MicroRNA-1284 Inhibits Cell Viability and Induces Apoptosis of Ovarian Cancer Cell Line OVCAR3

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Ovarian cancer is a malignancy with high mortality among women. Multiple reports show that microRNAs (miRs) act as regulators in ovarian cancer inhibition, while the role of miR-1284 in ovarian cancer is still unknown. This study aimed to investigate the effects of miR-1284 on ovarian cancer cells. Human ovarian cancer cell line OVCAR3 was cultured and transfected with miR-1284 mimics, inhibitors, or control. Viability and apoptosis of transfected cells were then determined by MTT assay, BrdU assay, and flow cytometry. Expression changes of p27, p21, and PI3K/Akt pathway-related proteins were measured by Western blot. Results showed that miR-1284 overexpression suppressed cell viability while increasing the apoptosis in OVCAR3 cells. Moreover, the expression level of p27 was upregulated by miR-1284 overexpression. Furthermore, miR-1284 overexpression and Akt inhibitor GSK690693 downregulated the levels of p-Akt and Bcl-2 while upregulating the levels of Bax and caspase 3. However, miR-1284 suppression attenuated the regulatory effects of GSK690693 on these proteins. In conclusion, miR-1284 could inhibit cell viability via regulating the expression of p27 and induce apoptosis via regulating the PI3K/Akt pathway in OVCAR3 cells.

Key words: MicroRNA-1284; Ovarian cancer; Cell viability; Apoptosis; PI3K/Akt pathway; p27

INTRODUCTION

Ovarian cancer is a gynecological malignancy that forms in the ovary. Among women, it is the seventh most common cancer and the eighth most common cause of death from cancer (1,2). The exact cause of ovarian cancer is still unclear, but most of the risk is related to ovulation, obesity, and hormones (3,4). In addition, a personal family history of ovarian cancer can increase the risk of developing it, as well as link to mutations in high-penetrance genes, such as early onset breast cancer 1/2 (BRCA1/BRCA2) (5). Treatment usually includes some combination of surgery, radiation therapy, and chemotherapy (6). Although tremendous advances have been made recently in the treatment of ovarian cancer, the overall 5-year survival rates for women with ovarian cancer at an advanced stage is only 30% (7). Thus, there clearly remains a need to better understand the molecular pathogenesis so that new gene targets for treating ovarian cancer can be investigated.

MicroRNAs (miRs) are small noncoding RNAs, with high conservative properties among a wide range of species (8). Currently, it is well known that miRs regulate various human cancers, and the abnormal expression of miRs is involved in oncogenic pathways (9,10). In ovarian cancer, numerous miRs are involved in malignant transformation and tumor progression (8). For instance, Yuan et al. reported that overexpression of miR-494 suppressed cellular proliferation and immigration (11). In addition, miRs can function as master coordinators, efficiently regulating and coordinating multiple cellular pathways and processes (12). For example, miR-199a regulates IKK expression to modulate the inflammatory microenvironment (13) and targets CD44 to suppress the tumorigenicity and multidrug resistance in ovarian cancer cells (14). Thus, miRs have been suggested as possible therapeutic armaments against ovarian cancer (8).

miR-1284 is in the miRs family and recently has been found to act as a diagnostic biomarker and auxiliary inhibitor in some cancers. A study by Patnaik et al. reported that the expression of miR-1284 of whole blood could be used to distinguish patients with lung adenocarcinoma (15). In addition, Cao et al. revealed that miR-1284 reversed chemoresistance of gastric cancer cells (16). However, the role of miR-1284 in ovarian cancer is still unknown. In the current study, we overexpressed and suppressed miR-1284 in OVCAR3 cells and investigated the influences of miR-1284 overexpression and suppression on cell viability and apoptosis in vitro.

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We also determined the expression changes of p27, p21, and PI3K/Akt pathway-related proteins [i.e., phosphorylated Akt (p-Akt), Akt, Bcl-2, BCL2-associated X protein (Bax), procaspase 3, and active caspase 3] in cells.

