

Original Article

MiR-494 inhibits proliferation and migration of human ovarian cancer cells by suppressing SIRT1 expression

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Abstract: Earlier studies have shown that miR-494 is critical in the pathogenesis of various cancers. However, its role in ovarian cancer is still untested. The present study aimed to investigate whether miR-494 is able to regulate the proliferative and migration activity of ovarian cancer cells. We firstly found that miR-494 expression was significantly decreased in ovarian cancer tissues and ovarian cancer cell line compared with the adjacent normal tissues. Ectopic expression of miR-494 in ovarian cancer cell line SKOV3 significantly inhibited the proliferative activity, whereas inhibiting endogenous miR-494 by its antisense inhibitor did the opposite. More importantly, overexpression of miR-494 decreased the wound healing activity, whereas miR-494 antisense inhibitor promoted the wound healing process, suggesting the inhibitory role of miR-494 in the cell migration activity of ovarian cancer cells. In further exploring the mechanism of the anti-cancer action of miR-494, we found that Sirtuin 1 (SIRT1) could be potentially targeted by miR-494. Western blot analysis showed that miR-494 inhibited the protein level of SIRT1. Furthermore, Luciferase activity assay showed that miR-494 inhibited the luciferase intensity in luciferase reporter carrying 3'UTR of SIRT1 but not in the reporter carrying the mutant 3'UTR. Our study therefore identified for the first time that miR-494 might be an important negative regulator of proliferation and migration in ovarian cancer cells by regulating SIRT1 expression. Modulating the level of miR-494 in ovarian cancers may be a potential treatment strategy for ovarian cancer.

Keywords: Ovarian cancer, miR-494, SIRT1, SKOV3 cells

Introduction

Ovarian cancer is one of the most common causes of cancer associated death in females over the world, and about 90% of the cases of malignant tumors in ovarian are originated from the ovarian epithelium [1]. Despite its high sensitivity to chemotherapy drugs, the prognosis of ovarian cancer is poor, with a 5 year survival rate of 25% [2]. Therefore, it is of great importance to address the issues related to the molecular mechanisms of ovarian cancer pathogenesis, which would potentially guide the treatment strategies.

Recent accumulating evidences have demonstrated that microRNAs, a class of non-coding RNAs broadly found in eukaryotes, play critical roles in multiple cellular processes such as proliferation, differentiation and apoptosis [3-5]. MicroRNA functions to complementarily bind to the 3'UTR region of targeted genes and in most cases plays a suppressive role for their expres-

sion. Several microRNAs including miR-26b, miR-302a, miR-126a, miR-448 and miR-498 have been shown to be aberrantly expressed in ovarian cancers by previous studies [6-10]. Numerous mechanisms have been proposed. For example, miR-26b is downregulated in ovarian cancers, and it modulates the proliferative and invasive activity by directly binding to Oct4 [6]. MiR-302a has been proposed to play an anti-tumor role by activating the apoptotic program [7]. MiR-126a and miR-448 have also been demonstrated to be anti-tumor microRNAs in ovarian cancers by targeting PAK-4 and CXCL12 [8, 9], respectively. MiR-494 has been previously identified as an anti-tumor microRNA in several cancers [5, 11]; however, it is not established whether it regulates the biological behaviors of ovarian cancer with regard to carcinogenesis, metastasis and invasion.

In the present study, we focused on the possible role of miR-494 in the proliferation and

migration of ovarian cancer cells. Our results showed that miR-494 is significantly downregulated in ovarian cancers, and we proved the anti-proliferative and anti-migration action of miR-494. Further mechanistic studies supported the deacetylase SIRT1 as a direct target of miR-494. Our study for the first time identifies the anti-tumor activity of miR-494 in ovarian cancers and suggests that miR-494 might be a useful tool in ovarian cancer treatment.

Materials and methods

Clinical sample collection

We collected tumor tissues and the adjacent normal tissues from 50 epithelial ovarian cancer cases aged between 23 and 71, who were admitted to our hospital from December 2013 to March 2015. Each diagnosis was histologically proved by two independent experienced pathologists, and the negative margins of resection in the adjacent normal tissues were also confirmed. All the patients did not receive any radiotherapy, chemotherapy and hormone-therapy pre-operation.

