Original Article
miR-126 inhibits epithelial ovarian cancer growth partially by repression of IRS2

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Abstract: MiR-126 has been reported to involve in the development and progression of various cancers. However its role in epithelial ovarian cancer (EOC) remains unclear. Therefore, the aims of this study were to investigate the miR-126 expression and its clinical diagnosis significance in patients suffering EOC and to analyze its role and potential molecular mechanism on the carcinogenesis of ovarian cancer by a series of molecular experiments. Here, we found that miR-126 was significantly decreased in ovarian cancer cell lines and tissues by quantitative RT-PCR (qRT-PCR), and its expression was negatively correlated with advanced FIGO stage, high histological grading and lymph node metastasis (all P<0.01). Functional study demonstrated that the restoration expression of miR-126 significantly inhibited EOC cell proliferation, migration and invasion, and induces apoptosis. Additionally, IRS2 was predicted the target gene of miR-126. Luciferase reporter assay further verified direct target association of miR-126 to specific sites of the IRS2 3'-untranslated regions. qRT-PCR and western blot assays showed that overexpression of miR-126 inhibited IRS2 expression. Importantly, IRS2 expression was upregulated in EOC tissues and inversely correlated with the expression of miR-126. Taken together, these results shows for the first time that miR-126 functions as a tumor suppressor in epithelial ovarian cancer partially by repression of IRS2.

Keywords: microRNAs, miR-126, ovarian cancer, epithelial ovarian cancer, IRS2

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological cancers worldwide, and it accounts for more than 90% of all forms of ovarian cancer [1, 2]. Primary treatment of EOC is surgical resection of visible disease, followed by adjuvant chemotherapy, which improved the quality of life in patients with EOC, however, the 5-year survival rate for all stages of ovarian cancer has been estimated to be 45.6% due to the high rate of recurrence and chemoresistance [3, 4]. Therefore, it is need to identify new biomarkers, treatments, and therapeutic targets for human epithelial ovarian cancer.

Micro-RNAs (miRNAs) are calss of single-stranded, small (18-25 nucleotides in length) non-coding RNA molecules that regulate the expression of target genes by pairing with sites in the 3' untranslated region (30-UTR) [5]. Increasingly evidence has suggested that miR-NAs play important role in many physiological and pathological processes, such as cell growth and differentiation, proliferation, apoptosis, vascular angiogenesis and embryonic development [6, 7]. It has been showed that miRNAs function as oncogenes or tumor suppressor, and involve in various processes of tumor progression including development, differentiation, apoptosis, proliferation, cell cycle, and metastasis [6, 8, 9]. Therefore, miRNAs are attractive candidates in study of cancer physiological and pathological processes.

MicroRNA-126 (miR-126), an important miRNA, has been reported to have important functions in hepatocellular carcinoma [10], renal cell carcinoma [11], osteosarcoma [12], gastric cancer [13], cervix cancer [14], non-small cell lung cancer [15], colorectal cancer [16], and colon cancer [17]. However, to our knowledge, the precise roles and mechanisms of miR-126 in epithelial ovarian cancer remain unclear. Thus, in the present study, we investigate the biological role

Table 1. Correlation between clinicopathological features and miR-126 expression in EOC tissues

Variables	No. of cases	miR-494 expression		P value
		Low (n %)	High (n %)	
Age (years)				P>0.05
<55	24	13 (59.1)	11 (40.9)	
≥55	16	10 (62.5)	6 (37.5)	
Tumor size				P>0.05
≥5	23	12 (52.2)	11 (47.8)	
<5	17	11 (64.5)	6 (35.5)	
FIGO stage				P<0.01
I-II	30	14 (46.7)	16 (53.3)	
III-IV	10	9 (90.0)	1 (10.0)	
Histological grading				P<0.01
1-2	28	13 (46.4)	15 (53.6)	
3	12	10 (83.3)	2 (16.7)	
Lymph node metastasis				P<0.01
No	33	16 (48.5)	17 (52.5)	
Yes	7	7 (100.0)	0 (0.0)	

and underlying molecular mechanism of miR-126 on the carcinogenesis of epithelial ovarian cancer.

Materials and methods

Clinical specimens

Epithelial ovarian cancer tissues and their paired adjacent normal tissues were obtained from the 40 patients with EOC who underwent surgery at the First Hospital of Jilin University (Changchun, China) from June 2010 to July 2015. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. All the patients were diagnosed as EOC and the samples were histologically confirmed by pathologist. None of patients had received either radiotherapy or chemotherapy. Clinical data including age, tumor size, FIGO stage, histological grading and lymph node metastasis was collected and listed in **Table 1**. Informed consent was obtained from each patient, and the study protocols were approved by the Ethics Committee of Jilin University (Changchun, China).