MATERIALS AND METHODS

Cell Culture and Transfection

Human ovarian cancer cell line OVCAR3 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C (17).

For transfection, cells were plated onto a 60-mm dish, and after the cells had grown to about 70% confluence, miR-1284 mimics, inhibitors, or controls (Gene Pharma, Shanghai, P.R. China) were transfected into cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 48 h of transfection, the cells were collected for further analysis. Cells were treated with the 10 nM Akt inhibitor GSK690693 (Selleck Chemicals).

MTT Analysis

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (18). In brief, the transfected cells were seeded at 2×10^3 cells per well in 96-well plates and incubated for 1, 2, 3, 4, or 5 days. Then 20 µl of 10 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) solution was added to each well and incubated for another 4 h. Formazan was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the absorbance was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm.

BrdU Assay

Cell viability was further detected by bromodeoxyuridine (BrdU) assay kit (Roche, Penzberg, Germany). The protocol used in the present analysis was recommended by the kit manufacturer. Briefly, the transfected cells were seeded at 2×10^3 cells per well in 96-well plates. Afterward, 10 µl of BrdU solution (final concentration of 10 µM) was added to each well, and the cells were incubated at 37°C for 4 h. After incubation, 200 µl of FixDenat was added to each well and incubated for another 30 min; 100 µl of anti-BrdU-POD working solution was added to each well and reincubated for 1.5 h at room temperature. Finally, 100 µl of substrate solution was added to each well to develop color, and immune complexes were measured by photometric detection at 690 nm.

Apoptosis Assay

Cell apoptosis was detected by Annexin-V-FITC/PI Kit (Dojindo Molecular Technologies, Kyushu, Japan), according to the manuals. Briefly, the transfected cells were harvested by trypsinization and resuspended in 200 µl of binding buffer containing 10 µl of annexin V-FITC and 5 µl of PI. The samples were incubated in the dark for 15 min and then analyzed with flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA) (19). The percentage of apoptosis cells was calculated by counting cells directly.

Western Blot

Protein levels in cells were measured using Western blot assay. The transfected cells were lysed in Triton lysis buffer (Solarbio, Beijing, P.R. China) for 30 min on ice, and the protein concentration was determined by Bicinchoninic Acid (BCA) Kit (Solarbio). The protein was resolved over 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (20). The membrane was blocked in blocking buffer (5% nonfat milk) for 2 h at room temperature, and then incubated with primary antibodies against p-Akt (1:1,000), Akt (1:1,000), Bcl-2 (1:1,000), Bax (1:1,000), p27 (1:1,000), p21 (1:1,000), procaspase 3 (1:1,000), cleaved caspase 3 (1:1,000), and actin (1:2,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. A secondary antibody (1:2,000) (Cell Signaling Technology, Danvers, MA, USA) was used for 1 h at room temperature. The bands were detected by chemiluminescence and autoradiography using an X-ray film (Applygen, Beijing, P.R. China), and densitometric measurements were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All experiments were conducted in triplicate, and all data are expressed as mean ± standard derivation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) in GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant with a value of $p < 0.05$.

