

Original Article

microRNA-29a repressed epithelial ovarian cancer progression by directly targeting SIRT1

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Abstract: Ovarian cancer is the leading cause of cancer-related deaths in gynecologic malignancies. Over the past 30 years, the death rates of ovarian cancer remain largely unchanged. Therefore, it is urgent to understand the mechanisms contributed to carcinogenesis and progression of ovarian cancer. In this study, we investigated the expression, effects and molecular mechanism of microRNA-29a in epithelial ovarian cancer cells. The expression levels of microRNA-29a in EOC tissues and cell lines were determined by qRT-PCR. The microRNA-29a mimic or NC was introduced into epithelial ovarian cancer cells using Lipofectamine™ 2000, followed by cell viability assay, migration and invasion assay. Subsequently, molecular mechanism underlying the suppressive effects of microRNA-29a overexpression in EOC was determined by bioinformatics analysis, qRT-PCR, Western blot and luciferase reporter assay. Moreover, the effects of SIRT1 downregulation on growth, migration and invasion in epithelial ovarian cancer cells were measured. Our results showed that the microRNA-29a expression levels were obviously decreased in epithelial ovarian cancer tissues and cell lines. Overexpression of microRNA-29a inhibited the proliferation, migration and invasion of epithelial ovarian cancer cells. Bioinformatics analysis predicted that the SIRT1 was a potential target gene of microRNA-29a. qRT-PCR, Western blot and luciferase reporter assay indicated that microRNA-29a repressed SIRT1 expression by binding its 3'UTR. SIRT1 siRNA has the similar suppressive effects with microRNA-29a overexpression in epithelial ovarian cancer cells, further confirming that SIRT1 was a direct target of microRNA-29a in epithelial ovarian cancer. Taken together, enforced microRNA-29a expression inhibited proliferation, migration and invasion of epithelial ovarian cancer cells via directly targeting SIRT1.

Keywords: microRNA-29a, SIRT1, progression, epithelial ovarian cancer, EOC

Introduction

Ovarian cancer, the fourth most common cancer among women worldwide, is the most lethal of the gynecologic malignancies [1]. In 2015, it is estimated that approximately 21,290 women will receive a new diagnosis of ovarian cancer and 14,180 women will die due to ovarian cancer [2]. In general, according to the morphological origin of ovarian cancer, it can be split into three major types: epithelial, stromal and germ cell [3]. Epithelial ovarian cancer (EOC), including serous adenocarcinoma, clear cell carcinoma and endometrial adenocarcinoma, is believed to originate from the surface epithelium of the ovary [4]. It is the most common kind of ovarian cancer and accounts for approximately 90% of ovarian cancer cases [5, 6]. Despite encouraging developments in diagnos-

tic method and treatments of EOC, the five-year overall survival rate is still low as a result of the high recurrence rate and metastasis [7]. It has been demonstrated that cancer metastasis is the leading cause of relapse and death from ovarian cancer [8]. Therefore, it is essential and of great interest to explore the molecular mechanism underlying EOC, and then investigate the effective therapeutic treatments to repress EOC progression. microRNAs (miRNAs) are an abundant group of endogenous, single-stranded and non-coding RNAs with regulatory functions that play important functions in carcinogenesis and progression of human cancers [9, 10]. They regulate target genes expression through targeting the 3'-untranslated region (3'-UTR) of target genes for translational repression and/or degradation [11, 12]. miRNAs have been identified to be involved in a wide reper-

toire of biological mechanisms, including cell proliferation, cell apoptosis, cell migration, cell invasion, tumorigenesis, and metastasis [13]. Increasing studies have demonstrated that abnormal expression of miRNAs has been found in many human cancers, also including EOC [14-16]. In these cancers, miRNAs can act as oncogenes or tumor suppressor genes depending on their target genes [17, 18]. Originally, miRNAs, down-regulated in cancers, act as tumor suppressors, inhibiting carcinogenesis and progression by negatively regulation of oncogenes. In contrast, miRNAs are up-regulated in tumors and generally participate in tumor suppressors over-expression [11, 19]. These findings indicated that well understanding of the expression patterns, roles and molecular mechanisms of miRNA-mediated processes may provide tremendous progress in the therapy for human cancers.

