Tumor suppressor role of miR-217 in human epithelial ovarian cancer by targeting IGF1R

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Abstract. Accumulating evidence shows that microRNA-217 (miR-217) is frequently dysregulated in various cancers, and plays crucial roles in tumorigenesis and metastasis; however, the role and underlying molecular mechanism of miR-217 in human epithelial ovarian cancer (EOC) remains unclear. Here, we report that miR-217 expression was downregulated in EOC tissue and inversely correlated with advanced FIGO stage, high histological grading and lymph node metastasis (P<0.01). Function analysis revealed that the ectopic expression of miR-217 in EOC cells inhibited cell proliferation, migration and invasion in vitro, as well as suppressed tumor growth in vivo. Bioinformatics analysis and dual luciferase assays identified insulin-like growth factor 1 receptor (IGF1R) as a direct target of miR-217 in EOC cells. Western blot assay showed that overexpression of miR-217 in EOC cells inhibited IGF1R expression. In addition, downregulation of IGF1R mimicked the tumor-suppressive effects of miR-217 in EOC cells, whereas the reintroduction of IGF1R partially abrogated the suppression effect induced by miR-217 on EOC cells. Collectively, these results demonstrated that miR-217 plays a tumor suppressor role in human epithelial ovarian cancer by directly targeting IGF1R gene, suggesting a new potential therapeutic target in EOC.

Introduction

Epithelial ovarian carcinoma (EOC) is the most common histological type of ovarian cancer, accounting for 80-90% of ovarian cancer cases (1,2). It represents the most fatal gynecological malignancy among women worldwide, causing over 140,000 deaths every year (1). Despite great progress in current multidisciplinary treatment, the 5-year survival rate of patients with advanced-stage EOC remains <30% due to the lack of diagnostic methods for early stage detection and effective strategies for treatment (3). Therefore, understanding the underlying molecular mechanisms involved in EOC occurrence and procession would facilitate early detection and optimize strategies for treatment, and further improve the survival of EOC patients.

miRNAs are small endogenous non-coding RNAs composed of ~19-25 nucleotides that can bind the 3'-untranslated region (3'-UTR) of specific genes of mRNA, causing either degradation or inhibition of protein translation, thus leading to effectively silence their targets gene (4). It has been shown that miRNAs are involved in various biological processes, such as cell proliferation, migration and invasion, differentiation, survival, and tumorigenesis (5,6). Growing body of evidence show that miRNAs can act as either oncogenes or as tumor suppressors (7), and play important roles in tumor growth and metastasis by regulating cancer cell proliferation, apoptosis, differentiation and invasion (8-10), suggesting that miRNAs could act as potent therapeutic targets or diagnostic marker for various cancers.

It has been shown that microRNA-217 (miR-217) was frequently dysregulated in many tumor types. For instance, miR-217 is decreased and functions as a tumor suppressor in gastric cancer by targeting polycomb group protein the enhancer of zeste homolog 2 (EZH2) (11), and it is downregulated and associated with poor survival in clear cell renal cell carcinoma (12), whereas, it is upregulated and functions as an oncogene in breast cancer by targeting the cell fate determination factor (DACH1) (13). However, the clinical significance and biological role of miR-217 in ovarian cancer remains unclear. Therefore, in this study, the miR-217 expression and its clinical diagnostic significance in patients with EOC were determined, and its role and underlying molecular mechanism in EOC procession were assessed.

Materials and methods

Patients and tissue samples. Human epithelial ovarian cancer (EOC) tissue samples and the corresponding adjacent ovarian tissue were obtained from the 36 EOC patients who underwent surgery at Beijing Obstetrics and Gynecology Hospital, Capital Medical University (Beijing, China) from September 2010 to October 2014. Normal ovarian tissues adjacent to the tumor were taken 5 cm from the tumor cells.
All samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. None of these patients received chemotherapy or radiotherapy before surgery. Clinical data on all subjects including age, tumor size, FIGO stage, histological grading and lymph node metastasis was collected and listed in Table I. All patients provided written informed consent for the use of their tissues. This project was approved by the ethics committee of Capital Medical University.

**Cell culture.** A human ovarian surface epithelial cell line (HOSEpiC) and three human ovarian cancer cell lines (SKOV3, OVCAR3 and A2780) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO₂.