RESULTS

Effects of miR-1284 on OVCAR3 Cell Viability

The miR-1284 mimics and miR-1284 inhibitors were used to respectively overexpress and inhibit miR-1284 in OVCAR3 cells, and the effects of the altered miR-1284 expression on ovarian cancer cell viability were measured by MTT assay. As shown in Figure 1, miR-1284 overexpression significantly inhibited the proliferation of OVCAR3 cells at 3 ($p < 0.01$), 4 ($p < 0.001$), and 5 ($p < 0.001$) days. However, miR-1284 suppression showed the opposite results; cell proliferation was
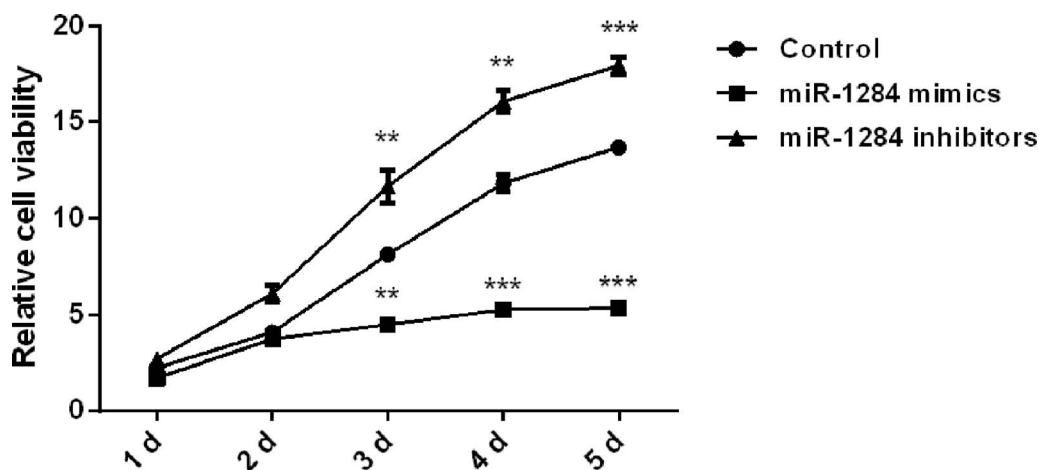
Figure 1. Effect of miR-1284 on OVCAR3 cell viability. miR-1284 mimics, inhibitors, or controls were transfected into OVCAR3 cells, and after being cultured for 1–5 days, the viability of transfected cells was measured by MTT assay. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. $**p<0.01$; $***p<0.001$.

inhibited at the same three time points ($p<0.01$, $p<0.01$, and $p<0.001$) when compared with the control group. These results reveal that overexpression of miR-1284 led to suppressed OVCAR3 cell viability and that inhibition of miR-1284 could promote cell viability.

The Effects of miR-1284 on Cell Viability Involved in the Expression of p27

In order to further confirm the inhibitive effects of miR-1284 on ovarian cancer cells, a BrdU assay was performed

to detect cell viability, and Western blot was used to measure protein expression of p27 and p21 in OVCAR3 cells. As shown in Figure 2A, cell proliferation was significantly reduced by miR-1284 overexpression ($p < 0.001$) and was significantly increased by miR-1284 suppression ($p < 0.05$) when compared with the control group. These results were consistent with the results shown in Figure 1. The results in Figure 2B showed that the protein level of p27 was remarkably upregulated by miR-1284 overexpression and downregulated by miR-1284 inhibition.
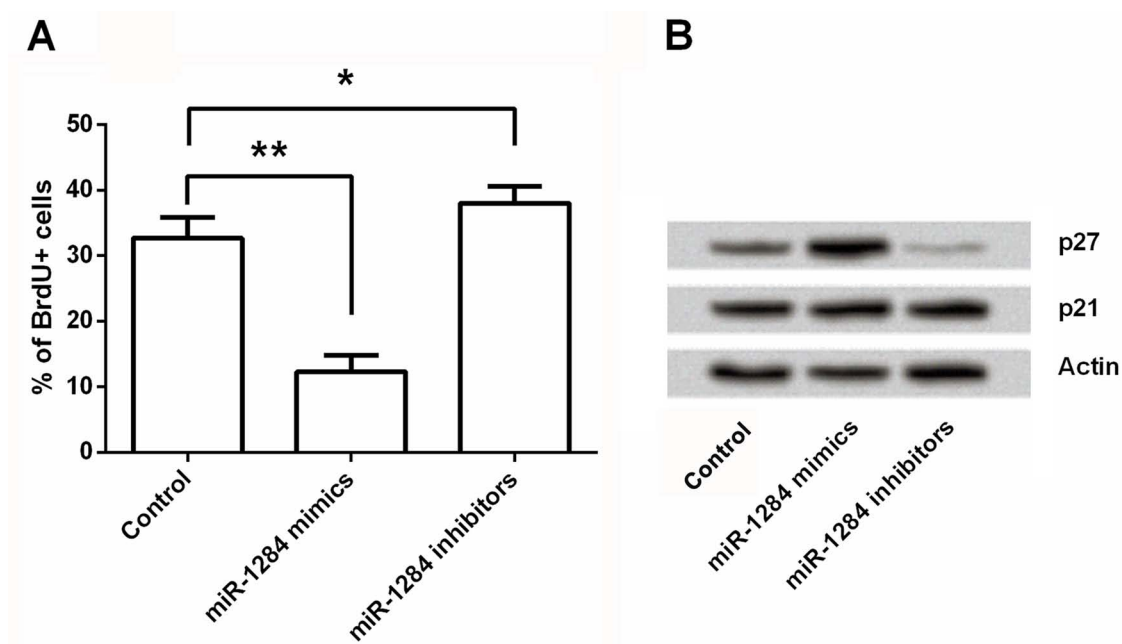

