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miR-21 down-regulation promotes apoptosis and inhibits invasion and migration abilities of OVCAR3 cells

Abstract

Purpose: To investigate the influence of miR-21 down-regulation on cell proliferation, apoptosis, invasion and migration of ovarian papillary adenocarcinoma cell lines (OVCAR3).

Methods: Short-hairpin RNA (shRNA), specifically targeting miR-21, was constructed and transfected into OVCAR3 cells using the pSIREN-RetroQ linear vector (pSIREN-miR-21). The expression of miR-21 was detected with stem-loop real-time RT-PCR in OVCAR3 cells. Cell proliferation and apoptosis were monitored using the MTT assay and flow cytometry, respectively. Cell migration and invasion were assessed using the transwell migration and scratch-wound assay, respectively. Western-blotting was used for PDCD4 protein expression.

Results: pSIREN-miR-21 suppressed miR-21 expression in OVCAR3 cells. miR-21 expression levels in pSIREN-miR-21 cells was 0.3 ± 0.1 , which was significantly lower when compared with pSIREN-miR-21-Neg and control groups ($P < 0.01$). Cell inhibition rate in the pSIREN-miR-21 group was higher than the control group (29.4% vs 9.0%, $P < 0.01$), as was the percentage of apoptotic and necrotic cells. By transwell migration assay, the number of cells migrating in the pSIREN-miR-21 group was significantly lower than in the control group. In addition, fewer cells were observed in the wounded area of the pSIREN-miR-21 group following the scratch-wound assay. PDCD4 expression was increased in OVCAR-3 cells transfected by pSIREN-miR-21 compared with vector-control transfected cells. Moreover, the optical density of the transfected cells was significantly lower than the two control groups.

Conclusion: Down-regulation of miR-21 dramatically increased apoptotic cell death and decreased cell proliferation, invasion and migration in OVCAR3 cells. MiR-21 may play an important role in the biological behaviors of epithelial ovarian carcinoma cells through negative control of the expression of PDCD4.

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Epithelial ovarian cancer (EOC), which accounts for 90% of ovarian cancers, continues to be the leading cause of death among gynecological malignancies. Approximately 70% of patients are diagnosed at the advanced stage when extensive cell invasion and migration in the abdominopelvic cavity has occurred, which is the main cause of death in EOC patients. Therefore, inhibiting malignant biological behaviors of tumor cells is critical for improvement of EOC patient prognosis. Recently, accumulating evidence suggested that microRNAs (miRNAs), a class of small non-coding RNA molecules regulating diverse biological processes, may play an important role in tumorigenesis and development of cancer [1]. Recent studies show that more than half of the miRNAs can be aligned to genomic fragile sites or regions associated with cancers [2]. Aberrant expression of miRNAs has been reported in various tumors such as breast, lung, liver, colon and gastric cancer, indicating that there is a close correlation between miRNAs and human malignancy [3,4].

miR-21 has been frequently observed to be overexpressed in various tumors and may function as an oncomiR in diverse tumors, including ovarian cancer [5-7]. In addition, miR-21 may play an important role not only in tumor growth but also in invasion and metastasis by targeting multiple tumor/metastatic suppressor genes including PTEN, PDCD4 and BCL-2 [8-10]. In our previous study, using stem-loop real-time PCR, miR-21 was shown to be significantly over-expressed in human EOC tissue and ovarian papillary adenocarcinoma cell lines (OVCAR3). Remarkably, the over-expression of miR-21 correlated with histological differentiation, clinicopathological stage and lymph node metastasis [11]. Therefore, it is possible that miR-21 may function as an oncomiR in tumorigenesis and ovarian cancer progression.