**RNA extraction and real-time PCR analysis.** Total RNA was extracted from tissues sample and cultured cells using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA USA) according to the manufacturer's instructions. For the measurement of miR-217, the All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, USA) was used following the manufacturer's instructions; U6 small RNA was used as the internal control. For the detection of IGF1R mRNA, first-strand cDNA was synthesized using PrimeScript RT reagent kit (Takara, Dalian, China). The expression levels of IGF1R were quantified by Real-time PCR Mixture reagent containing miR-217 binding site and a mutant variant were amplified by PCR and cloned into the pGL3-control vector (Ambion) at the NheI and XhoI sites, and termed as: IGF1R-Wt-3'-UTR reporter plasmid or IGF1R-Mut-3'-UTR reporter plasmid (100 ng/well) and the pRL-TK luciferase luciferase reporters (25 ng/well) using Lipofectamine 2000 (Invitrogen). Luciferase activity levels were determined using the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Renilla-luciferase was used for normalization.

**Cell transfection.** miR-217 mimic (miR-217) and corresponding miRNA negative control (miR-NC), the siRNAs targeting human IGF1R (si-IGF1R) and corresponding negative control (si-NC) were purchased from GenePharma Co., Ltd. (Shanghai, China). IGF1R overexpressed plasmids were obtained from Ribobio Co. (Guangzhou, China). Cell transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**MTT assay.** Transfected SKOV3 cells (5x10⁴ cells/well) were seeded into the 96-well plates and cultured in the DMEM medium including 10% FBS. At indicated time (24, 48 and 72 h), 5 µl MTT solution (5 mg/ml, Sigma-Aldrich, MO, USA) was added into each well and incubated for 4 h at 37°C, and then terminated by 200 µl DMSO. The absorption at 570 nm was measured under a microplate reader (Thermo Labsystems, Finland).

**Colony formation assay.** Cells (1x10⁵) were seeded in a 6-well plate at 24 h after transfection and cultured for two weeks in DMEM medium containing 10% FBS. Then colonies were washed three times with PBS, fixed with methanol and stained with 0.1% crystal violet for 30 min, and the number of colonies with >50 cells was counted.

**Wound healing assay.** Wound healing assays were performed to detect cell migration. Briefly, the transfected cells (1x10⁵ cells/well) were seeded in 6-well plates, and an artificial homogeneous wound was created using a sterile plastic micropipette tip, and then cells were cultured under standard conditions for 24 h. Cells were imaged at 0 and 24 h after the wounding. The cell migration was evaluated by counting cells that had migrated from the wound edge at five random fields under a microscope (Olympus, Tokyo, Japan).

**Cell invasion assay.** Cell invasion potential was evaluated using Transwell chambers (8-µm pore; BD Biosciences). Briefly, cell suspensions (5x10⁶ cells) in serum-free DMEM medium were placed into the upper chamber precoated with Matrigel (BD, USA) 24 h after transfection; the bottom chamber was filled with DMEM containing 10% FBS as chemoattractant. After 48 h, cells remaining on the upper surface of the membrane were removed using a cotton swab, and cells that had migrated to the lower surface of the membrane were fixed with 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. Photographs of five randomly selected fields of the fixed cells were taken and counted under a microscope (Olympus).

**Luciferase reporter assay.** The human IGF1R 3’-UTR containing miR-217 binding site and a mutant variant were amplified by PCR and cloned into the pGL3-control vector (Ambion) at the NheI and Xhol sites, and termed as: IGF1R-Wt-3’-UTR and IGF1R-Mut-3’-UTR, respectively. For luciferase assays, SKOV3 cells were cultured in a 6-well plate and then co-transfected with the miR-217 or miR-NC (100 nM/well) and IGF1R-Wt-3’-UTR reporter plasmid or IGF1R-Mut-3’-UTR reporter plasmid (100 ng/well) and the pRL-TK luciferase reporters (25 ng/well) using Lipofectamine 2000 (Invitrogen). Luciferase activity levels were determined using the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Renilla-luciferase was used for normalization.