Figure 2. Effect of miR-1284 on cell viability involving the expression of p27. miR-1284 mimics, inhibitors, or controls were transfected into OVCAR3 cells. Afterward, cell viability was measured by BrdU assay (A), and expression changes of p27 and p21 were detected by Western blot (B). Actin acted as an internal control. BrdU[+], bromodeoxyuridine positive. $*p<0.05$; $**p<0.01$.
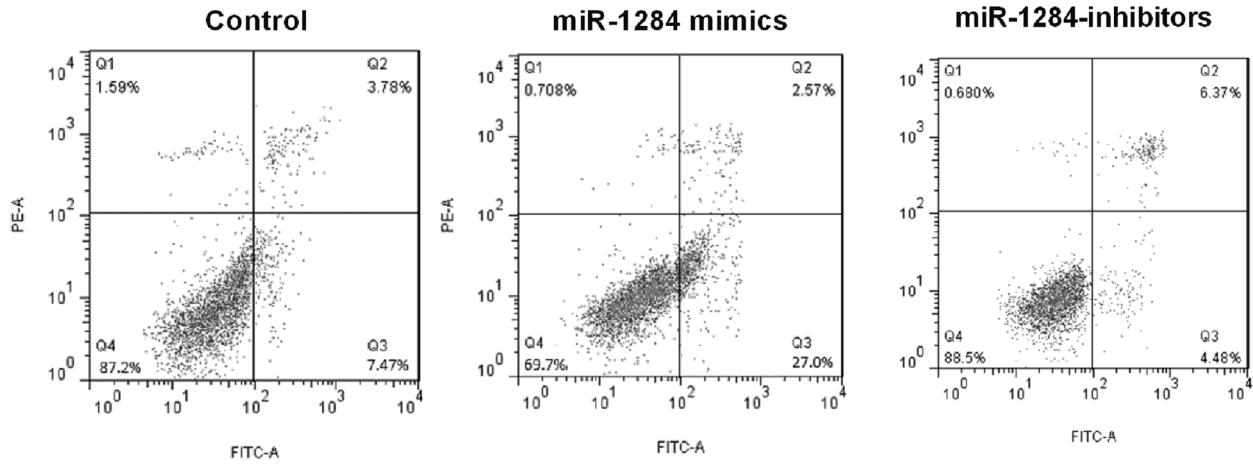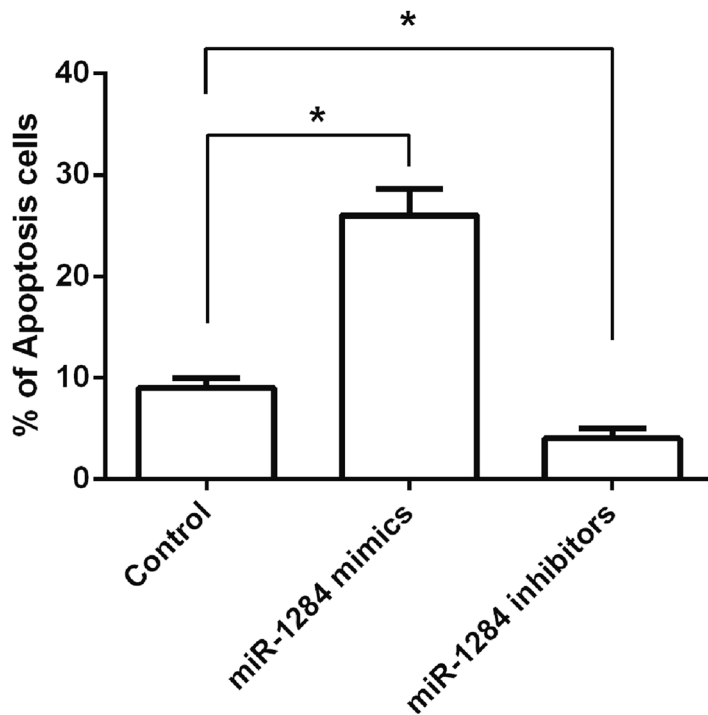
A