In the present study, a short-hairpin RNA (shRNA) was constructed that specifically targeted the mature fragment of miR-21 and effectively down-regulated the expression of miR-21 in OVCAR3 cells after transfection with pSIREN-miR-21. The cell growth, apoptosis rate and the invasion and migration of cells were then investigated after knockdown of miR-21. Furthermore, the expression of PDCD4 protein in the OVCAR3 cells was assessed using a Western blot. Results indicated that miR-21 was effectively and specifically suppressed in tumor cells. Moreover, the expression of PDCD4 protein was significantly increased and down-regulated changes occurred in malignant biological behaviours of OVCAR3 cells after knockdown of miR-21. Targeted down-regulation of miR-21 may, therefore, be a potential therapeutic target for recurrence and metastasis in EOC patients.

Materials and Methods

Construction of shRNA targeted miR-21 expression vector

A shRNA specifically targeting miR-21 was constructed as follows: the mature fragment sequence for miR-21 was obtained from the Microbase databank (<http://www.mirbase.org/cgi-bin/mirna>), and the following short anti-miR-21 RNA oligonucleotide (miR-21) was synthesized:

5'-gatccCAGCCCATCGACTGGTGTtAtcaagagaAACACCAGTCGATGGGCTGTTTTTg-3'

A control sequence, which was not specific for miR-21 (miR-21-neg), was also constructed:

5'-gatccGGCGCATAAGAAGCATATAttcaagagaTATATGCTTCTTATGCGCCTTTTTTg-3'

Both sequences were evaluated for sequence specificity by a BLAST search and did not show homology to other known genes. Annealed oligonucleotides were ligated within the Linear pSIREN-RetroQ vector, resulting in the pSIREN-miR-21 and pSIREN-miR-21-Neg recombinants. The recombinants were identified by restriction endonuclease analysis and DNA sequencing.

Cell lines, culture conditions and transfection

OVCAR3 cells were purchased from the Shanghai Cell Library of Academia Sinica Type Culture Collection. Cells were cultured in McCoy's 5A medium with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Twenty-four hours prior to transfection, cells were seeded in 6-well plates at a density of 5×10^5 cells/well and grown to 60% confluence. OVCAR3 cells were transfected using the lipofectamine2000 reagent according to the manufacturer's instructions. Briefly, pDsRed (7 µl/100 µl) was mixed with recombinant pSIREN-miR-21 (3.5 µl/100 µl). Diluted lipofectamine2000 reagent (100 µl; 1:100) was added to the pSIREN-miR-21 recombinant mixture (100 µl). Following 15 min incubation at room temperature, 200 µl of transfection mixture was added to each well, resulting in a siRNA concentration of 254 nm.

Isolation of total RNA

RNA was extracted from cell lines using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The dissolved RNA was stored at -70°C before use. RNA quality was assessed with a NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

TABLE 1. Primers used for real-time RT-PCR assay

Target	Primer	Sequence
hsa-miR-21	RT	5'GTCGTATCCAGTGC GTGTCTG TGGAGTCGGCAATTGCACTGGATACGACACAGCCCA3'
	Forward primer	5'GCGGCAACACCAGTCGATG3'
	Reverse primer	5'TGCGTGTCGTGGAGTC3'
U6	Forward primer	5'GCTTCGGCAGCACATATACTAAAAT3'
	Reverse primer	5'CGCTTCACGAATTTGCGTGT CAT3'

Real-time RT-PCR for miR-21 quantification

Gene-specific looped primers were designed for RT-PCR (listed in Table 1). Reverse transcription of RNA was performed using 1 µg of total RNA in a Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA) in a total volume of 20 µl. The reaction proceeded for 30 min at 16°C, 42 min at 42°C and 5 min at 85°C, and the cDNA was stored at -20°C. Real-time PCR was performed using SYBR Green I (Invitrogen) detection chemistry on a Rotor-Gene 3000 Real-time PCR machine (Corbett Research, Victoria, Australia). The reactions were amplified for an initial 5 min denaturation step at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 60°C, 20 s at 72°C and 20 s at 78°C. Real-time PCR for the U6 gene was performed in the same way with the corresponding primers. All the reactions were typically run in duplicate three times, simultaneously. The cycle number at which the fluorescent signal in each reaction tube crossed a threshold (CT) was determined for miR-21 and the relative amount of miR-21 in the pSIREN-miR-21 group (A) to that of the control group (B) was described using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT_{miR-21} - CT_{U6})_{group A} - (CT_{miR-21} - CT_{U6})_{group B}$.