Cell culture

A human ovarian surface epithelial cell line (HOSEpiC) and four human ovarian cancer cells SKOV3, A2780, OVCAR and HO-8910 were obtained primarily from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum(FBS, Gibco BRL), 100 U/mL of penicillin and 100 mg/mL of streptomycin at 37°C in a humidified chamber supplemented with 5% CO_2.

Real time quantitative RT-PCR

Total RNA from harvested cells or human tissues was isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. To detect miR-126 expression, cDNA was synthesized from 10 ng of total RNA using TaqMan™ miRNA hsa-miR-126 specific primers (Applied Biosystems Life Technologies, Beijing, China) and a TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems Life Technologies). Quantitative PCR were performed by the TaqMan miRNA assay kits (Applied Biosystems, Foster City, CA, USA) under ABI 7900 Fast system (Applied Biosystems). Expression of U6 was used as an endogenous control. To determine the mRNA levels of IRS2, cDNA was synthesized by PrimeScript RT reagent Kit (Takara, Dalian, China) following the manufacturer's instructions. The expression levels of IRS2 were quantified by Real-time PCR Mixture Reagent (Takara) under ABI 7900 Fast system. The primers for IRS2 and β-actin were described as previous study [18]. β-actin was used as internal control, The comparative $2^{-\Delta\Delta Ct}$ method was used for relative quantification and statistical analysis.

Transfection

miR-126 mimic or corresponding negative control (miR-NC) were purchased from GenePharma (Shanghai, China), and were respectively transfected into SKVO3 cells at final concentration 100 nM using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Transfection efficiencies were evaluated in every experiment by qRT-PCR at 48 h post-transfection.

Cell proliferation assay

Cell proliferation was assessed with a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. In briefly, SKVO3 cells were seeded in 96-well culture plates at a density of 5×10^3 cells per well 24 h after transfected with miR-126 or miR-NC. After 1 to 4 days, cells were stained with incubated with 20 µl MTT reagent (5 mg/ml, Sigma, St. Louis, MO) for 4 h at 37°C. The cell medium was carefully removed, and incubated with 200 µl of dimethyl sulfoxide (DMSO, Sigma) was added to each well to dissolve the crystals for 10 min at 37°C. The absorbance in each well was measured with a microplate reader (Tecan, Männedorf, Switzerland) set at 490 nm.

Cell apoptosis assay

Cells apoptosis analysis was performed on SKVO3 cells 48 h after transfection. Transfected cells were incubated with PE Annexin-V and 7AAD following the PE Annexin-V Apoptosis Detection Kit I (BD Pharmingen, CA, USA) protocol, and then were analyzed by under a flow cytometer (BD Biosciences San Jose, CA, USA). The apoptotic rate was analyzed using Cell-Quest software (BD Biosciences).

Migration and invasion assay

To examine the migration ability of cells *in vitro*, a wound-healing assay was performed. In briefly, transfected cells were seeded in 3.5-cm plates and grown to a density of 70 to 80%. Afterwards, cells were scratched using a sterile plastic micropipette tip to create an artificial wound. Cells were imaged at 0 and 24 h after the wounding, and the migrating distance was measured after 24 h.

For invasion assay, a transwell chamber assay was performed. Transfected cells (2×10^4 cells/well) were placed in the upper chamber of a 24-well Matrigel-coated Transwell unit with 8 µm -pore-size polycarbonate nucleopore filters (Corning Costar, Cambridge, MA), and cultured in serum-free DMEM medium. The lower chamber was filled with DMEM medium with 10% FBS to attract cells. After cells had been cultured at 37°C for 48 h, the cells adhering to the lower surface were fixed with 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. The invaded cells were photographed and were counted in five random fields of view

at 100 × magnification under light microscope (Olympus, Tokyo, Japan).

Luciferase assay

The 3'UTR of IRS2 was amplified using PCR and subcloned into pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and XhoI restriction sites. Mutant constructs of IRS2 were also generated by introducing mutated nucleotides within the seed region-binding sequences in the oligonucleotides, and inserted into pGL3-control vector at the NheI and XhoI restriction sites. For luciferase assays, the SKVO3 cells were plated in 24-well plates at a density of 2×10^4 cells per well and transfected with 100 ng of IRS2-3'UTR-WT or IRS-3'UTR-Mut reported plasmid, and 100 nM of miR-126 mimic or miR-NC, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h post transfection, both firefly and Renilla luciferases activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization.

