Anti-tumor Role of MicroRNA-4782-3p in Epithelial Ovarian Cancer

Ting An¹, Jie Liu¹, Qian Yang¹, Li Xiao²*, Xibiao Jia³*

¹Department of Pediatric Surgery, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, P.R. China
²Department of Human Anatomy, Chengdu Medical College, Chengdu, 610500, P.R. China
³Key Laboratory of Birth Defects and Related Diseases of Women and Children, Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

Abstract: Ovarian cancer ranks fifth in cancer death among women. The 5-year relative survival of all stages ovarian cancer is 47%. Identification of new molecular targets is required for the development of targeted therapy. MicroRNA (miRNA) are small, highly conserved RNA molecules involved in the regulation of gene expression. miR-4782-3p has shown tumor-suppressive activities in non-small cell lung cancer. Thus, it is possible that miR-4782-3p may play a role in the development of ovarian cancer. Herein, we analyzed the levels of miR-4782-3p in ovarian cancer tissue and cell lines and tested the function of miR-4782-3p in cell proliferation and cell apoptosis. We found that miR-4782-3p plays an inhibitory role in ovarian cancer growth. The data showed that ovarian cancer tissue and cell lines showed lower levels of miR-4782-3p, which inhibited cell growth and increased cell apoptosis. Moreover, PDGFRα was identified as a direct target of miR-4782-3p. In conclusion, our data indicated that miR-4782-3p has an inhibitory impact on ovarian cancer.

Keywords: MiR-4782-3p, Ovarian cancer, Cell growth, Apoptosis

1. Introduction

Ovarian cancer (OC) contributed approximately 239,000 new cases and 152,000 deaths worldwide annually[1]. It is the seventh most commonly diagnosed cancer among women worldwide[2-6] and the tenth most common in China. Epithelial ovarian cancer (EOC), a common type of ovarian cancer, develops from the cells that cover the outer surface of the ovary. In most cancer, EOC is a type of treatable solid cancer, which respond to excision and chemotherapy drug. This disease, however, frequently persists and recurs, having the highest fatality-to-case ratio of all the gynecologic cancers[7]. The identification of new molecular targets is required for the development of targeting therapy[8].

MicroRNAs (miRNAs) are a class of small, endogenous RNAs of 21-25 nucleotides (nts) in length. MiRNAs regulate the gene expression by targeting specific mRNA for degradation to achieve translational repression[9]. Aberrant expressions of miRNA are involved in tumorigenesis of various cancers[10-16]. In OC, many miRNAs were aberrantly expressed. For example, specific miRNAs such as miR-21, miR-203, miR-205, miR-141, miR-200a, miR-200b, and miR-200c were significantly up-regulated in the cancer tissues in relative to in the normal ovarian tissues. Besides, subtypes of OC show different miRNAs profiles, the expression of miR-200a and miR-200c were enhanced in serous,
endometrioid, and clear cell, whereas the expression of miR-200b and miR-141 are increased in endometrioid and serous subtypes [17].

MiR-4782-3p is a newly discovered microRNA. The function of miR-4782-3p has been investigated in hepatocellular carcinoma [18] and non-small cell lung cancer [19]. Two studies indicated that miR-4782-3p plays a tumor-suppressive role in hepatocellular carcinoma (HCC) and non-small cell lung cancer. Thus, it is possible that miR-4782-3p also plays an inhibitory role in OC.

Platelet-derived growth factor receptor alpha (PDGFRα) is a receptor located on the surface of various cell types [20]. Significantly, the PDGFRα expression rate in malignant ovarian tumors was higher than that in normal ovarian tissues or benign ovarian tumor [21]. Furthermore, a previous study showed that PDGFRα contributes to the progression of ovarian cancer [22]. PDGFRα is a targeted gene of miR-34a [23] in gastric cancer and was targeted by miR-219-5p in colorectal cancer [24].

Here, we tested the effect of miR-4782-3p in ovarian cancer and revealed the anti-tumor function of miR-4782-3p. Our data also indicated miR-4782-3p targeted PDGFRα at least in SKOV3 cells.