Flow cytometry analysis

Cells were seeded in 24 well plates at 5×10^4 cells/ml (1 ml/well). Following 48-72 h incubation at 37°C in a humidified atmosphere containing 5% CO₂, OVCAR3 cells were harvested, centrifuged at 300 g for 5 min. The supernatant was discarded, the pellet was resuspended in phosphate buffered saline (PBS) and cell concentration was adjusted to 1×10^5 cells/ml using PBS. The Annexin-V-FITC Apoptosis Detection Kit was used to assess apoptotic cell death.

Cell proliferation assays

Cell proliferation was assessed using the MTT assay. Cells (5.0×10^3 cells/well) were seeded on 96 well plates. At a series of time points, 10 µl of MTT was added to each well, and the cells were incubated at 37°C for 4 h. Dimethylsulfoxide (100

µl) was added to each well and the plate was shaken for 30 s. The optical density was measured at 570 nm using a microplate reader (Bio-Rad Model 680).

Scratch-wound assay

Cells were plated onto six well plates at a concentration of 1×10^5 cells/well. Cell monolayers were carefully wounded by scratching with a sterile plastic pipette tip. The cells were washed twice with cooled PBS and incubated in the McCoy's 5A medium with 2% fetal bovine serum for 48 h. For each wound, five fields were photographed at 0 and 48 h after injury.

Cell invasion assay

Cells (2.5×10^4 cells/well) were seeded onto the top chamber of a 24 well matrigel-coated polyethylene terephthalate membrane insert with 8 µm pores (Corning, USA). The bottom chamber was filled with 0.6 ml McCoy's 5A with 10% (v/v) FBS as a chemoattractant. After incubation for 24 h, the filter membrane was fixed with 100% (v/v) methanol and stained with hematoxylin and eosin. The degree of invasiveness was quantified by counting the number of cells in 10 random fields of view per filter, using a $\times 400$ objective lens. Data obtained from three separate inserts are shown as mean $\pm$ SD.

Western blot

Cells were washed twice with ice-cold PBS and lysed on the culture dishes using lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% protease inhibitors). The protein concentration was determined using the BCA method and 20 µg of each sample was separated by SDS-PAGE (10% and 4%) and transferred to PVDF membranes. Nonspecific binding sites were blocked by incubating with 5% BSA solution for one hour at room temperature. The membranes were then incubated with rabbit antihuman PDGFR4 antibody (1:6000) and rabbit anti-β-actin polyclonal antibody (1:1000) overnight at 4°C. Anti-rabbit secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:2000. The protein

FIGURE 1. (A) Identification of recombinant plasmid pSIREN-miR-21 by restriction endonucleases. (A1) pSIREN-miR-21; (A2) pSIREN-miR-21-neg. Lane 1: DNA Marker Lane 2: pSIREN-miR-21 digested by BamHI/BglII, yielding a fragment of 263 bp; Lane 3: pSIREN-miR-21 digested by EcoRI/BglII, yielding a fragment of 322 bp; Lane 4: pSIREN-miR-21 digested by BamHI; Lane 5: pSIREN-miR-21 digested by EcoRI; Lane 6: Recombinant plasmid pSIREN-miR-21. (B) The expression of DsRed fluorescence in OVCAR-3 cells co-transfected with pDsRed and pSIREN-PSPRKs after 72 h ($\times 100$, exposure time: 500 ms, plus value: 2500). (B1) recombinant plasmid pDsRed co-transfected into OVCAR-3 cells with pSIREN-miR-21; (B2) recombinant plasmid pDsRed co-transfected into OVCAR-3 cells with pSIREN-miR-21-neg.

was visualized using ECL Western blot detection reagents and analyzed by scanning densitometry by Smart View Analysis Software (Shanghai FURI Science & Technology Co., China).