Western blotting

Protein extracts were performed from cultured cells or tissues through RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations were measured by using a BCA assay kit (Beyotime). Equal quantities (30 µg) of protein samples were loaded on 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Wisconsin, USA), and Blocking was performed with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 2 h, Then membrans incubated at 4°C overnight with following primary antibody: anti-human IRS2 (1:1000, Santa Cruz, USA) and anti-human β-actin (1:5000, Santa Cruz, USA), followed incubated with horseradish peroxidase (HRP)-conjugated second antibody (1:5000, Santa Cruz, USA) for 1 h at room temperature. β-actin was used as an internal control for protein loading. The protein bland was observed by enhanced chemiluminescence (ECL, Cell Signaling Technology).

Statistical analysis

All the statistics were expressed as the mean ± SD (standard deviation) of at least three sepa-
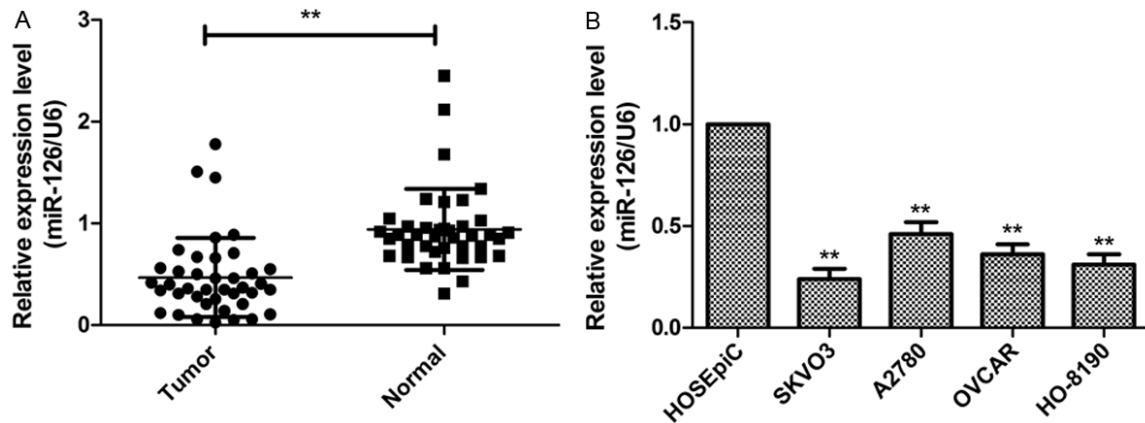
Figure 1. miR-126 expression is downregulated in epithelial ovarian cancer (EOC) tissues and cell lines. A. miR-126 expression in 40 EOC tissues and their corresponding adjacent normal tissues were detected by quantitative RT-PCR (qRT-PCR) *P<0.05; **P<0.01 versus normal tissue. B. miR-126 expression in four ovarian cancer cell lines (SKOV3, A2780, OVCAR and HO-8910) and human ovarian surface epithelial cell line (HOSEpiC) were detected by qRT-PCR. *P<0.05; **P<0.01 versus HOSEpiC.

rate experiments, and processed using SPSS 19.0 statistical software (Chicago, IL, USA). A P<0.05 was considered statistically significant.

Results

miR-126 is down-regulated in EOC tissues and cell lines

To determine whether miR-126 was involved in ovarian tumorigenesis, we evaluated the expression levels of miR-126 in EOC tissues and cell lines. qRT-PCR assay showed that expression of miR-126 was significantly decreased in EOC tissues compared with adjacent normal tissue, as well as was differentially decreased in four ovarian cancer cell lines compared with human ovarian surface epithelial cell line (HOSEpiC) (**Figure 1A** and **1B**). SKOV3 cells exhibited the lowest expression of miR-126, and were selected for next studies (**Figure 1B**).