**In vivo nude mouse tumorigenesis assay.** Twenty female BABL/c nude mice (4-5-week-old) were purchased from the Experimental Animal Center of Beijing, and maintained under specific pathogen-free conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University. For tumor growth assays, SKOV3 cells stably overexpressing miR-217 or miR-NC were resuspended in DMEM medium, and 2x10⁶ cells (200 µl) were subcutaneously injected into the dorsal flank of nude mice. Tumor volume was measured every 5 days and was calculated with the formula: volume = (a x b²)/2, in which ‘a’ refers to the longest diameter and ‘b’ the shortest. Mice were sacrificed at day 30, and tumors were dissected and weighed. Parts of the tumor tissues were snap-frozen in liquid nitrogen and stored at -80°C for analysis for IGF-1R expression.

**Western blot assays.** Cells or tissue were lysed in RIPA buffer with protein inhibitors cocktail (Complete Mini;
Roche Diagnostics, Basel, Switzerland). Concentrations of total cellular protein were determined using a BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Total proteins (30 µg) from each sample were electrophoresed on 10% by sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% non-fat milk and probed with the antibodies: anti-IGF1R (1:1,000, Santa Cruz, USA) and anti-GAPDH (1:5,000, Santa Cruz) overnight at 4˚C. The membranes were washed and probed with the secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. The protein band was detected with the BioMax MR-1 radiographic film (Kodak, Xiamen, China) and enhanced chemiluminescence system (Amersham Pharmacia Biotech, Bucks, UK). The GAPDH were used as internal control.

Statistical analysis. The data are reported as mean ± standard deviation (SD), and all experiments were repeated at least three times independently. The SPSS software package (version 19.0, SPSS Inc; Chicago, IL, USA) was used for the statistical analysis. Student’s t-test, ANOVA or a Chi-square test was employed to determine statistical significance as appropriate. A P-value of <0.05 was considered statistically significant.

Results

miR-217 is downregulated in EOC tissues and cell lines. The expression of miR-217 level in 36 EOC tumor tissues and paired adjacent normal ovarian tissues were tested by quantitative RT-PCR (qRT-PCR). As shown in Fig. 1A, the expression of miR-217 in EOC tissues was significantly downregulated compared with corresponding adjacent normal ovarian tissues (P<0.01) (Fig. 1A). In addition, the levels of miR-217 expression in three human ovarian cancer cell lines (the SKOV3, OVCAR3 and A2780) and human ovarian surface epithelial cell line (HOSEpiC) were examined by qRT-PCR (Fig. 1B). As expected, in all three ovarian cancer cell lines, the miR-217 expression level was lower than that in a control human ovarian surface epithelial cell line (HOSEpiC). The SKOV3 cell line was selected for further studies since it possessed the lowest levels of miR-217 expression among the three cell lines. To investigate the clinical relevance of miR-217 in EOC, the median (0.47) of all 36 cases was chosen as the cutoff point dividing the cases into two groups: low-miR-217 expression (<0.47, 20 cases) group and high-miR-217 expressing group

<table>
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<th>High (n%)</th>
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<td>&gt;0.05</td>
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<td>≥5</td>
<td>19</td>
<td>10 (52.6)</td>
<td>9 (47.4)</td>
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Figure 1. miR-217 is downregulated in EOC tissues and cell lines. (A) Relative expression level of miR-217 in 36 pairs of EOC tissue samples (tumor) and matched adjacent normal tissue samples (normal) was analyzed using quantitative RT-PCR (qRT-PCR). U6 was used as an endogenous control. *P<0.05, **P<0.01 compared to normal. (B) Relative expression of miR-217 in three human ovarian cancer cell lines (the SKOV3, OVCAR3 and A2780) and human ovarian surface epithelial cell line (HOSEpiC). *P<0.05, **P<0.01 compared to HOSEpiC.
miR-217 inhibits EOC cell growth and metastasis. The above results showed that miR-217 expression was significantly negatively associated with lymph node metastasis (P<0.01) and advanced FIGO stage (P<0.01). Therefore, miR-217 mimic and miR-NC were transfected into SKOV3 cells. As shown in Fig. 1A, SKOV3 cells expressed high level of miR-217 after transfection with miR-217 compared to cells transfected with miR-NC (P<0.01). To further examine the miR-217 role in EOC cell growth, cell proliferation and colony formation assays were performed in SKOV3 cells after transfected with miR-217 mimic or miR-NC (P<0.01). It was found that the restored expression of miR-217 resulted in significant decrease of proliferation (Fig. 2B) and colony formation (Fig. 2C) in SKOV3 cells. Furthermore, to test the biological role of miR-217 on EOC cell metastasis, cell migration and cell invasion were determined in SKOV3 cells after transfected with miR-217 mimic or miR-NC by wound healing assay and invasion chamber assay, respectively. The results showed that restoration of miR-217 could significantly suppress the capacity of migration (Fig. 2D) and invasion (Fig. 2E) in EOC cells. These results suggested that miR-217 inhibited EOC cell growth and metastasis.

