MiR-124 inhibits invasion and induces apoptosis of ovarian cancer cells by targeting programmed cell death 6

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Abstract. Epithelial ovarian cancer remains the most common type of malignant tumor of the female reproductive system worldwide. Routine surgery and chemotherapy are the best treatments available for patients with ovarian cancer; however, almost 40% of ovarian cancer cases are intractable, with poor 5-year survival rates. MicroRNAs (miRNA) are endogenous small non-coding RNA molecules that function in transcriptional and post-transcriptional regulation of gene expression in various cellular processes. Recent studies demonstrated that microRNA (miR)-124 was downregulated in numerous types of tumors; however, the function and mechanism underlying miR-124 in epithelial ovarian cancer remain unclear. The present study revealed that miR-124 may be significantly downregulated in epithelial ovarian cancer. Using prediction algorithms and luciferase reporter gene assays, the present study identified and confirmed programmed cell death 6 (PDCD6) as a novel, direct target of miR-124. Overexpression of miR-124 suppressed PDCD6 expression, inhibited cell proliferation, migration and invasion, and induced apoptosis in SKOV3 and OCVAR3 cells in vitro. In the present study, overexpression of PDCD6 in epithelial ovarian cancer cells co-transfected with miR-124 effectively reversed the miR-124-induced apoptosis. Therefore, the results of the present study suggested that miR-124 is a tumor suppressor miRNA and a potential target for future treatment of ovarian malignant neoplasms.

Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs molecules that trigger transcriptional or post-transcriptional regulation by binding to the 3'-untranslated region (UTR) of target genes (1-3). In recent studies, miRNAs were revealed to function as either oncogenes or tumor suppressor genes in biological processes, including cell proliferation, migration, apoptosis and differentiation and in tumorigenesis of a number of types of human cancer (3-7). In females worldwide, ovarian cancer is a common gynecological malignancy and remains a major problem, with high mortality due to diagnosis at an advanced stage and distant metastases (8,9). Various miRNAs are post-transcriptionally deregulated in ovarian cancer (10-13). Although evidence has supported miRNAs as a potential target in cancer diagnosis, therapy and prognosis, the role of miRNAs in ovarian cancer remains unclear. miR-124, a tumor suppressor miRNA, is significantly downregulated in a number of human malignant tumors (14-17), including hepatocellular carcinoma and gastric carcinoma; however, the possible effect of miR-124 in ovarian cancer has not been fully explored.

Programmed cell death 6 (PDCD6) is a calcium-binding protein that regulates cell proliferation and death and belongs to the penta-EF-hand protein family (18,19). Previous studies have indicated that PDCD6 is critical in T-cell receptor-, Fas- and glucocorticoid-induced programmed cell death (19,20). Furthermore, PDCD6 was demonstrated to be a pro-apoptotic protein involved in cell viability functions (19,21); however, its role in regulating migration and invasion in ovarian cancer is unclear.

The present study aimed to determine whether miR-124 and PDCD6 are involved in the apoptosis, migration and invasion of ovarian cancer. It was revealed that miR-124 was downregulated in ovarian cancer cell lines and revealed an association between miR-124 expression level and ovarian cancer cell metastasis. The present study further identified PDCD6 as a novel, direct target of miR-124 that suppressed apoptosis in ovarian cancer progression, and miR-124 rescue experiments restored PDCD6 expression level. The results of the present study demonstrated that miR-124 suppressed cell motility, migration and invasion and induced cell apoptosis by downregulating PDCD6. Thus, miR-124 may provide a novel target for effective treatment of ovarian cancer.

Materials and methods

Patients and tissue samples. Human ovarian tumor tissues were collected from the 30 patients who had ovarian epithelial carcinoma and non-neoplastic ovarian tissues from 30 healthy...
individuals under 60 years of age who received surgical resection between January 2009 and December 2012 at the Department of Gynecologic Oncology at Chongqing Cancer Hospital (Chongqing Cancer Institute, Chongqing, China). The present study was approved by the Ethics Committee of the Chongqing Cancer Hospital (Chongqing Cancer Institute, Chongqing, China). Written informed consent was provided by all patients prior to enrollment in the present study.