To investigate the clinical relevance of miR-126 in EOC, the median (0.426) of all 40 patients was chosen as the cutoff point to divide two groups of all cases: low-miR-126 (<0.426, 23 cases) group and high-miR-126 expressing group (>0.426, 17 cases). It was found that miR-126 expression were negatively correlated with FIGO stage, histological grading and lymph node metastasis (all P<0.01), which are all indicators of poor prognosis (**Table 1**). Meanwhile, we did not find any correlation of miR-126 expression and age and tumor size. These studies suggest that miR-126 is down-regulated in EOC tissues and cell lines.

miR-126 inhibits EOC cell proliferation and induces cell apoptosis

To investigate the function of miR-126, we restored it expression by transfected miR-126 mimic in SKOV3 (**Figure 2A**). Proliferation of SKOV3 cells was determined by MTT assay at the indicated time (1-4 days). With time longer, difference in the proliferation rate became more significant in SKOV3 cells with miR-126 restoration compared with miR-NC group (**Figure 2B**), which suggested that restoration of miR-126 inhibits SKVO3 cell proliferation. In addition, we also investigate effect miR-126 on cell apoptosis. Flow cytometer assay showed that restoration of miR-126 induced cells apoptosis relative to miR-NC group (P<0.05, **Figure 2C**).
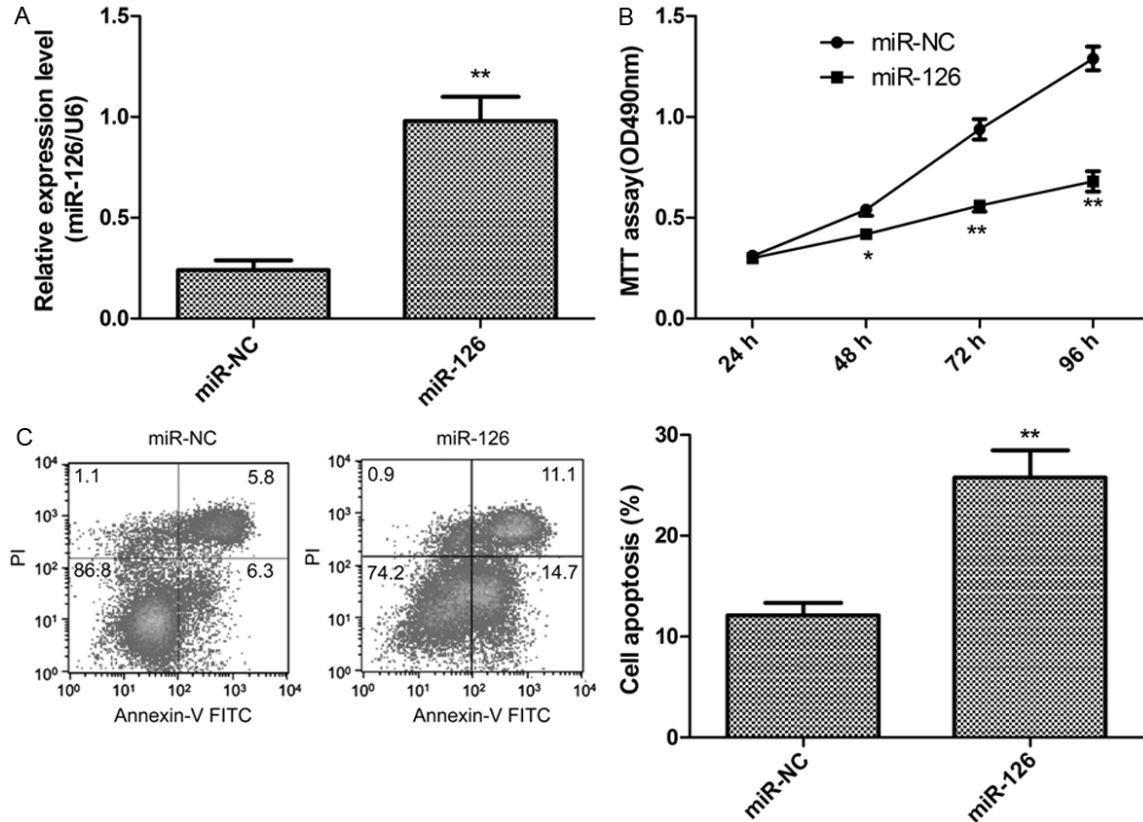
miR-126 inhibits EOC cell migration and invasion

We also investigate whether miR-126 effect on EOC migration and invasion in SKOV3 cells transfected with miR-126 mimic or miR-NC by wound heal and transwell chamber assay, respectively. Our results demonstrated that restoration of miR-126 expression could significantly suppress SKVO3 cell migratory (**Figure 3A**) and invasive (**Figure 3B**) capabilities.