Knockdown of IGF1R has similar effect with miR-217 overexpression. To explore the biological functions of IGF1R in EOC cells, endogenous expression of IGF1R was knocked down in SKOV3 cells with specific siRNA (si-IGF1R). The qRT-PCR and western blot assay confirmed that IGF1R expression on mRNA level and protein level (Fig. 4A and B) were significantly decreased in SKOV3 cells after transfected with si-IGF1R. Furthermore, knockdown of IGF1R significantly inhibited cell proliferation (Fig. 4C), colony formation and migration (Fig. 4D) in EOC cells.

IGF1R is a direct target of miR-217. To investigate the potential target gene which miR-217 could regulate in ovarian cancer cells, we used two publicly available algorithms (Targetscan6.2 and miRanda) to predict miR-217 targets in EOC. Among the candidate target genes, IGF1R attracted our attention since it functions as an oncogene in various cancers, including ovarian cancer (15). As shown in Fig. 3A, miR-217 has one predicted binding site in the 3’-UTR of IGF1R mRNA. To further confirm whether IGF1R is a direct target of miR-217, luciferase reporter assay was performed, and found that SKOV3 cells transfected with miR-217 mimic decrease IGF1R-Wt-3’-UTR reporter activity (P<0.01, Fig. 3B), while transfection of miR-217 mimic had no inhibition effect on the IGF1R-Mut-3’-UTR reporter activity in EOC cells (Fig. 3B), indicting the direct regulation of miR-217 in the 3’-UTR of IGF1R mRNA. To determine whether overexpression miR-217 downregulated endogenous IGF1R expression, IGF-1R mRNA and protein expression were determined in SKOV3 cells after transfected with miR-217 mimic or miR-NC by qRT-PCR and western blotting, respectively. As expected, overexpression of miR-217 in SKOV3 cells significantly inhibited IGF1R expression on mRNA level (Fig. 3C) and protein level (Fig. 3D). In addition, the expression of IGF1R in 36 OS tissues and corresponding normal tissues were tested by qRT-PCR. It was found that IGF1R expression levels were upregulated in EOC tissues compared with adjacent normal tissues (Fig. 3E), and was inversely correlated with miR-217 expression in EOC tissue (Fig. 3F; r=-0.650, P<0.01). These results indicate that miR-217 directly bindings to the 3’-UTR of IGF-1R and inhibits its expression.
(Fig. 4D), migration (Fig. 4E) and invasion (Fig. 4F), suggesting that inhibition of IGF1R had similar effect with miR-217 overexpression in EOC cells.

IGF1R overexpression attenuated the effect of miR-217. To examine a possible role for IGF1R in miR-217-mediated suppression of EOC growth and metastasis, SKOV3 cells were transfected with miR-217 mimic or miR-NC, followed by transfection with overexpression of IGF1R plasmids. The expression levels of IGF1R on mRNA level and protein level were increased in SKOV3 cells co-transfected IGF1R overexpression plasmid and miR-217 compared to cells transfected only with miR-217 (Fig. 5A and B). In addition, reintroduction of IGF1R partially abrogated inhibition effect of miR-217 on
miR-217 inhibits EOC growth by targeting IGF1R.

Cell proliferation, colony formation, migration and invasion (Fig. 5C-F). These results indicated that miR-217 inhibits EOC growth and metastasis partially by targeting IGF1R.

miR-217 suppresses tumor growth in vivo. To determine whether miR-217 was responsible for EOC tumorigenicity, we subcutaneously injected SKOV3 cells stably overex-
pressing miR-217 or miR-NC into the dorsal flank of nude mice. Compared with miR-NC group, the tumor volume and weight of the miR-217 group were markedly reduced (Figs. 5B and 6A). Furthermore, we also determined IGF1R expression on mRNA level and protein level in tumor tissue by qRT-PCR and western blotting, respectively. It was found that IGF1R expression on mRNA and protein levels were markedly reduced in miR-217 group compared to miR-NC group (Fig. 6C and D). These data indicated that miR-217 suppressed tumor growth of EOC in vivo by targeting IGF1R.