Cell lines and cell culture. SKOV3 and OVCAR3 human ovarian cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Transient transfection of miR-124. The miR-124 mimics and the miR-124 mimics scramble were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). The sequences (5'-3') were as follows: hsa-miR-124-5p mimics forward, 5'-CGUGUU CACACGGGACCUUGAU-3' and reverse, 5'-AUCAAGGUC CGCGUGUAAACG-3'; miR-124 mimics scramble forward, 5'-UUUCUCCAGUUGACGAGU-3' and reverse, 5'-ACG UGACCGUUCGGAGAG-3'. The pcDNA3.1/pCD6 and pcDNA3.1/control were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were harvested and plated in six-well plates at a density of 5x10⁴ cells/ml. All cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. In the rescue experiment, cells were co-transfected with miR-124 mimics and plasmids.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) from clinical tumor samples and transfected cells according to the manufacturer's instructions. RNA concentration and quality were evaluated, and 0.5 μg RNA was used for RT. MMLV reverse transcriptase (Takara Bio., Inc., Otsu, Japan) was used to synthesize complementary DNA (cDNA). For synthesis of miR-124 cDNA, a miR-124 RT primer was used and U6 small nuclear RNA was used as the internal control. cDNA for other target genes was amplified using the primer oligo(dT) with β-tubulin as the internal control. Target genes and controls were analyzed by RT-qPCR using SYBR Premix Ex Taq (Promega Corporation, Madison, WI, USA). The following primers were used for qPCR: miR-124 forward, 5'-TGGCGTGAAGGCGACCGGTG-3' and reverse, 5'-CCA GTGCCAGGTCGCGGTG-3'; U6 forward, 5'-TGGCGGTGTC TGCGTTCCCGACG-3' and reverse, 5'-CAGTCGAGGCT CCGAGGTG-3'; PDCD6 forward, 5'-TCCAGAGGTCGATA AAGACA-3' and reverse, 5'-TCTGCCAGCTCCGTGATG T-3'; β-tubulin forward, 5'-TGGCCAGATCTTTAGACCCAC AACA-3' and reverse, 5'-CGCGGCTACAGGACAGCAGA T-3'. PCR was conducted at 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec in an iQ5 Real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the samples were assessed by relative quantification (2^ΔΔCq method) (22).

Target prediction. The prediction of the PDCD6 3'-UTR as a miR-124 binding target was determined using TargetScan (www.targetscan.org) and PicTar (www.pictar.org).

Dual-luciferase reporter assay. A luciferase reporter assay was used to monitor the direct interaction between the miRNA and its target mRNA. The binding site of miR-124 in the 3'-UTR of the target mRNA was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation), according to the manufacturer's instructions. Cells were subsequently co-transfected with miRNA and the wild type or mutant 3'-UTR for the luciferase assay. Following transfection for 48 h at room temperature, the cells were harvested for protein extraction. Luciferase intensity was examined using the Dual-Luciferase Reporter Gene Assay kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. Renilla luciferase intensity was used as an internal control.

Cell migration and invasion assays. Cell migration was evaluated using a wound-healing assay. At 24 h after transfection, a wound was created by 20 μl pipette tips in all culture wells. Cultures were imaged at 0, 24 and 48 h to assess cell migration. Light microscopy was undertaken at x100 magnification at 0, 24 and 48 h to assess cell migration.

The present study used a Transwell assay with a Matrigel-coated membrane matrix (BD Biosciences, Franklin Lakes, NJ, USA) to assess cell invasion. After transfection, cells (5x10⁴) were seeded in the upper chamber of a Millicell Transwell chamber (EMD Millipore, Billerica, MA, USA) with 200 μl RPMI-1640 medium without FBS. Cells that migrated through the membrane were fixed with 4% methanol for 10 min at room temperature, and stained with 0.1% crystal violet for 15 min at room temperature. Cells were then imaged at x100 magnification and counted using an inverted microscope.

Cell survival assay. SKOV3 and OVCAR3 cells were plated onto 96-well plates 48 h after transfection at a density of 5x10⁴ cells/ml. A total of three replicate wells were examined for each group. When cells reached 60-70% confluency, 10 μl 5 mg/ml MTT was added to each well. After 4 h, the culture medium was discarded, 100 μl dimethyl sulfoxide was added to each well and the plates were agitated at room temperature for 1 h. Absorbance (optical density) was evaluated using a microplate reader at a wavelength of 490 nm. This experiment was repeated three times.