IRS2 is a direct target of miR-126

To investigate the underlying mechanism of growth inhibition by miR-126 in EOC cells, we
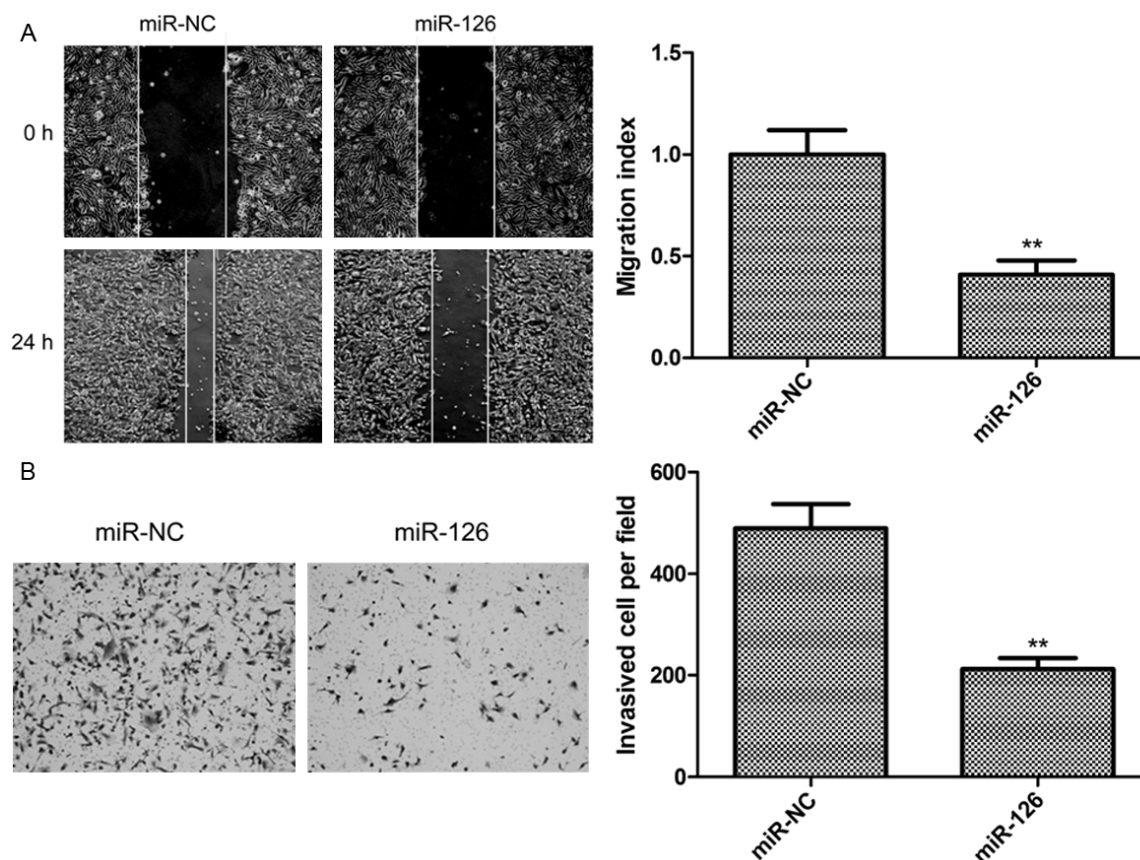
Figure 2. miR-126 inhibits EOC cell proliferation and induced cell apoptosis. A. miR-126 expression was restored in SKOV3 cells after transfected with miR-126 mimic, assessed by qRT-PCR. B. Cell proliferation was determined by MTT assay in SKVO3 cells transfected with miR-126 or miR-NC. C. Cell apoptosis was determined by flow cytometer assay in SKVO3 cells transfected with miR-126 or miR-NC. P<0.05; **P<0.01 versus miR-NC.

looked for its potential downstream targets using two publicly available algorithms (Targetscan6.2 and miRanda) to help identify miR-126 targets in EOC cells. Based on the predicted binding region of miR-126 in the 3'-UTR of IRS2, we propose that IRS2 is a direct target of miR-126 (**Figure 4A**). To further confirm this propose, dual-luciferase reporter assay were performed, and found that restoration of miR-126 significantly decreased the luciferase activity of the IRS 3'-UTR-WT in SKVO3 cells (**Figure 4B**), while had no inhibition effect on the mutant IRS2-3'UTR reporter activity in SKVO3 cells (**Figure 4B**), indicting the direct regulation of miR-126 in the 3'UTR of IRS2 mRNA. In addition, qRT-PCR and western blotting showed that restoration of miR-126 in SKOV3 cells significantly decrease IRS2 expression on mRNA level (**Figure 4C**) and protein level (**Figure 4D**). These results suggest that IRS2 is a direct target of miR-126.