In this paper, we determined frequent down-regulation of miR-29a in EOC tissues and cell lines. Overexpression of miR-29a inhibited cell growth, migration and invasion of EOC cells. Furthermore, SIRT1 was validated as the direct target of miR-29a in EOC and confirmed that miR-29a acted as a tumor suppressor by down-regulation of SIRT1. Therefore, our findings showed important functions for miR-29a in the progression of EOC, and indicated its possible application in EOC treatment.

Material and methods

Ethics statement

This study was approved by the Ethics Committee of The first affiliated hospital of Zhejiang Chinese Medical University and complied with the Declaration of Helsinki. All patients participated into this study gave written informed consent.

Tissue samples

A total of 36 EOC tissues and paired adjacent specimens (normal) were used in this study. All tissue samples were acquired from patients with primary EOC who underwent surgery at the first affiliated hospital of Zhejiang Chinese Medicine University. EOC tissues and paired adjacent specimens were snap-frozen in liquid nitrogen after surgical resection and then stored at -80°C until RNA extraction.

Cell lines, culture conditions and transfection

Human EOC cell lines OVCAR3, SKOV3, A2780, ES-2, CAOV3 and human normal ovarian epithelial cell line NOEC were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HEK293T cell line was also obtained from ATCC. NOEC cells were cultured in cell culture medium consisting of Ham's F-12 medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 120 mg/ml streptomycin (Gibco, Grand Island, NY) and 120 mg/ml penicillin (Gibco, Grand Island, NY). OVCAR3, SKOV3, A2780, ES-2, CAOV3 and HEK293T cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin. All cells were cultured in a humidified 5% CO₂ cell incubator at 37°C.

The miR-29a mimics and negative control mimics (NC) were purchased from GenePharma (Shanghai, China). SIRT1 siRNA and control siRNA were obtained from RiboBio Co., Ltd (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was adapted to the transfection of miRNA mimics, NC or siRNA in EOC cells, following to the manufacturer's instructions.

Total RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA of tissues and cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of total RNA was determined using the ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, USA). For miR-29a expression, total RNA was reverse transcribed into cDNA with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), followed by qRT-PCR using Taqman microRNA assay kit (Applied Biosystems, Foster City, CA, USA). To detect SIRT1 mRNA level, RNA was first reverse-transcribed with M-MLV reverse transcriptase (Promega, Beijing, China) and qRT-PCR was performed with SYBR Green Mix Taq™ Kit (TaKaRa, Japan). U6 snRNA and β-actin mRNA were used as an endogenous control for miR-29a and SIRT1 mRNA, respectively.