Western blot analysis. At the indicated time, SKOV3 and OVCAR3 whole-cell lysates were extracted using radioimmunoprecipitation assay buffer and incubated for 30 min on ice. Cell lysates were prepared from cells seeded on 6-well plates at 10⁵ cells per well using radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1 mM MgCl₂) containing protease inhibitors at 48 h post-transfection.

Proteins were quantified using the BCA method, according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Proteins (10 μg) were separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes.
(EMD Millipore). The membranes were blocked in 5% non-fat milk/TBST for 1 h at room temperature. Thereafter, they were incubated with rabbit monoclonal anti-PDCD6 antibody (1:1,000; ab133326) and anti-β-tubulin antibody (1:1,000; ab6046) (Abcam, Cambridge, UK) overnight at 4˚C. After adding peroxidase-conjugated goat anti-rabbit antibody (1:5,000; BA1055; Boster Biological Technology, Pleasanton, CA, USA) for 1 h at room temperature, the membranes were visualized by Western Lightning®-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer, Inc., Waltham, MA, USA, and X-ray film was applied to analyze the image and intensity of band.

Cell cycle and apoptosis assays. At 48 h after transfection, 1x10⁶ cells were seeded onto 75-mm dishes. To analyze the cell cycle stage, the cells were harvested by trypsin and washed twice with cold PBS, fixed in ice-cold 70% ethanol and stained with 100 μg/ml propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 1 mg/ml RNase A for 30 min at room temperature in the dark. Subsequently, the cell cycle stages were analyzed using a flow cytometer and Cell Quest software version 3.3 (BD Biosciences). To analyze apoptosis, the cells were harvested from the culture dishes by trypsinization. After washing twice with cold PBS, cells were resuspended in 200 μl binding buffer and incubated with 10 μl Annexin V-R-PE and 10 μl 7-ADD (SouthernBiotech, Birmingham, AL, USA) in the dark at 4˚C for 30 min. Thereafter, 380 μl binding buffer was added to each tube and the samples were analyzed by flow cytometry.

Statistical analysis. All data were presented as the mean ± standard deviation. Error bars represent standard error. One-way analysis of variance, followed by Fisher’s least significance test or two-tailed Student’s t-test were used to analyzed the statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-124 is downregulated in ovarian cancer. To analyze the expression level of miR-124 in ovarian cancer progression, RT-qPCR was performed using TaqMan probes. Ovarian cancer tissue and normal ovarian tissue have distinct
miR-124 suppresses the migration and invasion of ovarian cancer cell lines. To investigate the biological role of miR-124 in ovarian cancer, the present study observed its effect on the migration and invasion of the ovarian cancer SKOV3 and OCVAR3 cell lines by transient transfection with miR-124 mimics, miR-124 mimics scramble or negative control (NC). The wound-healing assay indicated that transfection with miR-124 mimics significantly inhibited migration in both cell lines relative to the scramble control or NC at 24 and 48 h (Fig. 2A and B). Furthermore, ectopic expression of miR-124 markedly decreased invasion of the cell lines compared with the scramble control or NC (Fig. 2C-F).

PDCD6 is a direct target of miR-124 in ovarian cancer. To investigate the potential targets of miR-124, the present study performed bioinformatics analysis using TargetScan and Pictar (Fig. 1E), which predicted that miR-124 targets the PDCD6 3'-UTR region. To determine whether the 3'-UTR of PDCD6 mRNA is a direct target of miR-124 in ovarian cancer cells, the wild-type (wt) full-length 3'-UTR of PDCD6 or a mutant (mt) sequence was cloned into a luciferase reporter vector (Fig. 1G). Cells were co-transfected with wt 3'-UTR or mt 3'-UTR vectors and miR-124 mimics. The results indicated that overexpression of miR-124 significantly decreased the luciferase activity of reporter genes with wt 3'-UTR compared with the controls. The luciferase activity for the mt 3'-UTR vector was not affected by transfection with miR-124 (Fig. 1F).