IRS2 expression was upregulated and inversely correlated with miR-126 expression in EOC tissues

Since it has been confirmed that IRS2 is a direct target of miR-126, we determined the expression of IRS2 expression in EOC tissues and corresponding normal tissue. We found that IRS2 expression on mRNA levels (**Figure 5A**) and protein levels (**Figure 5B**) were upregulated compared with matched normal tissue. Using Spearman's correlation analysis, we found that IRS2 mRNA expression was inversely correlated with miR-126 expression in EOC tissues (**Figure 5C**; r=-0.421, P<0.05). Meanwhile, we also detected the IRS2 expression in four ovarian cancer cell lines (SKOV3, A2780, OVCAR and HO-8910) and human ovarian surface epithelial cell line (HOSEpiC). Western blot assay showed that IRS2 protein expression was obviously upregulated in four ovarian cancer

Figure 3. miR-126 inhibits EOC cell migration and invasion. A. Cell migration was determined by wound healing assay in SKVO3 cells transfected with miR-126 or miR-NC. B. Cell invasion was determined by invasion chamber assay in SKVO3 cells transfected with miR-126 or miR-NC. P<0.05; **P<0.01 versus miR-NC.

cell lines compared with human ovarian surface epithelial cell line (HOSEpiC) (**Figure 5D**).

Discussion

Accumulating evidence had shown that microRNAs (miRNAs), a class of small non-coding RNAs, are important regulators involved in cell proliferation, cycle, apoptosis, invasion and migration of multiple types of human cancers, including ovarian cancer [19, 20]. A larger number of miRNAs has been identified to play a role in control cell proliferation, metastasis, and cell cycle in ovarian cancer [19, 20]. For example, Wen *et al* [21] found that that miR-338-3p functions as a tumor suppressor and suppresses tumor growth of EOC *in vitro* and *in vivo* through PI3K/AKT signaling pathways by targeting Runx2. Zhu *et al* [22] reported that miR-661 functions as tumor promoter by targeting the INPP5J gene, and then promoting cell proliferation of ovarian cancer. Lan *et al* [23] showed that miR-140-5p inhibited ovarian cancer cell

proliferation and induces apoptosis partially by repression of PDGFRA. In the present study, our results first showed that miR-126 expression was obviously decreased in EOC tissue and cell lines, and its expression was negative associated with FIGEO stage, histological grading and lymph node metastasis. Our results also showed that restoration of miR-126 significantly inhibited EOC cell proliferation, migration and invasion, and induced cell apoptosis. These results suggested that miR-126 might play crucial roles in the carcinogenesis of EOC.