miR-29a in epithelial ovarian cancer

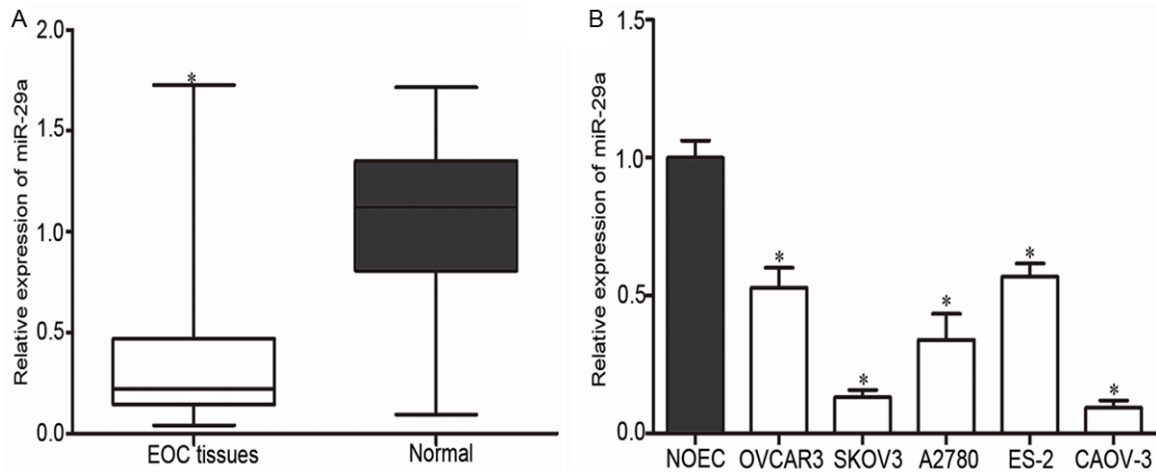


Figure 1. miR-29a was down-regulated in EOC. A. The expression of miR-29a was determined by qRT-PCR in EOC tissues and paired adjacent specimens. B. The expression of miR-29a was determined by qRT-PCR in EOC cell lines and human normal ovarian epithelial cell line. * $P < 0.05$ compared with their respective controls.

Cell viability assay

To evaluate cell viability, 4,000 cells/well were plated into 96-well plates. After being cultured for 24 h, cells were transfected with miRNA mimics or siRNA using Lipofectamine 2000. At different time points after transfection, cell viability assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay. In briefly, 20 μ l MTT assay reagent (5 mg/mL) was added into each well, and the plates were incubated for another 4 h. Then, the medium containing MTT assay reagent was carefully removed and 150 μ l of dimethyl sulfoxide (DMSO) (Sigma St. Louis, MO) was added into each well to dissolve the formazan precipitates. The absorbance at 490 nm was detected with a spectrophotometer.

Migration and invasion assay

24-well transwell chambers with 8 μ m-pore-size filter (Corning Costar, Cambridge, MA) were applied to perform migration assay and invasion assay. The chambers were precoated with or without Matrigel (BD Biosciences, San Jose, CA, USA) for invasion and migration assay, respectively. In briefly, 1×10^5 transfected cells were suspended into 100 μ l serum-free medium and seeded into upper chambers.

500 μ l culture medium containing 20% FBS was added into the lower chamber. At 24 h

after incubation, cells that remained in the upper surface of the filter were carefully removed with cotton swabs. Migrated and invaded cells were fixed with 100% methanol and stained with 0.5% crystal violet. The numbers of migrated or invaded cells were counted with a microscope.

Western blot

Total protein of transfected cells was isolated by using ice-cold lysis buffer with Protease inhibitors. Equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) on 10% gel, followed by transferring to poly vinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were immunoblotted overnight at 4°C with primary antibodies: mouse anti-human monoclonal SIRT1 (sc-74504; Santa Cruz Biotechnology, CA, USA) and GADPH (sc-51907; Santa Cruz Biotechnology, CA, USA). The membranes were then incubated with their respective horseradish peroxidase-conjugated secondary antibodies and visualized using an ECL-plus reagents (Amersham Biosciences Corp., USA).

Luciferase report assay

The 3'UTR and mutant 3'UTR of SIRT1 into the pGL3-promotor vector was obtained from GenePharma (Shanghai, China). Cells were seeded into 24-well plates and transfected with miR-