2. Materials and methods

2.1. Patients and sample

Ten EOC tissue samples and adjacent normal tissue were acquired from the Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Chengdu. Ethics committee of Sichuan University assessed and approved the use of human EOC tissues in this study. Informed consent of all patients was obtained in accordance with the regulation and law in China. The pathological diagnosis was performed by the senior pathologist of West China Second University Hospital.

2.2. Cell culture and reagent

The OC cell lines, such as SKOV3 and HO8910 cells, and normal human ovarian epithelial cells were obtained from the cell bank of Sichuan University. The SKOV3 cell line was derived from ovarian adenocarcinoma, whereas the HO8910 cell line was derived from ovarian serous cystadenocarcinoma. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; cat. No. 11965092, Thermo Fisher, Waltham, MA) according to the standard protocol. Cells were maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere.

2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The levels of miR-4782-3p in human EOC tissue samples, SKOV3, and HO8910 cells were analyzed by qRT-PCR. Firstly, Trizol™ reagent (cat. No. 15596026, Thermo Fisher, Waltham, MA) was applied to isolate the total RNAs from OC tissue or SKOV3 and HO8910 cells. U6 small nuclear (sn) RNA was used as the internal control. qRT-PCR was performed using QuantStudio 3 Real-Time PCR System (Thermo Fisher, Waltham, MA). PCR was performed using 0.1 μg of cDNA and SYBR-Green PCR Master Mix (Applied Biosystems, Grand Island, NY). Furthermore, appropriate primers were applied. The Applied Biosystems StepOnePlus Real-Time PCR System was used for transcription analysis with the StepOne software version 2.1. Comparative ΔΔCT method was used for data analysis. The primers were synthesized and tested by the ShengRui Company (ShengRui, Chengdu, China) [25-28]. The primer sequences of miR-4782-3p were as follows: Forward 5'-TGCCGTGATGTCTTCCAT ATC-3'; reverse 5'-GTGCTATCCAG TGCAGGTCGAGTCAC TGATAGCAGTCTAG-3'. The primer sequences of U6 snRNA were as follows: Forward 5'-CTCGCTTCTGGAGCA TATATCT-3'; reverse 5'-ACGTTCAGAATTTGCGTG TC-3'. The primer sequences of PDGFRα were as follows: Forward 5'-CCCCACCGTGT CTTCGACA-3'; reverse 5'-CGGATGGTCA CTCTTTAGGAAG-3'. The primer sequences of GAPDH were as follows: Forward 5'-CTCGACACCCAAACTGCTTGA-3'; reverse 5'-GGCCATCCACGTCTCAGAG-3'. The relative levels of miR-4782-3p was normalized to U6 snRNA, and the relative levels of PDGFRα were normalized to GAPDH.

2.4. miR-4782-3p mimics, miR-4782-3p ASO, and PDGFRα siRNA transfection in OC cells

The miR-4782-3p levels in SKOV3 and HO8910 cells were up-regulated and down-regulated by miR-4782-3p mimics and miR-4782-3p antisense oligonucleotides (ASO), separately. The miR-4782-3p mimics and ASO were designed and constructed by Sangon Biotech (Shanghai, China). PDGFRα siRNA was constructed by Sangon Biotech (Sangon, Shanghai, China). SKOV3 and HO8910 cells were seeded at a density of 1×10^4 per well into 6-well plates and cultured overnight. Then, miR-4782-3p mimics and ASO, PDGFRα siRNA were transfected into cells using the Lipofectamine 2000 (cat. no. 11668019, Thermo Fisher, Waltham, MA) according to the standard protocol.

2.5. Cell growth

The CyQUANT MTT Cell Viability Assay (cat. no. V13154) was applied to determine the cell growth. Briefly, the MTT stock solution and sodium dodecyl sulfate-hydrochloride (SDS-HCl solution) were prepared. In this experiment, we added 10 μl of 12-mM MTT stock solution to each well. Then, the whole plates were incubated at 37°C for 4 h. Then, 100 μl of SDS-HCl solution was added to each well and mixed well with a pipet. Then, the plates were incubated at 37°C for 4 h in a humidified chamber.
At last, optical density was measured using a microplate reader (Multiskan Fc, Thermo Scientific, Waltham, MA, USA) with a 570-nm filter.29.