The sequence encoding miR-126, located at intron 7 of the EGFL7 gene, had been showed that it expression could be epigenetically regulated with the EGFL7 gene [24]. Downregulation of miR-126 expression has been found in hepatocellular carcinoma [10], renal cell carcinoma [11], osteosarcoma [12], gastric cancer [13], cervix cancer [14], non-small cell lung cancer [15], colorectal cancer [16], and colon cancer [17]. An increasing number of studies have
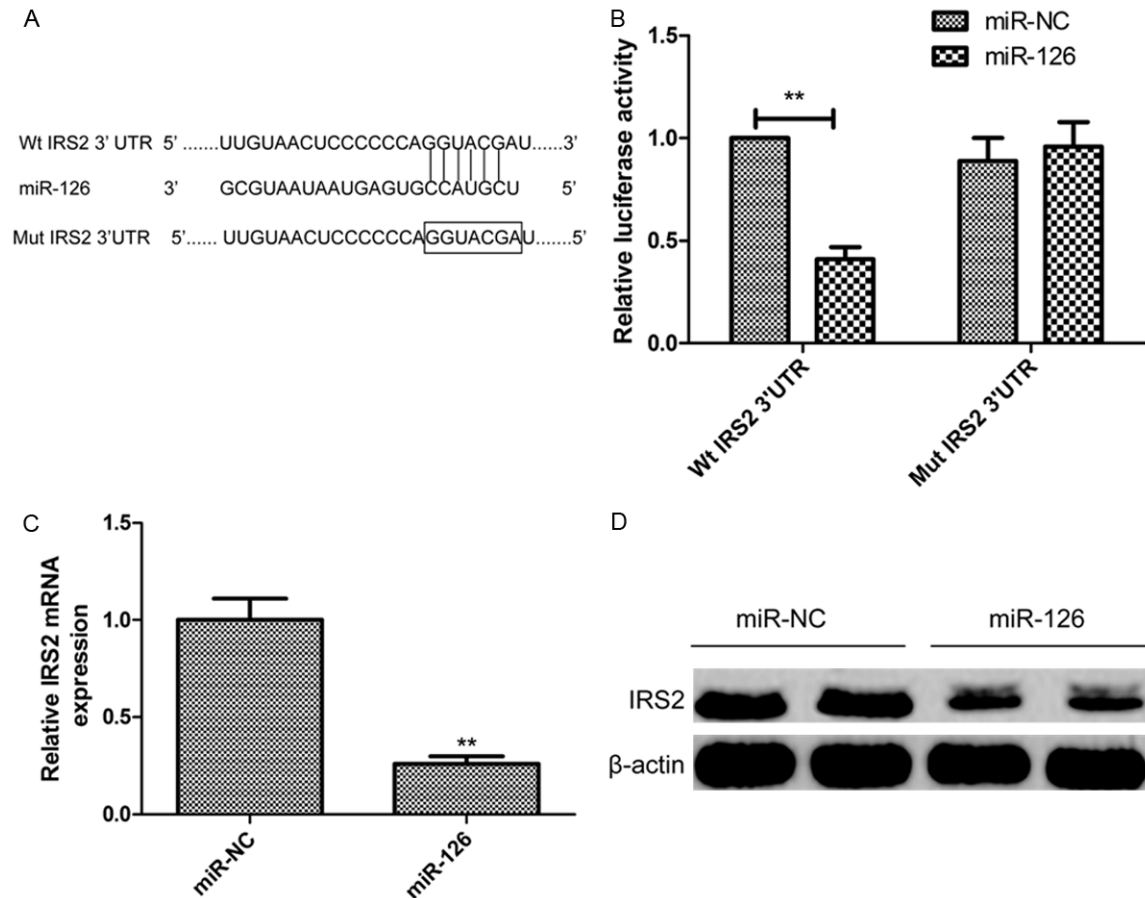
Figure 4. IRS2 is a direct target of miR-126. A. The putative miR-126-binding sites and mutant (Mut) 3'-UTR IRS2 sites was shown. B. Luciferase assay were measured in SKVO3 cells cotransfected with miR-126 or miR-NC and IRS2 3'UTR (Wt) or a mutant (Mut) reported plamid. Wt: Wide type; Mut: Mutant type. C. IRS2 mRNA expression was analyzed in SKVO3 cells transfected miR-126 mimic or miR-NC by qRT-PCR assay. β-actin was used as an internal control. D. IRS2 protein expression was determined in SKVO3 cells transfected miR-126 mimic or miR-NC by Western blot assay. β-actin was used as an internal control. $P < 0.05$; $**P < 0.01$ versus miR-NC.

showed that miR-126 plays a potential role as a tumor suppressor in many kinds of cancers [10-17]. For ovarian cancer, only a study showed that miR-126 could inhibit serine/threonine p21-activated kinase 4 (PAK4) protein expressions in ovarian cancer cells [25]. However, the expression and function of miR-126 in ovarian cancer remains unclear. In this study, our results showed that miR-126 expression level was decreased in EOC tissue and cell lines, and that restoration of miR-126 significantly inhibited EOC cell proliferation, migration and invasion. These results suggested that miR-126 functions as tumor suppressor in epithelial ovarian cancer.

IRS2, located in the 13q34 region, is important member of the insulin receptor substrates (IRSs) family [26]. IRSs are cytoplasmic scaf-