Cell culture and reagents

The ovarian cancer cell line SKOV3 was purchased from American Type Culture Collection (ATCC). The cells were cultured at 37°C in DMEM (Gibco) containing 10% FBS (Gibco) in a CO₂ incubator.

Electrotransfection was used to deliver the nucleotides into cells, the reagents for electrotransfection were purchased from Lonza (USA). TRIzol, miR-494, anti-miR-494 and their negative control were purchased from Invitrogen (USA). Dual luciferase detection system was purchased from Promega (USA). The antibody for human SIRT1 was obtained from Cell Signaling Technology (USA).

Real-time PCR

Total RNA of ovarian cancer cells SKOV3 was isolated with TRIzol reagent and was then transcribed to the first cDNA strand with Takara reverse transcription kit (Dalian, China). MiR-494 PCR primer set was purchased from Sangon biotechnology (Shanghai, China). The real-time amplification was processed with a 7500 Fast Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA). The primer sequences for amplification were as follows [12]: miR-494 forward 5'-TGACCTGAAACATACACGGGA-3', miR-494 reverse 5'-TATCGTTGACTCCACTCCTTGAC-3', U6 forward 5'-GCTTCGGCAGCACATATACTAAAAT-3' and U6 reverse 5'-CGCTTCACGAATTTGCGTGCAT-3'. The relative expression of miR-494 was calculated by the 2^{-ΔΔCt} method.

MTT assay

Cells were seeded into a 96-well plate at the concentration of 2.5×10⁴/ml. After transfection, cells were cultured as the experiment design. MTT (5 mg/ml) was used at the volume of 20 ul per well and was cultured with cell for 4 h to form the formazan. The formazan product was dissolved and visualized with 150 ul DMSO. The accurate O.D. value at 490 nm was obtained by a spectrophotometer.

Wound healing assay

The protocol for wound healing assay was described in previous literature [13]. Briefly, transfected cells cultured in 24-well plated were allowed to grow to the confluence of 70%-80%. A 1 ml sterilized pipet was used to make an identical scratch in each well. The width of the wound was measured 48 h after the experiment.

Target prediction and luciferase reporter assay

Target for miR-494 was predicted with the online software TargetScan (www.targetscan.org). We found a potential target for miR-494 in the 3'UTR of miR-494. A fragment containing the putative binding site (765-772 of the 3'UTR of SIRT1) was cloned into pMIR-reporter. The luciferase reporter was transfected into SKOV3 cell along with miR-494. Luciferase activity was measured by the Dual luciferase kit according to the instructions provided by Promega.

Western blot

Protein of SKOV3 cells were isolated by RIPA lysis buffer (Thermo, USA). Whole cell lysates were isolated by SDS-PAGE electrophoresis, followed by transferring onto a PVDF membrane. Primary antibody was incubated at 4°C overnight. The band for each lane was detected by an ECL method.