Statistical analysis

Data analyses were performed with the statistical software package SPSS 13.0. All the experiments were repeated at least three times. Results are presented as the mean $\pm$ standard deviation (SD). The Student's t-test was used to assess miR-21 expression and other data in different groups. The level of statistical significance was set at $P < 0.05$.

Results

Construction and identification of shRNA expression vectors

When the annealed oligos (59 bp) were inserted downstream of the H1 promoter of the empty vector pSIREN-RetroQ, the recombinant vectors could be digested by BamHI/BglII and EcoRI/BglII restriction enzymes to produce 263 bp and 322 bp fragments, respectively. When the recombinants were digested by BamHI or EcoRI only, no fragment was produced (Figure 1). The recombinant vectors were then identified by DNA sequencing.

miR-21 expression suppression by pSIREN-miR-21

All pSIREN-miR-21 recombinants were mixed with pDsRed and transfected for 72 h into OVCAR-3 cells (Figure 1). miR-21 expression levels were confirmed by stem-loop real-time RT-PCR in the transfected cells. Data showed that miR-21 expression levels (0.3 ± 0.1) decreased significantly in pSIREN-miR-21 transfected cells compared with pSIREN-miR-21-Neg transfected and control group cells (Figure 2).

Knockdown of miR-21 increases OVCAR3 cell apoptosis in vitro

Flow cytometry using Annexin V-FITC and PI stained cells was used to distinguish and quantitatively determine the percentage of viable and apoptotic cells at 48 and 72 h after transfection with pSIREN-miR-21. Figure 3 indicates that $25.8 \pm 0.8\%$ of the cells underwent early stages of apoptosis at 48 h, but only 0.01% of the cells in the negative control. Moreover, $30.4 \pm 0.8\%$ and $3.6 \pm 0.2\%$ of the cells were viable and

FIGURE 2. miR-21 expression was significantly downregulated in OVCAR-3 cells transfected with pSIREN-miR-21. Relative expression of miR-21 was detected with stem-loop real-time RT-PCR, and the relative amount of miR-21 was described using $2^{-\Delta\Delta CT}$. A Student's t-test was used to compare the pSIREN-miR-21-neg transfected group and control group. * $P < 0.01$

FIGURE 3. Detection of apoptosis in OVCAR-3 cells transfected with pSIREN-miR-21 using flow cytometry following Annexin V-FITC staining. (A) 48 h after transfection; (B) 72 h after transfection a: control group b: pSIREN-miR-21-neg group c: pSIREN-miR-21 group. Early apoptotic cell death rate in pSIREN-miR-21 group at 48 h and 72 h after transfection was $25.8 \pm 0.8\%$ and $30.5 \pm 0.8\%$, respectively, which was significantly higher than in the control groups ($P < 0.01$). Necrotic cell death rate at 72 h was $3.6 \pm 0.2\%$ and also higher than other groups, $P < 0.05$.

necrotic respectively at 72 h, which were higher than $7.8 \pm 0.3\%$ and $1.6 \pm 0.1\%$ of the cells in the negative control. In addition, the percentage of apoptotic cells gradually increased from 48 to 72 h after transfection in the pSIREN-miR-21 group. The percentage of viable apoptotic cells and necrotic cells at 72 h were higher than that at 48 h ($p < 0.05$ and $p < 0.01$, respectively).

Knockdown of miR-21 inhibits OVCAR-3 cell proliferation in vitro

Cell viability was assessed using the MTT assay after transfection for 72 h with pSIREN-miR-21. As shown in Figure 4, the inhibition rate for proliferation in the pSIREN-miR-21 group was higher than that in the control group (29.4% and 9.0%, respectively), indicating that proliferative ability was suppressed by transfection of pSIREN-miR-21. There was no significant difference for light absorption values between the pSIREN-miR-21-neg group and the control group.

miR-21 affects OVCAR3 cell migration and invasion in vitro

Following the scratch-wound assay, scratched areas were seen to be covered with fewer cells in the pSIREN-miR-21 group than

FIGURE 4. MTT assay for the evaluation of cell proliferation of OVCAR-3 transfected cells with pSIREN-miR-21. The optical density and inhibition rate in the pSIREN-miR-21 group was 0.66 and 29.4%, respectively, which was higher than the negative control (0.8 and $9.0 \pm 1.3\%$, respectively). Statistical analysis was performed using the chi square test; * $P < 0.05$; ** $P < 0.01$.