miR-29a in epithelial ovarian cancer

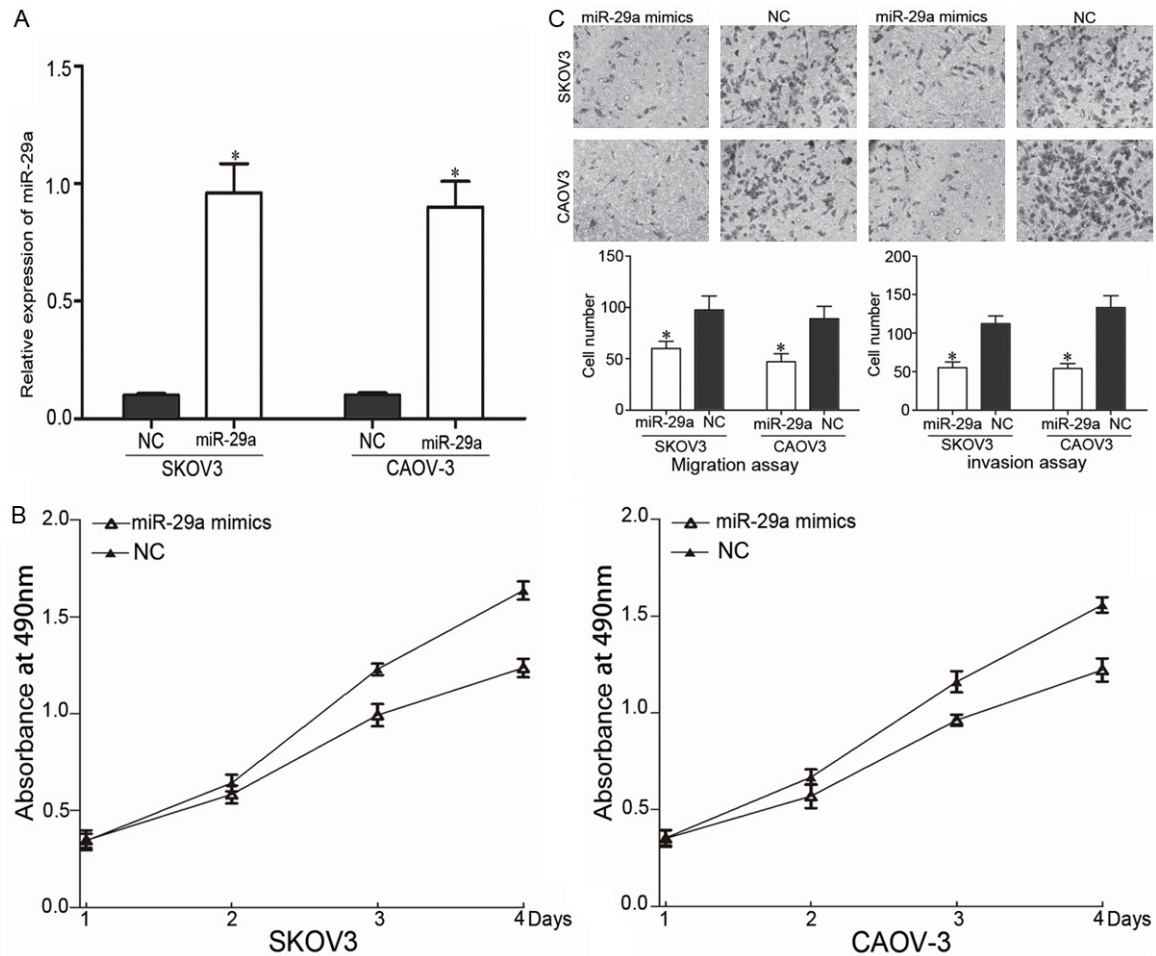


Figure 2. Overexpression of miR-29a inhibited proliferation, migration and invasion of EOC cells. A. The expression of miR-29a was determined by qRT-PCR in SKOV3 and CAOV3 cells transfected with miR-29a mimics or NC. B. The effect of miR-29a on cell proliferation was determined by cell viability assay. C. The effect of miR-29a on cell migration and invasion abilities was determined with migration and invasion assays. * $P < 0.05$ compared with their respective controls.

29a mimics or NC and then co-transfected with pGL3-SIRT1-3'UTR Wt or pGL3-SIRT1-3'UTR Mut. After incubation 48 h, cells were collected and luciferase report assay was performed with Dual-luciferase assay system (Promega, Madison, USA). Renilla luciferase activity was used as control for luciferase activity.

Statistical analysis

All experimental data from independent experiments were analyzed by GraphPad Prism 5.0 and results were expressed as mean \pm SD (standard deviation, SD). The differences between groups were compared using SPSS 17 software (SPSS Inc., Chicago, IL, USA). Double-tailed $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-29a was down-regulated in EOC

In order to investigate the expression of miR-29a in clinical tissues, miR-29a expression in EOC tissues and paired adjacent specimens was detected using qRT-PCR. The results showed that miR-29a was significantly down-regulated in EOC tissues in contrast to paired adjacent specimens (**Figure 1A**).

