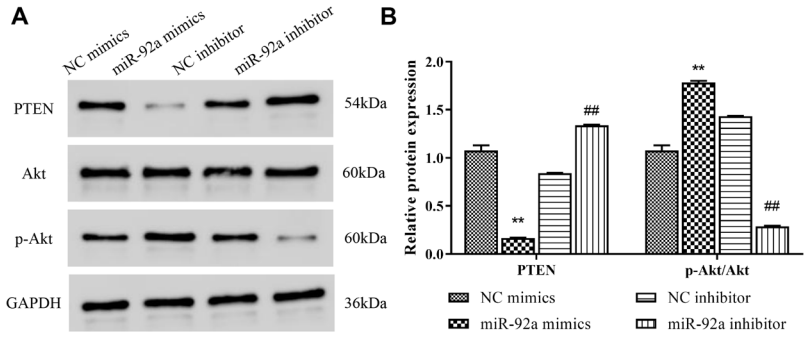

Figure 4. MiR-92a regulates PTEN/Akt signaling pathway in SKOV3-Tax cells.
(**A/B**) Western blot to detect the protein level of PTEN, p-Akt and Akt in SKOV3-Tax cells of each group and grayscale analysis was performed for the relative expression level of PTEN and p-Akt/Akt, $^{**}P<0.01$ *vs.*, NC mimics, $^{\#\#}P<0.01$ *vs.*, NC inhibitor.

and the age of onset of malignant tumors is increasingly younger. Therefore, how to improve the overall health of mankind is a concern and attention in the world today (Vasan *et al.*, 2019). As an important component of cell signaling pathways, miRNAs have been suggested by increasing evidence that are major regulators of many life activities, such as cell proliferation, differentiation, apoptosis, stress response, and angiogenesis. These activities function by binding the 3'UTR region of multiple target genes (Ji *et al.*, 2017). It has been revealed that miRNAs can act as suppressor genes and oncogenes to regulate the development of tumors (Schwarzenbach *et al.*, 2014). In addition, several researchers have reported that miRNAs also play a crucial role in the migration, differentiation, apoptosis, and other processes of OC (Li *et al.*, 2019; Ye *et al.*, 2019). MiR-92a, a novel miRNA discovered in recent years, is highly expressed and exerts a cancer-promoting effect in a variety of tumors (Zhou *et al.*, 2015; Lin *et al.*, 2013; Ohyagi-Hara *et al.*, 2013). In this study, miR-92a was remarkably highly expressed in OC cells *via* a series of in vitro cell experiments. Furthermore, high miR-92a expression promoted OC cell proliferation, colony formation, and paclitaxel resistance, while inhibiting apoptosis.

Peptidyl-prolyl cis/trans isomerase (NIMA-interacting 1, PTEN) is a tumor suppressor gene with bispecific phosphohydrolase function and a high mutation rate in various human cancers (Li *et al.*, 1997). The PTEN gene is located on the short arm of chromosome 10 (10q23.3), with a complex encoded protein structure and a variety of physiological functions. PTEN gene can act not only on the nucleus to regulate the cell cycle, but also on the cell membrane to participate in cell-cell interaction and adhesion functions (Leslie den Hertog, 2014). Studies have manifested that PTEN presents low expression in some human tumors (Yeh Means, 2007). For example, PTEN was down-regulated in various tumors such as lung cancer (Malaney *et al.*, 2018), liver cancer (Liu *et al.*, 2018) and breast cancer (Ngeow *et al.*, 2017). Loss of PTEN function has been demonstrated to stimulate cell growth and survival by excessively activating the PI3K/AKT signaling pathway (Salmena *et al.*, 2008; Hollander *et al.*, 2011; Xie *et al.*, 2021). In this study, PTEN was found to be a downstream target gene of miR-92a through a miRNA-mRNA interaction database. Additionally, luciferase reporter assay and expression analysis revealed that miR-92a could target and regulate the PTEN expression, and down-regulation of miR-92a expression greatly promoted the PTEN expression. More importantly, we discovered that overexpression of miR-92a notably increased the p-Akt level and the ratio of p-Akt/Akt in OC cells; however, inhibition of miR-92a expression considerably lowered the p-Akt level and the ratio of p-Akt/Akt in OC cells. The above is consistent with the results of previous studies that miR-92a promotes the malignant behavior of tumors such as prostate cancer, nasopharyngeal carcinoma and osteosarcoma through the PTEN/Akt signaling pathway (Zhang *et al.*, 2016; Xiao *et al.*, 2017; Yanshen *et al.*, 2021).

The present study experimentally demonstrated that miR-93a promoted the proliferation, colony formation and paclitaxel resistance of OC cells, and inhibited apoptosis. However, only one OC cell line was tested in this study without mutual confirmation of multiple cell lines. Moreover, this study was an experimental exploration only in cells and did not validate whether miR-92a exerts the same function *in vivo*. Besides, only PTEN/Akt signaling pathway has been investigated in mechanism exploration, and it is not certain whether miR-92a also acts through other pathways. The above issues need to be further experimentally validated and explored.

CONCLUSIONS

In summary, miR-92a was up-regulated in OC cell line SKOV3, and high miR-92a expression not only promoted the proliferation, colony formation and resistance to paclitaxel chemotherapy of SKOV3-Tax cells, but also inhibited apoptosis. Additionally, miR-92a may play a cancer-promoting role by regulating the PTEN/Akt signaling pathway. And miR-92a/PTEN/Akt signaling is possibly a potential therapeutic target for OC patients in the future.

Declarations

Disclosure. No author has any potential conflict of interest.

Data availability statement. The data that support the findings of this study are available from the authors upon reasonable request.

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