FIGURE 5. Effect of miR-21 down-regulation on OVCAR-3 cell invasion and migration. (I) Effect of miR-21 down-regulation on OVCAR-3 cell migration 48 h after transfection ($\times 100$) A: control group, B: pSIREN-miR-21-neg group, C: pSIREN-miR-21 group; (II) The transverse metastasis length of OVCAR3 cells in pSIREN-miR-21 group was 0.62 ± 0.12 mm which was significantly shorter than the controls (* $P < 0.01$); (III) Effect of miR-21 down-regulation on OVCAR-3 cell invasion 48 h after transfection ($\times 400$) A: control group, B: pSIREN-miR-21-net group, C: pSIREN-miR-21 group; (IV) The number of migrating cells in pSIREN-miR-21 group was 29.5 ± 2.8 , which was significantly lower than the controls (* $P < 0.05$).

in the blank and negative control group, in which the scratched areas were mostly covered. The transverse metastasis length of OVCAR3 cells transfected with pSIREN-miR-21 was obviously shorter than the controls (Figure 5, I-II). To further quantitatively evaluate the effect of suppression of miR-21 in cell invasion, a transwell invasion assay was performed. As shown in Figure 5, III-IV, the number of migrating cells in the pSIREN-miR-21 group was 29.5 ± 2.8 , which was significantly lower than the control group (73.0 ± 2.2) ($P < 0.05$). No significant difference was observed between the control group and the pSIREN-miR-21-neg group (69.0 ± 1.8). This result indicates that cell migration and invasion were significantly suppressed after pSIREN-miR-21 transfection.

miR-21 Modulates PDCD4 Expression in OVCAR-3 cells

To test whether PDCD4 was directly modulated by miR-21, Western blotting was used to detect PDCD4 expression in OVCAR-3 cells after transfection. The data showed that PDCD4 expression was increased in OVCAR-3 cells transfected by pSIREN-miR-21 compared with vector-control transfected cells (Figure 6), and the optical density of the cells transfected by pSIREN-miR-21 was 0.661 ± 0.015 ; significantly lower than the two control groups (0.848 ± 0.150 for negative control, 0.935 ± 0.133 for blank control, $P < 0.01$).

Discussion

MicroRNAs, approximately 22 nucleotides long, which negatively regulate gene expression in a variety of eukaryotic organisms, are important regulators of several cellular processes, including proliferation, differentiation, apoptosis and development, as they simultaneously control the expression levels of hundreds of genes [12]. In recent years, the emerging significance of miRNAs in cancer has evoked great interest, resulting in numerous cancer profiling studies. Several studies have shown that miRNA expression profiles are altered in ovarian cancer, acting as oncogenes or tumor-suppressor genes [4,13]. miR-21 is one of the most common miRNAs up-regulated in a variety of solid tumors, including glioblastoma, breast, liver and stomach cancers [8, 14]. In our previous study, the up-regulated expression of miR-21 in EOC tissue was observed and correlated with the clinicopathological stage and lymph node metastases, indicating that miR-21 may have a role in cell migration and invasion.

In this study, the link between miR-21 expression, proliferation, apoptosis and migration in OVCAR3 cell lines was evaluated using a loss-of-function approach. Although miR-21 antisense oligonucleotide (ASO) can effectively reduce the