**Discussion**

Recent studies have shown that miRNAs play a fundamental role in the growth and metastasis of malignant cancers (16), including EOC (17). A larger number of miRNAs has been reported to be involved in EOC procession and development. For example, Wen et al found that miR-338-3p functions as tumor suppressor in EOC cells that blocks the growth of ovarian cancer cells through PI3K/AKT signaling pathways by targeting Runx2 (18). Lin et al reported that miR-26b inhibited EOC cell viability, migratory ability and sphere-forming capacity in vitro and in vivo by targeting karyopherin α2 (KPNA2) (19). Zhang et al showed that ectopic overexpression of miR-373 in human EOC cells suppressed cell invasion in vitro and metastasis in vivo, and the epithelial-mesenchymal transition process by targeting Rab22a (20). Here, we found that miR-217 expression was downregulated in both EOC cell lines and human EOC tissues relative to corresponding normal tissue and human ovarian surface epithelial cell line (HOSEpiC), respectively, and that low miR-217 expression was significantly associated with high histological grading, advanced FIGO stage, and lymph node metastasis. In addition, we also showed that restoration of miR-217 significantly inhibit cell proliferation, colony formation, migration and invasion in vitro, and suppressed tumor growth in vivo. These results suggested that miR-217 might be used as a potential therapeutic agent for treatment of EOC.

miR-217 has been reported to be downregulated and to function as a tumor suppressor in gastric cancer (11), pancreatic ductal adenocarcinoma (21), hepatocellular carcinoma (22), osteosarcoma (23), colorectal cancer (24), clear renal cell carcinoma (12) and chronic myelogenous leukemia (25), while it is overexpressed and acts as an oncogene in B-cell lymphomas (26) and breast cancer (13). These controversial findings suggest that the role miR-217 in tumor progression.
depends on the tumor type and its targets gene. Here, we investigated the biological roles of miR-217 in EOC, and found that miR-217 expression was downregulated in EOC tissues and cell lines. In addition, we also found that overexpression of miR-217 in EOC cells inhibited cell proliferation, colony formation, reduced cell migration and invasion capabilities in vitro, and that restoration of miR-217 suppressed tumor growth in a nude mouse xenograft model system. These results might suggest that miR-217 functions as a tumor suppressor in EOC.

In view of the vital importance of miR-217 in EOC, we further investigated the mechanism mediating the role of miR-217. Using bioinformatics analysis combined with subsequent experimental confirmation, we demonstrated IGFI R1 was a direct target of miR-217. To investigate whether miR-217 inhibits EOC cell growth and metastasis via targeting IGFI R1, we first knocked down IGFI R1 expression using specific siRNAs, and found that inhibition of IGFI R1 decreased cell proliferation, colony formation, migration and invasion of SKOV3 cells, yielding very similar effect as that of restored miR-217 expression in SKOV3 cells. Accordingly, we also found that reintroduction of IGFI R1 partially abrogated the suppression effect induced by miR-217. IGFI R1, located on chromosome 15q25-26, is an important member of the insulin receptor family of receptor tyrosine kinases (27). It was frequently found to be overexpressed in cancer, including ovarian cancer (14). By regulating its downstream signaling, including PI3K/akt and MAPK/ERK signaling pathways, IGFI R1 plays an important role in cancer cell growth, survival, metabolism and transformation (28-30). Recent reports have demonstrated that IGFI R1 overexpression was associated with disease progression, poor prognosis and treatment resistance in ovarian cancer (31), and that IGFI R1 functions as an oncogene in EOC, and promoted carcinogenesis in EOC (32). In addition, IGFI R1 has been reported to be regulated by several miRNAs, such as miR-133a (14), miR-30a (33), miR-145 (34), and miR-139-5p (35). In the present study, we reported that miR-217 inhibited EOC cell growth and metastasis by targeting IGFI R1.

Taken together, to our knowledge, the present study is the first showing that miR-217 expression was significantly decreased in EOC cell lines and tissues, and its expression was inversely correlated with advanced FIGO stage, high histological grading and lymph node metastasis; and that miR-217 exerts a tumor suppressor role in EOC by partially targeting IGFI R1, suggesting that miR-217 might act as a potential target for miRNA-based EOC therapy.

References