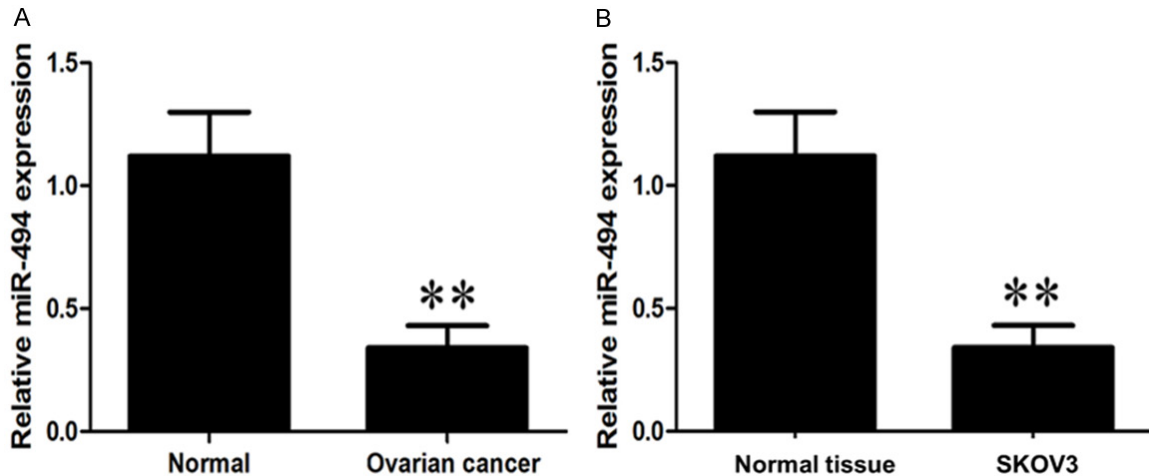


Figure 1. Decreased expression of miR-494 in ovarian cancer tissues and SKOV3 cell lines. MiR-494 was detected by real-time PCR. A. The expression of miR-494 in ovarian cancer tissues and the adjacent normal tissue. B. The expression of miR-494 in ovarian cancer tissues compared with normal tissue. ** $P < 0.01$, $n \geq 5$.

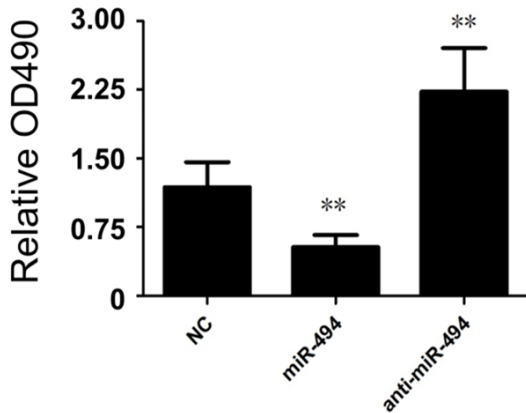


Figure 2. MiR-494 inhibits the proliferation of SKOV3 cell. Cell viability was determined by MTT assay 96 h after transfection. $n = 5$, ** $P < 0.01$ vs. NC group.

Statistical analysis

All data were expressed as means \pm SD. Independent t test was used for the comparison between 2 groups, and analysis of variants (ANOVA) was used for multiple comparisons. Turkey's test was used to compare the means between two groups in the context of ANOVA. Two sided $P < 0.05$ was considered as statistical significance.

Results

MiR-494 is downregulated in ovarian cancers

To examine the possible role of miR-494 in ovarian cancer, we firstly detected its expres-

sion level in normal tissues and tumor tissues. As shown in **Figure 1A**, we observed a marked decrease of miR-494 in tumor tissues. In addition, the expression of miR-494 was also down-regulated in ovarian cancer cell line SKOV3 compared with normal tissues (**Figure 1B**). These results indicated that miR-494 might play a role in the development or progression of ovarian cancers.

MiR-494 inhibits the proliferation of ovarian cancer cells

We next transfected cells with synthetic miR-494 or its antisense inhibitor (anti-miR-494) and tested its action on the proliferative activity of cancer cells by MTT assay. SKOV3 cells overexpressing miR-494 showed decreased cell viability compared with the negative control transfected cells. In contrast, inhibition of endogenous miR-494 by anti-miR-494 increased the proliferation (**Figure 2**).

MiR-494 inhibits the migration of ovarian cancer cells

We also tested whether miR-494 has an effect on the migration activity of SKOV3 cells. Cells transfected as described above were subjected to wound healing assay and cultured for 48 h post transfection. A significant increase or decrease of wound width was observed after miR-494 or anti-miR-494 transfection, respectively, suggesting that miR-494 exerted an adverse effect on ovarian cancer cell migration (**Figure 3**).