Subsequently, expression levels of miR-29a were also determined among EOC cell lines OVCAR3, SKOV3, A2780, ES-2, CAOV3 and human normal ovarian epithelial cell line NOEC. Strikingly, the results had confirmed that miR-29a was expressed at a lower level in all the

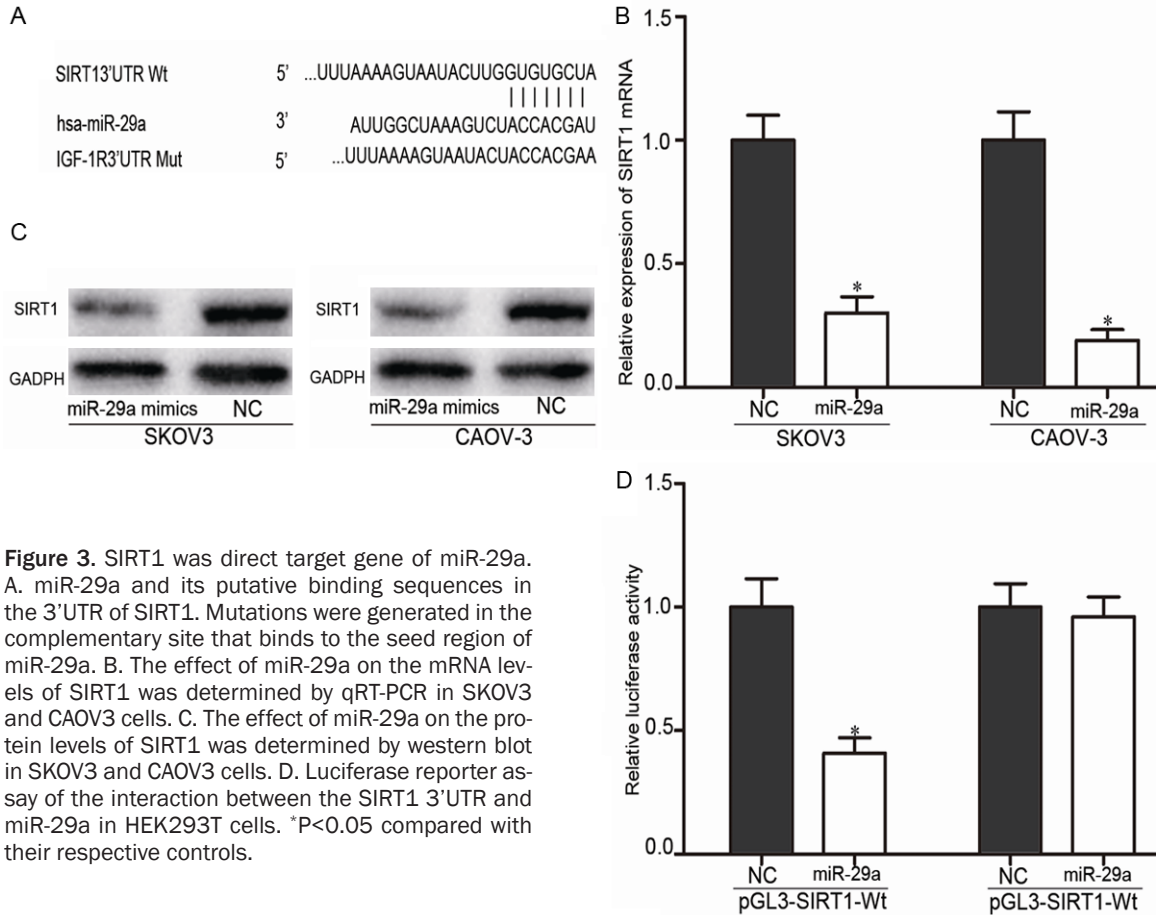


Figure 3. SIRT1 was direct target gene of miR-29a. A. miR-29a and its putative binding sequences in the 3'UTR of SIRT1. Mutations were generated in the complementary site that binds to the seed region of miR-29a. B. The effect of miR-29a on the mRNA levels of SIRT1 was determined by qRT-PCR in SKOV3 and CAOV3 cells. C. The effect of miR-29a on the protein levels of SIRT1 was determined by western blot in SKOV3 and CAOV3 cells. D. Luciferase reporter assay of the interaction between the SIRT1 3'UTR and miR-29a in HEK293T cells. *P<0.05 compared with their respective controls.