2.6. Annexin V staining protocol for flow cytometry

First, cell for apoptosis detection was digested and harvested. The cell apoptosis rate was analyzed using the Dead Cell Apoptosis Kit with Annexin V FITC and propidium iodide (PI) (cat. no. V13242, Thermo Fisher, Waltham, MA). Briefly, the cells were washed once in 1× phosphate-buffered saline (PBS), then once in 1× binding buffer. Next, the cells were suspended in 1× binding buffer at 1×10⁶ cells/ml, and annexin V-FITC was added. The mixture was incubated for 10-15 min at room temperature in the absence of light. Then, 2 ml of 1× binding buffer was added to the mixture, which was then centrifuged at 400 ×g for 5 min. The supernatant was discarded afterward. Then, cell pellet was resuspended in 200 μl of 1× binding buffer containing 3 μl of PI and incubated for 5 min on ice. The apoptosis rate was analyzed with a BD FACSuiteTM flow cytometer (BD Biosciences, Franklin Lakes, NJ) with a 488-nm excitation laser. The data were analyzed using BD FACSuiteTM version 1.01 (BD Biosciences). Cells in the right quadrant were chosen for apoptosis assay for quantification.

2.7. Prediction of the putative targets of miR-4782-3p

The putative target genes of miR-4782-3p were predicted by the bioinformatics method. Here, we used TargetScan (http://www.targetscan.org/7.0/) to calculate the possible mRNA targets of miRNAs by calculating the chance of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA.

2.8. Dual-luciferase reporter assays

The activity of PDGFRα was assayed by Dual-Luciferase® Reporter Assay System (cat. no. E1910, Promega, Madison, WI). Briefly, we cloned the 3'-untranslated region (3'-UTR) of PDGFRα genes and the mutated version of 3'-UTR of PDGFRα genes. The GeneArtTM Site-Directed Mutagenesis System (cat. no. A13282, Thermo Fisher, Waltham, MA) was used for the generation of the 3'-UTR of mutant PDGFRα. Then, these clones were inserted into the reporter plasmid and the control. Next, miR-4782-3p mimics were transfected to SKOV3 cells by lipofectamine 2000 according to the protocol. Twenty-four hours later, SKOV3 cells were collected for the luciferase activity measurement. Firefly luciferase activity was measured first, then Renilla luciferase assay buffer 2.0 was added to simultaneously quench firefly luciferase activity and measure Renilla luciferase activity. Then, the ratio of firefly luciferase/Renilla luciferase of each tube was calculated, and the value in control groups was arbitrarily defined as 100%.

2.9. Western blotting

SKOV3 cells were collected and washed by PBS for three times. Then, cells were transferred to cells lysis buffer (cat. no. ab152163, Abcam, Cambridge, UK). The protease inhibitor cocktail (cat. no. ab65621, Abcam, Cambridge, UK) was also added. The mixture was agitated for 30 min at 4°C. Then, the tube was centrifuged at 12,000 rpm. The supernatant was collected for protein amount determination by Pierce™ BCA Protein Assay Kit (cat. no. 23225, Thermo Fisher, Waltham, MA) according to the protocol. We of total protein from the cell lysate (30 μg) were loaded into each well of the stacking gel, and the SDS-PAGE was run for 2 h at 80 V. Upon completion of electrophoresis, the protein was transferred from the gel to the membrane. The membrane was blocked in a blocking buffer (cat. no. ab126587, Abcam, Cambridge, UK) for 1 h at room temperature. The primary antibodies used in this experiment were anti-PDGFRα (cat. no. ab5460, Abcam, Cambridge, UK) and anti-β-actin (cat. no. ab8227, Abcam, Cambridge, UK). The anti-PDGFRα and anti-β-actin antibodies were prepared in 1:1000 and 1:2000 dilutions, respectively. The membrane was incubated with the primary antibodies at 4°C overnight. Subsequently, the membrane was incubated with a secondary antibody (goat anti-rabbit IgG, 1:2000 dilution, cat. no. ab97080, Abcam, Cambridge, UK) for 1 h at room temperature. At last, the protein levels (PDGFRα and β-actin) were detected by ECL Western Blotting Substrate Kit (cat. no. ab65623, Abcam, Cambridge, UK).