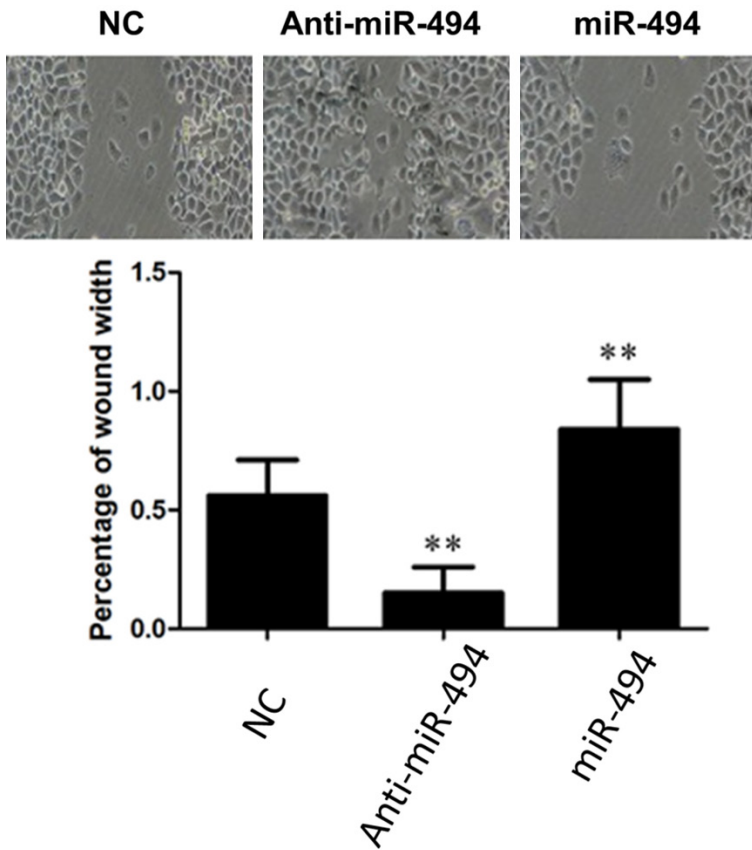


Figure 3. MiR-494 inhibits migration of SKOV3 cell. Migration activity was determined by wound healing assay, cells transfected with miR-494 or its inhibitor were given a scratch with a pipet tip, the photos were taken 48 h after transfection. n=5, **P<0.01 vs. NC group.

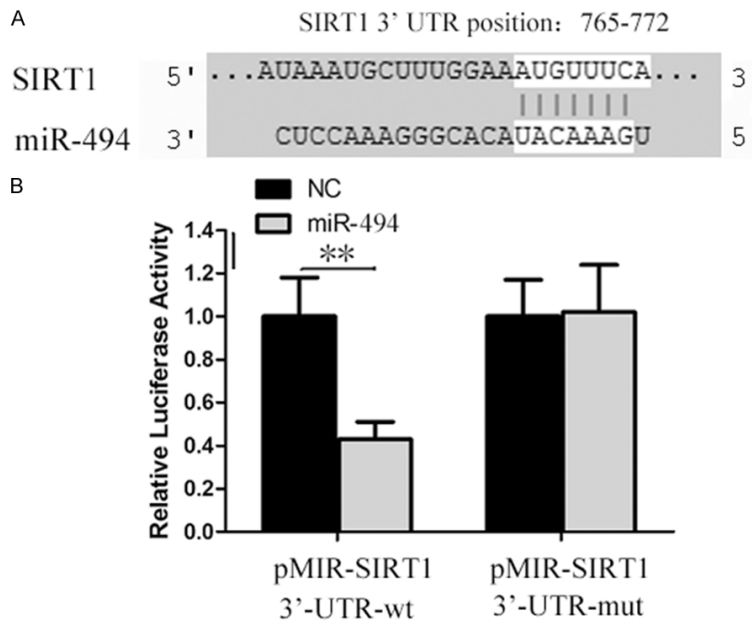


Figure 4. MiR-494 targets SIRT1. A. The putative binding site of miR-494 in the 3'UTR SIRT1 mRNA. B. The relative luciferase activity after miR-494 transfection. **P<0.01 vs. NC group, n=5.