fold proteins that act as signaling intermediates through which downstream intracellular signals are generated, which in turn allows initiation of intracellular signaling cascades, such as the Wnt/ß-catenin pathway [27]. Overexpression of IRS2 has been found in many types of cancers, including ovarian cancer [28]. It has been showed that IRS2 play an oncogenic role in various cancers, including ovarian cancer [29]. In this study, we confirmed IRS2 was a direct target of miR-126 by luciferase reporter assay. Restoration of miR-126 expression in SKVO3 cells inhibited IRS expression on mRNA level and protein level. Of note, our results showed that IRS2 expression was upregulated in EOC tissue and cell lines and its expression inversely correlated with miR-126 expression in EOC tissues. These results might suggest that
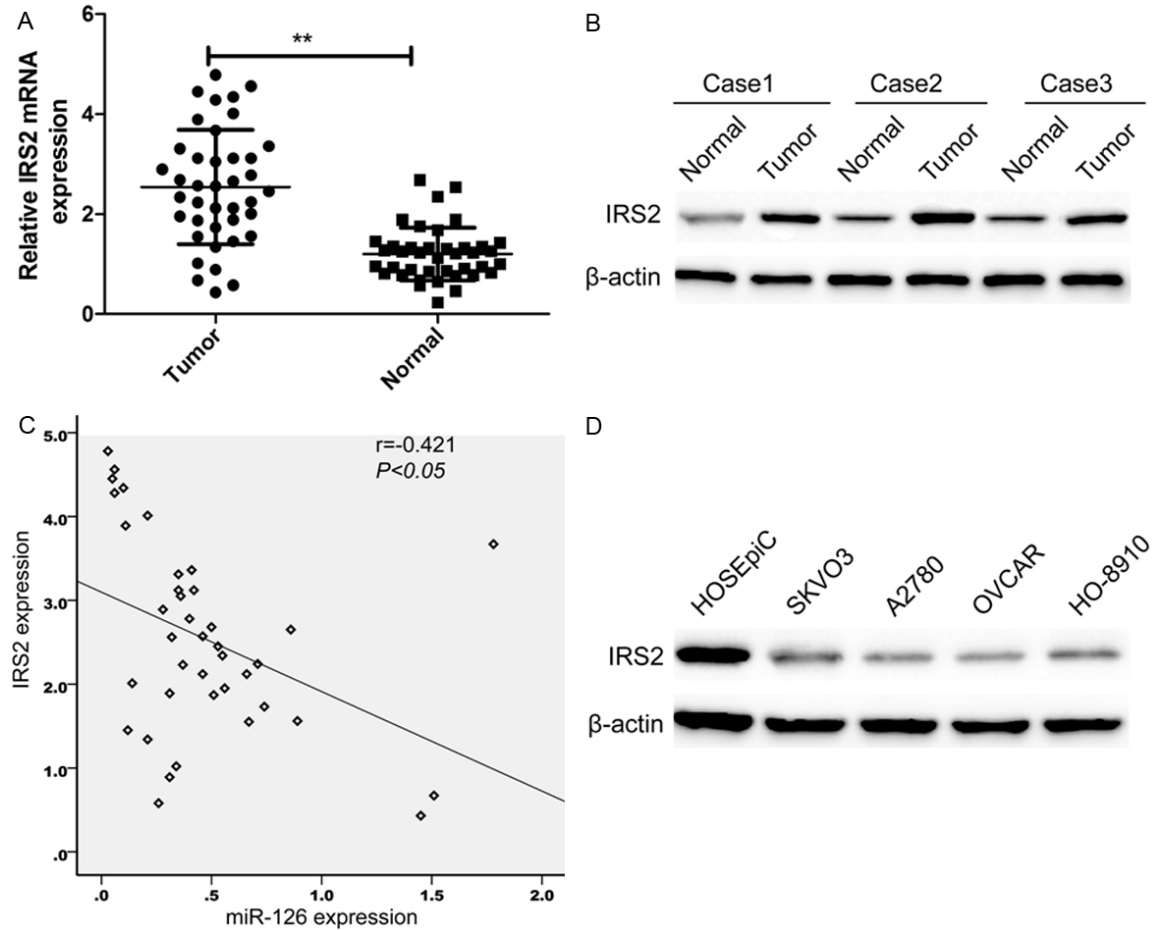
Figure 5. IRS2 was up-regulated and inversely correlated with miR-126 expression in EOC tissues. A. IRS2 mRNA expression in human EOC tissues and their corresponding normal tissues was determined by qRT-PCR. β-actin was used as an internal control. *P<0.05; **P<0.01 versus normal tissue. B. IRS2 protein expression in human EOC tissues and their corresponding normal tissues was determined by Weston bolt. β-actin was used as an internal control. *P<0.05; **P<0.01 versus normal tissue. C. The reverse relationship between IRS2 mRNA expression and miR-126 expression was explored in EOC tissues by Spearman's correlation. D. IRS2 protein expression was determined in four ovarian cancer cell lines (SKOV3, A2780, OVCAR and HO-8910) and human ovarian surface epithelial cell line (HOSEpiC). β-actin was used as an internal control.

miR-126 exerted tumor suppressor in epithelial ovarian cancer by repression of IRS2.

In summary, the results presented here first demonstrate that miR-126 expression level was decreased in EOC tissue and cell lines, and its expression was negatively correlated with advanced FIGEO stage, high histological grading and lymph node metastasis. Our finding also demonstrated that miR-126 inhibited EOC cell proliferation, migration and invasion, as well as induced cell apoptosis via directly targeting IRS2, suggesting that miR-126 might be developed as a new therapeutic target in epithelial ovarian cancer.

Disclosure of conflict of interest

None.

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