Expression of PDCD6 reversed the miR-124-induced suppression of cellular migration and invasion, and induction of cellular apoptosis. Transfection with miR-124 mimics or pcDNA3.1/ vector and miR-124 mimics significantly decreased ovarian cancer cell proliferation and migration compared with the controls (Fig. 3). Conversely, co-transfection with miR-124 mimics or pcDNA3.1/PDCD6 in SKOV3 or OCVAR3 cells induced recovery of cell migration and invasion. To investigate whether miR-124 affects cancer cell migration and invasion through PDCD6, the present study performed rescue experiments by upregulating PDCD6 expression in SKOV3 and OCVAR3 cells using pcDNA3.1/PDCD6. The expression level of PDCD6 mRNA was evaluated by qPCR (Fig. 4A and B). The expression level of miR-124 significantly increased subsequent to transient transfection with miR-124 mimics; however,
the expression level of PDCD6 mRNA markedly decreased, thus miR-124 downregulated PDCD6 mRNA expression level. Transient co-transfection of miR-124 mimics together and pcDNA3.1/PDCD6 rescued the expression level of PDCD6 mRNA. Similar results were obtained by western blot analysis of SKOV3 and OVCAR3 cells co-transfected with miR-124 mimics or NC and pcDNA3.1/PDCD6 or pcDNA3.1/vector (Fig. 4C). The incidence of apoptosis and cell cycle arrest following overexpression of miR-124 and overexpression of PDCD6 were confirmed by flow cytometry. The results demonstrated that transfection of miR-124 mimics markedly increased cell death (Fig. 4D) and the proportion of SKOV3 and OVCAR3 cells in the G0/G1 cell cycle phase, but decreased the proportion of cells in S phase (Fig. 4E). However, these effects were reversed by co-transfection of miR-124 mimics and pcDNA3.1/PDCD6.

**Discussion**

Currently, the majority of patients with ovarian cancer are diagnosed with distant metastases at an advanced stage, which challenges effective treatment (9,11,12,23). Therefore, more research into potential therapeutic strategies for ovarian cancer is required. Previous studies revealed that miRNAs may...
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Regulate migration and invasion, acting as tumor suppressors or oncogenes in human cancer types (16,17,24,25). Further investigation of the roles of miRNAs in cancer development is therefore important for understanding the molecular mechanisms underlying cancer metastasis. Accumulating evidence has suggested miR-124 may function as a tumor suppressor in ovarian cancer (26). The present study revealed that miR-124 was distinctly downregulated in clinical ovarian cancer tissues samples compared with non-neoplastic ovarian tissues from healthy individuals. Furthermore, miR-124 expression level was lower in ovarian cancer lines, exhibiting greater migration and invasion abilities, suggesting that it may be important in ovarian cancer carcinogenesis. Thus, miR-124 appears to be gradually downregulated during ovarian cancer progression, and its upregulation may decrease proliferation and metastasis of ovarian cancer. However, the mechanism underlying miR-124 regulation in ovarian cancer has not been fully elucidated.

PDCD6 is a calcium binding protein in the penta-EF-hand family, and is involved in T-cell receptor-, Fas- and glucocorticoid-induced apoptosis (20,27-30). PDCD6 was identified in rat liver hepatoma and human lung cancer tissues, suggesting that it may be involved in survival pathways, in addition to its pro-apoptotic function (19). Although numerous studies have investigated the association between PDCD6 expression level and disease risk, the results are controversial (19,21,31-34). A previous study indicated that PDCD6 was involved in T-cell receptor-induced apoptosis as a pro-apoptotic factor and that Alix and PDCD6 interaction with pro-caspase-8 significantly induced cell death via tumor necrosis factor receptor 1 (21). However, another study demonstrated that PDCD6 expression was upregulated in 7,371 tumor tissue samples from lung, breast or colon cancer, which indicated that PDCD6 may be involved in maintenance of cellular viability (19,35,36). Similarly, PDCD6 has been suggested to have a role in cancer cell pathology and contribute to cancer cell viability.

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In future studies, the upstream or downstream modulators of PDCD6 and the effect of miR-124 on signaling pathways of tumor progression by targeting PDCD6 will be investigated. In addition, as chemotherapy resistance is one of the main obstacles in treatment of ovarian cancer, the regulation and function of miR-124 and PDCD6 in chemotherapy resistance in ovarian cancer will continue to be investigated.

In conclusion, the present study provides evidence that the aberrant expression of miR-124 is important in cancer cell migration and invasion. Furthermore, miR-124 induced apoptosis in ovarian cancer cell by regulating PDCD6, a novel and direct target of miR-124. The upregulation of miR-124 markedly inhibited ovarian cancer cell growth, invasion and migration. These results provided a foundation for future studies exploring a new approach to suppressing cancer by targeting miR-124 and suggested that it may provide a novel therapeutic target for ovarian cancer.

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