B

Figure 3. Effect of miR-1284 on OVCAR3 cell apoptosis. (A, B) miR-1284 mimics, inhibitors, or controls were transfected into OVCAR3 cells, and cell apoptosis was determined by flow cytometry. $*p < 0.05$.

However, the expression of p21 was not significantly regulated by miR-1284 overexpression or miR-1284 inhibition. Therefore, miR-1284 overexpression might reduce OVCAR3 cell proliferation via regulating the expression of p27, but not p21.

Effects of miR-1284 on OVCAR3 Cell Apoptosis

After OVCAR3 cells were transfected with miR-1284 mimics, inhibitors, or control, flow cytometry was used to detect the apoptotic cells, aimed to reveal the effects of miR-1284 on ovarian cancer cell apoptosis.
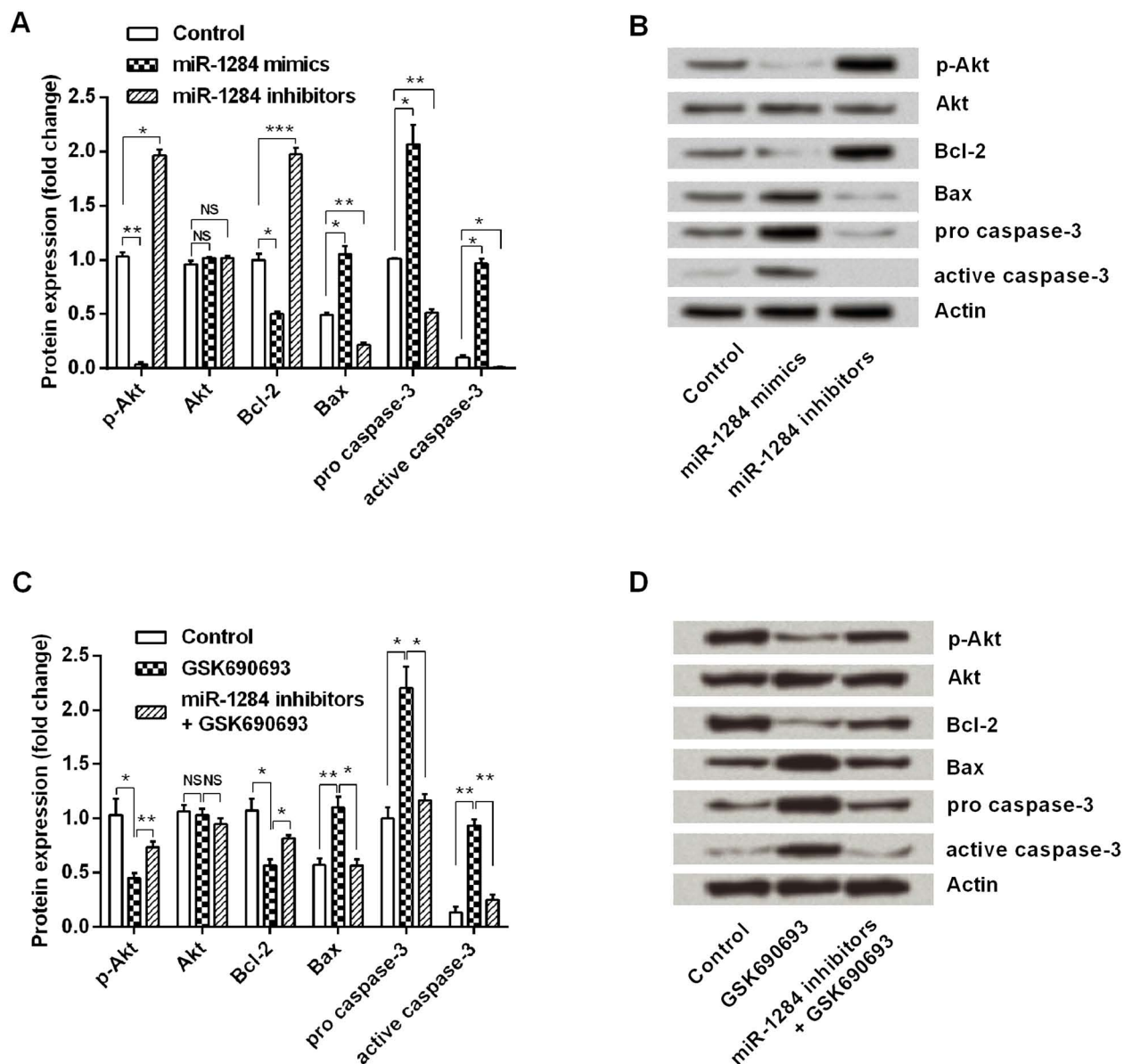
Figure 4. Effect of miR-1284 on cell apoptosis involved in the PI3K/Akt pathway. miR-1284 mimics, inhibitors, or controls were transfected into OVCAR3 cells, and then expression changes of p-Akt, Akt, Bcl-2, Bax, procaspase 3, and active caspase 3 in cells were detected by Western blot (A and B). OVCAR3 cells were treated with 10 nM Akt inhibitor GSK690693 and/or transfected with miR-1284, then the expressions of those six proteins were measured again (C and D). Actin acted as an internal control. PI3K/Akt, phosphoinositide 3-kinase/Akt; p-Akt, phosphorylated Akt; Bax, BCL2-associated X protein. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, no significance.