2.10. Statistical analysis

Every experiments were performed at least three time. Data were presented as mean±standard deviation (SD). The means between the two groups were compared using the two-tailed t-test. Means among three groups were compared using one-way analysis of variance (ANOVA), with post-hoc Student-Newman-Keuls test. Results with p<0.05 were considered statistically significant. The statistical analyses were performed using the SPSS software (version 15.0).

3. Results

3.1. Low levels of miR-4782-3p in OC tissues samples

To test the role of miR-4782-3p in OC, we firstly determined the levels of miR-4782-3p in 10 OC tissues compared with that in adjacent normal tissue. The data showed that the expression of miR-4782-3p in tumor tissue is lower than the matched normal adjacent tissue (Figure 1). Furthermore, OC tissues showed lower miR-4782-3p mean levels.

3.2. miR-4782-3p inhibited cells growth and increased cell apoptosis rate

SKOV3, HO8910, and normal human ovarian epithelial cells were tested for the miR-4782-3p levels by
An T, et al.

qRT-PCR. SKOV3 and HO8910 cells showed a lower level of miR-4782-3p (Figure 2A). Then, the miR-4782-3p mimics were transfected into SKOV3 and HO8910 cells to alter the miR-4782-33p levels (Figure 2B). We tested the cell growth and apoptosis following the transfection of miR-4782-3p mimics (Figure 2C). The data showed that miR-4782-3p mimics inhibited cell growth. Moreover, up-regulation of miR-4782-3p due to miR-4782-3p mimics increased the cell apoptotic rate (Figure 2D and E).

3.3. Down-regulation of miR-4782-3p promoted cell growth

Next, we tested the effect of down-regulation of miR-4782-3p by miR-4782-3p ASO transfection to SKOV3 and HO8910 cell. The decrease of miR-4782-3p was confirmed by qRT-PCR (Figure 3A). We found that down-regulation of miR-4782-3p increased cell growth (Figure 3B). Next, SKOV3 and HO8910 cells were treated with cisplatin following miR-4782-3p ASO transfection. We found that cisplatin increased the cell apoptosis rate and the down-regulation of miR-4782-3p decreased the level of apoptosis induced by cisplatin (Figure 3C and D).

3.4. miR-4782-3p targeted PDGFRα

To elucidate the underlying mechanism of miR-4782-3p, the bioinformatics method was applied. Bioinformatics data showed that miR-4782-3p may bind the 3’-UTR of PDGFRα (Figure 4A). To confirm this, we cloned the 3’-UTR of PDGFRα genes and the mutated version of 3’-UTR of PDGFRα genes. Then, these clones were inserted into the reporter plasmid and the control. Luciferase assay was performed 48 h following transfection. We found that in the wild-type 3’-UTR of PDGFRα, miR-4782-3p mimics reduced the luciferase activity, indicating that miR-4782-3p mimics could bind the 3’-UTR of PDGFRα, whereas, in the mutant 3’-UTR of PDGFRα, miR-4782-3p mimics could not reduce the luciferase activity. This data indicated that PDGFRα is one of the targeted genes of miR-4782-3p (Figure 4B). Moreover, at 48 h post-transfection, PDGFRα protein levels were determined by Western blot analysis, and the results indicated that miR-4782-3p mimics could inhibit the expression of PDGFRα in SKOV3 cells (Figure 4C). Next, we tested the PDGFRα level in the normal adjacent tissues and tumor tissues and found that PDGFRα expression was increased in the latter (Figure 4D). Next, we transfected PDGFRα siRNA to SKOV3 and HO8910 cells. After 24 h, the levels of PDGFRα were tested by qRT-PCR, and we found that PDGFRα siRNA decreased the PDGFRα levels (Figure 4E). Then, the cell growth was tested by MTT assay, and we found that PDGFRα siRNA decreased the cell growth of SKOV3 and HO8910 cells (Figure 4F).