five EOC cell lines than in NOEC, in accordance with the result of clinical analysis (Figure 1B).

miR-29a overexpression reduced proliferation, migration and invasion of EOC cells

Given that miR-29a was down-regulated in EOC, we hypothesized that miR-29a may act as a tumor suppressor in carcinogenesis and progression of EOC. To analyze the functions of miR-29a, SKOV3 and CAOV3 cells were transfected with miR-29a mimics or NC. Compared with the NC groups, transfection of miR-29a mimics obviously upregulated miR-29a expression in SKOV3 and CAOV3 cells (Figure 2A). Cell viability assay was performed following the transfection of miR-29a mimics or NC. The results showed that miR-29a overexpression reduced the cellular viability in SKOV3 and CAOV3 cells (Figure 2B). In addition, the effects of miR-29a on EOC cell migration and invasion ability was analyzed. The results showed that miR-29a overexpression inhibited the cellular migration and invasion ability in SKOV3 and

CAOV3 cells (Figure 2C). All these results indicated that miR-29a acted as a tumor suppressor in EOC.

miR-29a directly represses SIRT1 by binding its 3'UTR

To investigate the molecular mechanism for miR-29a's suppressive effects on EOC, TargetScan (<http://www.targetscan.org>) was used to predict potential target genes of miR-29a. Bioinformatics analysis showed the presence of miR-29a binding site on the 3'UTR of SIRT1 (Figure 3A). To confirm the correlation between miR-29a and SIRT1, qRT-PCR, western blot and luciferase reporter assays were performed to explore whether miR-29a could repress SIRT1 expression by binding its 3'UTR. The results showed that the SIRT1 expression levels were lower at both mRNA and protein levels in miR-29a-transfected SKOV3 and CAOV3 cells than that in NC groups (Figure 3B and 3C). Luciferase reporter assays indicated that luciferase activity was reduced by miR-29a in the pGL3-SIRT1-