Results in Figure 3A and B showed that cell apoptosis was significantly increased by miR-1284 overexpression ($p < 0.05$), while cell apoptosis was significantly decreased by miR-1284 suppression. These results indicated that miR-1284 overexpression could induce OVCAR3 cell apoptosis, while miR-1284 suppression could inhibit apoptosis.

The Effects of miR-1284 on Cell Apoptosis Involved in the PI3K/Akt Pathway

To investigate the possible molecular mechanisms of miR-1284 in ovarian cancer cells, the expression levels of p-Akt, Akt, Bcl-2, Bax, procaspase 3, and active caspase 3 in the transfected cells were detected by Western blot. Results in Figure 4A and B showed that miR-1284

overexpression significantly downregulated the expression of p-Akt ($p<0.01$) and Bcl-2 ($p<0.05$) and significantly upregulated the expression of Bax ($p<0.01$), procaspase 3 ($p<0.01$), and active caspase 3 ($p<0.05$) when compared with the control group. However, the expression changes of these proteins in miR-1284 suppression cells were absolutely in contrast with miR-1284 overexpression. These results suggested that the PI3K/Akt pathway was constitutively downregulated by miR-1284 overexpression.

To examine whether the PI3K/Akt pathway was involved in the effects of miR-1284 on ovarian cancer cells, OVCAR3 cells were treated with an Akt inhibitor GSK690693 and/or transfected with miR-1284 inhibitors and then the expression changes of these factors were measured again. As shown in Figure 4C and D, GSK690693 significantly downregulated the expressions of p-Akt ($p<0.05$) and Bcl-2 ($p<0.05$) while significantly upregulating Bax ($p<0.01$), procaspase 3 ($p<0.05$), and active caspase 3 ($p<0.01$). However, miR-1284 suppression significantly attenuated the regulative effects of GSK690693 on these protein expressions ($p<0.05$ or $p<0.01$). Collectively, the PI3K/Akt pathway might contribute to the apoptosis of ovarian cancer cells.