4. Discussion

In this study, we tested the effect of miR-4782-3p in ovarian cancer and discovered that miR-4782-3p showed lower levels in tumor tissues. In addition, miR-4782-3p inhibited cellular proliferation and promoted cell apoptosis. We also found that PDGFRα is a direct target of miR-4782-3p.

Previous research indicated that miR-4782-3p plays a tumor-suppressive role in liver cancer[33] and lung cancer[34]. This study confirmed the suppressive role of miR-4782-3p in epithelial ovarian cancer. Previous studies showed that miR-4782-3p inhibits tumor growth through ubiquitin-specific protease 14 (USP14). Herein, obtained results indicate that miR-4782-3p inhibited EOC growth through PDGFRα. In our opinion, miR-4782-3p played anti-tumor role, not tumor-promoting role in three types of cancer. It is possible that miR-4782-3p may show tumor inhibitory roles in other types of cancer.

PDGFR and platelet-derived growth factor (PDGF) participate in multiple normal physiological processes, such as embryogenesis, wound healing, and the development of the vascular system.[31] In addition, PDGF and PDGFR have been shown to participate in the progressive growth of ovarian cancer.[22] Interestingly, a previous study showed that PDGF-D could promote ovarian cancer invasion by up-regulating matrix metalloproteinases.[35]

More importantly, PDGFRα is an independent indicator of a poor prognosis in epithelial ovarian neoplasms.[34] PDGFRα and PDGF are an important novel potential target for ovarian cancer.[35] Our data showed that inhibition of PDGFRα through siRNA could inhibit the cells growth of ovarian cancer cells, which is consistent with a previous study.[22] Thus, our data indicated that inhibition of PDGFRα through siRNA or miR-4782-3p could both inhibit OC cell proliferation.

Interestingly, another study showed that inhibition of USP14 promotes connexin 32 internalization and

Figure 1. miR-4782-3p levels in OC tissues. miR-4782-3p levels in ten ovarian cancer tissue samples were analyzed by qRT-PCR. miR-4782-3p levels in adjacent normal tissue were arbitrarily normalized. Data are presented as mean ± SEM. Each experiment was performed at least for three times.
counteracts cisplatin cytotoxicity in human ovarian cancer cells\cite{36}. Remarkably, we found that USP14 negatively regulates lung tumorigenesis through not only apoptosis but also the autophagy pathway\cite{37}. Thus, it is possible that USP14 may be targeted by miR-4782-3p in ovarian cancer, and it is possible that miR-4782-3p could regulate autophagy. This warrants further investigation in future.

In this study, obtained results revealed that miR-4782-3p could target PDGFRα, indicating that miR-4782-3p is also a potential therapeutic target. It is known that USP14 is targeted by miR-4782-3p. Previous data showed that USP14 could promote cellular proliferation and inhibit apoptosis in ovarian cancer\cite{38}. Thus, we suspect that both PDGFRα and USP14 are targets of miR-4782-3p.

A growing number of studies revealed the importance of microRNAs in ovarian cancer and showed that differential expression profiles of miRNAs in the serum, plasma, exosomes in serum, ascites fluids, and tissues of ovarian cancer patients could help identify potential diagnostic and prognostic biomarkers\cite{39}. Furthermore, substantial evidence showed the dysregulation of miRNA expression in the early stages of ovarian cancer transformation\cite{40-42}. It is also important to note that miRNAs also regulated proliferation, cell cycle, and survivability of ovarian carcinomas\cite{43-45}.

In conclusion, our data showed the suppressive function of miR-4782-3p in ovarian cancer. Overexpression of miR-4782-3p leads to the inhibition of cancer cell growth and increased apoptosis rate, probably mediated by PDGFRα, the target of miR-4782-3p.