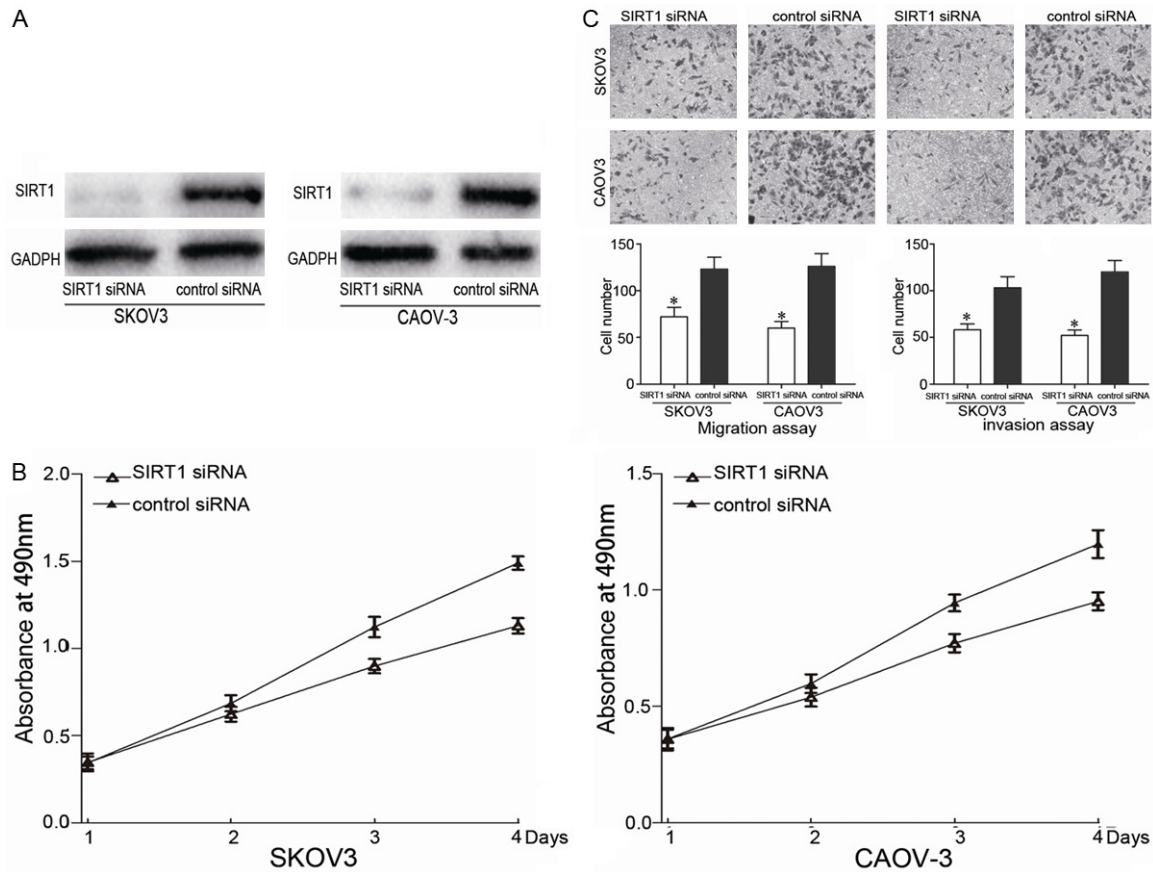


Figure 4. Knockdown of SIRT1 by siRNA inhibited proliferation, migration and invasion of EOC cells. A. The expression of SIRT1 protein was determined by western blot in SKOV3 and CAOV3 cells transfected with SIRT1 siRNA or control siRNA. B. The effect of SIRT1 siRNA on cell proliferation was determined by cell viability assay. C. The effect of SIRT1 siRNA on cell migration and invasion abilities was determined with migration and invasion assay. *P<0.05 compared with their respective controls.

3'UTR Wt group, but not in the pGL3-SIRT1-3'UTR Wt group (Figure 3D). Taken together, SIRT1 was identified as a direct target gene of miR-29a in EOC.

Knockdown of SIRT1 by siRNA inhibited proliferation, migration and invasion of EOC cells

The above results indicated that miR-29a directly targeted SIRT1 and reduced proliferation, migration and invasion of EOC cells. If these effects of miR-29a on EOC were mediated by SIRT1, knockdown of SIRT1 should have similar effects to miR-29a overexpression. In this study, SIRT1 siRNA was used to decrease SIRT1 expression in EOC cells. After transfection, SIRT1 was strongly down-regulated in SKOV3 and CAOV3 cells (Figure 4A). Cell viability assays, migration and invasion assays revealed that knockdown of SIRT1 by siRNA sig-

nificantly reduced proliferation, migration and invasion ability of SKOV3 and CAOV3 cells compared with those in the control siRNA group (Figure 4B and 4C). These results suggested that SIRT1 siRNA has the similar suppressive effects with miR-29a overexpression in EOC cells, further confirming that SIRT1 was a direct target of miR-29a in EOC.

Discussion

Ovarian cancer is the leading cause of cancer-related deaths in gynecologic malignancies. Over the past 30 years, the death rates of ovarian cancer remain largely unchanged, with a 5-year overall survival rate of only 30-39% [20]. Therefore, it is urgent to understand the mechanisms contributed to carcinogenesis and progression of ovarian cancer. Abnormal expression of miRNAs was involved in tumorigenesis

and development of malignant tumors through regulation of tumor suppressor genes and oncogenes [21, 22]. More importantly, accumulated studies have demonstrated that miRNAs play important functions in ovarian cancer [23-25]. In this study, we investigated the expression, biological function and mechanism of miR-29a in EOC. Our results showed that miR-29a was obviously reduced in EOC tissues and cell lines compared with that in paired adjacent specimens and human normal ovarian epithelial cell line, respectively. Over-expression of miR-29a suppressed growth, migration and invasion of EOC cells. SIRT1 was demonstrated as the direct target gene of miR-29a in EOC, and miR-29a acted as tumor suppressive roles in EOC via down-regulation of SIRT1. These findings indicated that miR-29a may be a potential therapeutic target for EOC.