DISCUSSION

Ovarian cancer is a gynecological malignancy with high mortality among women. Recently, an increasing number of studies reveal that miRs can inhibit the progress of ovarian cancer. However, the effects of miR-1284 on ovarian cancer are still unknown. In this study, miR-1284 was overexpressed or suppressed by transfection with miR-1284 mimics or miR-1284 inhibitors in OVCAR3 cells. We found that miR-1284 overexpression reduced cell viability and induced apoptosis. In addition, we discovered that the expression level of p27 was upregulated by miR-1284 overexpression, whereas it was downregulated by miR-1284 suppression. Furthermore, the PI3K/Akt pathway was aberrant in miR-1284 overexpressed or suppressed cells. An Akt inhibitor, GSK690693, remarkably suppressed Akt activation, while miR-1284 suppression attenuated this suppressive effect.

It is well known that cell growth and the proliferation of cancer cells are mediated via cell cycle progression (21), and many inhibitive effects of miRs on ovarian cancer cell viability are associated with cell cycle. Jin and Lian (22) demonstrated that miR-23a inhibited ovarian cancer cell proliferation by arresting more cells in the G_0/G_1 phase, and Xia et al. reported that miR-211 suppressed ovarian cancer proliferation by regulating cyclin D1 and cyclin-dependent kinase 6 (CDK6) (23). In cell cycle progression, p27 and p21 are two important members of cyclin-dependent kinase inhibitors (CDKIs). They prevent cell cycle progression from the G_1 to the S phase (24). In the current study, the expression level of p27

was upregulated by miR-1284 overexpression, while the expression of p21 was unaffected. Thus, miR-1284 overexpression could regulate p27, but not p21, and arrest cells in the G_0/G_1 phase and then inhibit ovarian cancer cell viability.

Another promising therapeutic strategy for treating cancer is manipulating apoptosis by modulation (25,26). Increasing evidence shows that the PI3K/Akt pathway is one of the most central molecular signaling pathways implicated in ovarian cancer cell apoptosis (27,28). Akt is a central protein in the PI3K/Akt pathway, and p-Akt could inhibit apoptosis by regulating its downstream targets, such as Bcl-2 family proteins (29). Moreover, Datta et al. reported that p-Akt enabled antiapoptotic Bcl-2 to bind to proapoptotic proteins Bax and BCL2 antagonist/killer (Bak) and then suppress apoptosis (30). In addition, PI3K/Akt can activate many other proapoptotic proteins, such as caspase 3 (31). Caspase 3 also plays a dominant role in apoptosis, and its active form (active caspase 3) initiates apoptosis by extrinsic and intrinsic pathways (32). A study in rat hepatocytes suggested that cell apoptosis was promoted by regulating Bax/Bcl-2 ratio, caspase 3 expression, and the PI3K/Akt pathway (31).

In the current study, miR-1284 overexpression and Akt inhibitor GSK690693 downregulated the levels of p-Akt and Bcl-2 while upregulating the levels of Bax and caspase 3. However, miR-1284 suppression attenuated the regulatory effects of GSK690693 on these proteins. Thus, we inferred that miR-1284 overexpression might induce cell apoptosis via regulating the PI3K/Akt pathway. Similarly, Zi et al. (33) reported that danusertib induced apoptosis involving the PI3K/Akt signaling pathway in human ovarian cancer cells. Our study provided the first in vitro evidence that miR-1284 could induce apoptosis through the PI3K/Akt pathway.

To sum up, this study revealed that overexpressed miR-1284 could inhibit cell viability and promote apoptosis in ovarian cancer cells via regulating p27 expression and the PI3K/Akt signaling pathway. miR-1284 might be a potential therapeutic target for ovarian cancer treatment. However, deep studies are still needed in order to investigate the regulatory mechanism of miR-1284 in ovarian cancer.

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