Acknowledgments

This study was supported by the Public Health and Preventive Medicine Provincial Experiment Teaching Center at Sichuan University and Food Safety Monitoring and Risk Assessment Key Laboratory of Sichuan Province, The Project of Development and Regeneration Key Laboratory of Sichuan Province (SYS16-010), and The Project of Sichuan Provincial Department of Education (18ZB0179).

Figure 2. Up-regulation of miR-4782-3p suppressed proliferation and increased apoptosis in SKOV3 and HO8910 cells. (A) The expression value of miR-4782-3p in normal human ovarian epithelial cells (NOE) and SKOV3 and HO8910 cells were determined by qRT-PCR. The miR-4782-3p levels of SKOV3 and HO8910 cells were respectively compared with the miR-4782-3p levels of NOE. (B) The level of miR-4782-3p in NOE was normalized and defined as 100%. SKOV3 and HO8910 cells were placed in plate; 12 h later, miR-4782-3p mimic transfected was performed. After 24 h, miR-4782-3p levels in the SKOV3 and HO8910 cells were assayed by qRT-PCR. (C) miR-4782-3p levels in the control group were normalized as 100%. In addition, cellular proliferation was assessed by the MTT method. (D) Finally, SKOV3 and HO8910 cells were respectively pretreated with miR-4782-3p. Then, SKOV3 and HO8910 cells were stained with annexin V-FITC and PI and the cell apoptotic rate was evaluated by FACS analysis. (E) Representative images of flow cytometry for apoptosis of SKOV3 cells. Data are indicated as mean ± SEM. Each experiment was performed for at least three times. *P < 0.05
Figure 3. Suppression of miR-4782-3p promoted SKOV3 and HO8910 proliferation. (A) SKOV3 and HO8910 cells were placed into 6-well plates and then transfected with miR-4782-3p ASO. After 24 h, miR-4782-3p level was tested by qRT-PCR. miR-4782-3p levels in NC ASO were normalized as 100% in both SKOV3 and HO8910 cells. (B) After miR-4782-3p ASO transfection, MTT assay was performed to test the cell growth. Apoptosis rates were tested by annexin V-FITC/PI staining. (C) Twenty-four hours following miR-4782-3p ASO transfection, miR-NC ASO was used as control. (D) Representative images of flow cytometry for apoptosis of SKOV3 cells. The data are presented as mean da. Each experiment was performed for at least three times. *P < 0.05; NC, negative control.
Figure 4. PDGFRα was targeted by miR-4782-3p in SKOV3 cells. (A) The binding site of miR-4782-3p in PDGFRα and its mutated version is listed. miR-4782-3p mimics and plasmids containing the mutated 3’-UTR sequence of PDGFRα were transfected to SKOV3, whereas miR-NC and plasmids containing wild-type transfection were used as controls. (B) 48 h later, the luciferase activity was analyzed. (C) miR-4782-3p mimics were transfected into SKOV3 cells, and the level of PDGFRα protein was determined by Western blotting. PDGFRα levels in ten OC tissue samples were analyzed by qRT-PCR. (D) PDGFRα levels in adjacent normal tissue were arbitrarily normalized as 1. SKOV3 and HO8910 cells were seeded separately and then transfected with PDGFRα siRNA. After 24 h, PDGFRα expression was tested by qRT-PCR. (E) PDGFRα levels in NC ASO were normalized as 100% in control. (F) The cellular proliferation was tested by MTT assay following PDGFRα siRNA transfection. Each experiment was performed for at least three times. *P < 0.05; NC, negative control.
Conflicts of interest
The authors declare that they have on conflicts of interest.

Author contributions
T.A. and J.L. collected patient data. T.A., J.L., and Q.Y. performed PCR, Western blotting, and other molecular experiments. L.X. and X.J. conceived the idea of the study design and wrote the manuscript. X.J. analyzed data and revised the manuscript for important intellectual content. All the authors have read and approved the manuscript.

References