miR-29a, a member of miR-29 family, has been found up-regulated in colorectal cancer [26]. However, the vast majority of studies indicated that miR-29a was down-regulated in human cancers, including prostate cancer [27], gastric cancer [28], esophageal carcinoma [29], pancreatic cancer [30], lung adenocarcinoma [31], oral squamous cell carcinoma [32], acute myeloid leukemia [33], and hepatocellular carcinoma [34]. These conflicting studies suggested that miR-29a expression in human cancers has tissue specificity. In the present study, we showed that miR-29a was significantly down-regulated in EOC tissues and cell lines. Our work expanded the expression of miR-29a in cancers.

miR-29a was defined as a tumor suppressor in many human cancers. Han et al. reported that miR-29a inhibited proliferation, migration and invasion of lung adenocarcinoma cells through targeting carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) [31]. Lu et al. found that up-regulation of miR-29a suppressed invasion and anti-apoptosis ability of oral squamous cell carcinoma cells via negatively regulation of matrix metalloproteinase 2 (MMP2). Zhao and his colleagues showed that miR-29a inhibited gastric cancer growth and metastasis by down-regulation of CDK2, CDK4, and CDK6 [35]. Liu also reported that miR-29a decreased migration and invasion ability of gastric cancer cells via blockade of Roundabout homolog 1 (Robo1) [28]. However, miR-29a was

demonstrated as an oncogene in colorectal cancer. It targeted KLF4 to enhance migration and invasion of gastric cancer cells. These conflicting studies indicated that the functions of miR-29a in human cancers also have tissue specificity. This phenomenon could be explained by the 'imperfect complementarity' of the interactions between miRNAs and targeted mRNAs [36]. The detailed molecular mechanisms underlying the tumor suppressive roles of miR-29a in EOC were investigated. SIRT1 was demonstrated as a direct target gene of miR-29a in EOC, and this conclusion could be supported by several lines of experimental evidences from this study. Firstly, bioinformatics analysis showed the presence of miR-29a binding site on the 3'UTR of SIRT1. Secondly, qRT-PCR and western blot showed that miR-29a over expression reduced SIRT1 expression at both mRNA and protein expression. Thirdly, luciferase reporter assay also showed that luciferase activity was reduced by miR-29a in the pGL3-SIRT1-3'UTR Wt group, but not in the pGL3-SIRT1-3'UTR Mut group. Finally, Knock-down of SIRT1 by siRNA has the similar suppressive effects with miR-29a overexpression in EOC cells, rendering SIRT1 was a functional direct target of miR-29a in EOC.

SIRT1, a member of the sirtuin family, has been reported to be up-regulated in various kinds of human cancers, such as breast cancer, colon cancer and prostate cancer [37-39]. Subsequently studies indicated that SIRT1 acted as an oncogene in human cancers by improving cancer cells cellular survival, migration, invasion and apoptosis [40-43]. In EOC, SIRT1 was also found up-regulated in tumor tissues, and expression levels of SIRT1 was correlated with increased overall survival in SIRT1 positive serous carcinoma [44]. SIRT1 has been reported to be modulated by multiple miRNAs in cancers. For example, in gastric cancer, miR-543 inhibited cancer cell growth by down-regulation of SIRT1 [45]. In renal cell carcinoma, miR-22 targeted SIRT1 to function as a tumor suppressor [46]. Moreover, in osteosarcoma, miR-204 inhibited growth, metastasis and epithelial-mesenchymal transition via targeting SIRT1. However, in EOC, this is the first study to demonstrate a direct correlation between miRNA and SIRT1 expression. These studies also indicated that miR-29a/SIRT1 based gene therapy could be a novel treatment for EOC.

In conclusion, the present study demonstrated that miR-29a was down-regulated in EOC, and inhibited proliferation, migration and invasion of EOC cells. SIRT1 was validated as a direct target gene of miR-29a in EOC. These findings indicated that miR-29a and its target gene SIRT1 might have potential roles in target therapy, and may be a promising novel target for the therapeutic treatment of EOC.

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Disclosure of conflict of interest

None.

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