FIGURE 6. Expression of PDCD4 protein in infected OVCAR-3 cells by Western blot. The protein levels of PDCD4 in pSIREN-miR-21 infected cells were much higher than those in the two control groups. Lane 1, Untransformed OVCAR-3 cells; Lane 2, OVCAR-3 cells transfected with pSIREN-miR-21-neg; Lane 3, OVCAR-3 cells transfected with pSIREN-miR-21

expression of miR-21, it does not significantly nor consistently change the viability of tumor cells and is therefore a less potent miR-21 inhibitor [15]. In contrast, the RNAi method using an shRNA expression vector offers several distinct advantages over the conventional miR-21 ASO, such as consistent and specific targeting of mature miRNAs and greater transfection efficiency [16]. When miR-21 levels were decreased, a significant increase in apoptotic cell death as also observed. Moreover, the percentage of apoptotic cells gradually increased after 48 and 72 h of transfection, demonstrating a consistent inhibition of pSIREN-miR-21 targeting miR-21. Cellular mechanisms that control apoptosis, cell growth and cell cycle function are efficient fail-safe mechanisms for preventing cancer development. Cancer cells, therefore, only survive if the apoptotic response is hindered or disrupted by miR-21, otherwise unrestricted cell proliferation occurs [17]. Consistent with this hypothesis, the present study showed that down-regulation in miR-21 leads to both an increase in apoptotic cell death and a significant inhibition of cell proliferation, migration and invasion of OVCAR3 cells.

Numerous studies have reported that miR-21 plays an important role in tumor growth, invasion and metastatic process by targeting multiple tumor/metastatic suppressor genes [9, 18]. PDCD4 (programmed cell death 4) has been identified as a suppressor of tumorigenesis with lost or reduced expression in cancers of epithelial origin, including the lung, breast and ovary [19-21]. Previous studies have shown that PDCD4 is one of the important target genes of miR-21 [22, 23]. In ovarian tumors, decreased or lost PDCD4 expression was associated with disease progression [21]. In addition, a consistent decrease in PDCD4 expression levels was associated with the steps from normal to borderline to malignant ovarian tissues, and PDCD4 over-expression in ovarian cancer cells resulted in malignant growth inhibition [21]. The regulatory mechanisms

of PDCD4 in ovarian cancer, however, are still not completely understood.

In our study, PDCD4 over-expression in miR-21-knockdown OVCAR3 cell suggests a potential mechanism of PDCD4 regulation by miR-21, which leading to PDCD4 under-expression in epithelial ovarian carcinoma. Recent research indicates that PDCD4 regulates translation by interacting with the translation initiation factors eIF4A, directly interacting with eIF4A via its MA3-c domain and inhibiting eIF4G helicase activity, which results in decrease of protein synthesis, inhibition of cell proliferation and promotion of apoptosis [24, 25]. PDCD4 down-regulation has also been shown to enhance invasion of colon cancer cells, by down-regulation of the transcription factor AP-1 components. And PDCD4 knockdown can also activates β -catenin/Tcf-dependent transcription and acts as a promoter of tumor cell invasion [26]. In our study, down-regulation of miR-21 significantly inhibited migration and invasion abilities of OVCAR3 cells, in addition to the inhibition of cell proliferation and increase in apoptotic cell death. All these results supported our conclusion that miR-21 down-regulating PDCD4 led to an increase in cell proliferation, invasion and metastasis of OVCAR3 cells.

Our previous study showed 41.7% of EOC samples were positive for PTEN expression and 58.3% were negative, which has a negative correlation with the expression of miR-21 in EOC tissues [27]. Moreover, the PTEN expression was enhanced and the migration and invasion abilities of OVCAR3 cells were suppressed when we down regulated the expression of miR-21, suggesting that EOC may enhance malignant biological behaviors by inactivating PTEN genetically and up-regulating miR-21 [27].

These experiments with knocked down miR-21 suggest that miR-21 lies embedded in a link of tumor suppression genes and has multiple specific targets in the network that lead to the observed phenotypes. Aberrant expression of miR-21 is likely to destroy the interconnected tumor-suppressive network and result in dysregulation of network functions. These broad effects enable miR-21 to act as a key oncogene modulating cell growth, desensitizing cells to apoptosis and cell metastasis.

Metastasis is a major cause of cancer-related death; hence, identifying the role of miR-21 in invasion and metastasis has direct clinical implications. Targeted down-regulation of miR-21 in human epithelial ovarian carcinoma could provide great therapeutic value.

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