SIRT1 is a direct target of *miR-494*

To further investigate the potential mechanism by which miR-494 regulates the behavior of SKOV3 cells, TargetScan database was then utilized to search the putative binding site of miR-494. As shown in **Figure 4A**, we found a conserved 8mer seed match within 3'UTR of SIRT1 (position 765-772 of SIRT1 3'-UTR). Luciferase assay was performed to validate this prediction, miR-494 significantly decreased the luciferase activity of reporter carrying wild type 3'UTR, which was completely abolished in cells expressing reporter carrying mutant 3'UTR (**Figure 4B**). Moreover, overexpression of miR-494 in SKOV3 cells suppressed the expression of SIRT1, and the opposite effect was observed when transfected cells with anti-miR-494 (**Figure 5**). These results confirmed that SIRT1 is indeed targeted by miR-494.

Discussion

Ovarian cancer is one of the serious health threats to women's lives over the world, and about 90% of the tumors in ovary originate from the epithelia [1]. Despite the development of systematic treatment that utilizes radical resection in combination of chemotherapy drugs such as cisplatin and paclitaxel over the past decades, the average 5-year survival rate for ovary cancer patients is only 25% [2]. Therefore, the unsatisfactory prognosis remains to be one of the biggest challenges in clinical practice, and exploring the molecular mechanism may help to establish more effective strategies for ovary cancer treatment.

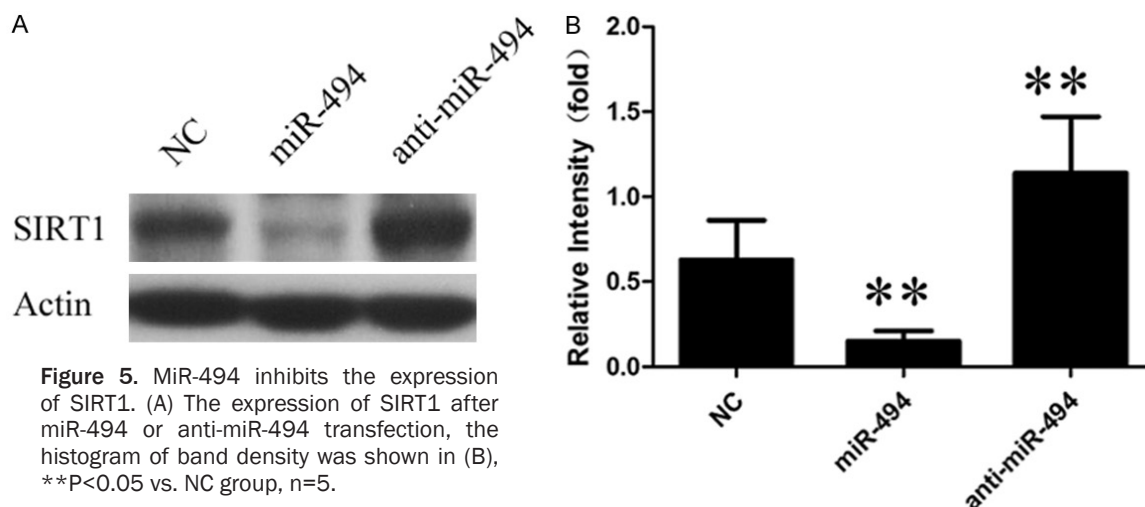


Figure 5. MiR-494 inhibits the expression of SIRT1. (A) The expression of SIRT1 after miR-494 or anti-miR-494 transfection, the histogram of band density was shown in (B), ** $P < 0.05$ vs. NC group, $n = 5$.

MicroRNAs (miRs), which refer to a class of non-coding RNAs and are about 21-25nt long, have recently emerged as critical molecular regulators of cancer development and progression [14, 15]. Large quantities of microRNAs have been proved to be aberrantly expressed in human cancers. Previous investigations have identified several important microRNAs in ovary cancers including miR-26b, miR-302a, miR-126a, miR-448 and miR-498. Although miR-494 has been previously demonstrated to be closely related to cancer development, the existing evidences do not reach a consensus. In glioma and hepatocellular carcinoma, miR-494 enhanced cancer cell proliferation, migration and invasion by regulating PTEN/Akt signaling pathway [5, 16]. On the contrary, the tumor suppressive effect of miR-494 was reported in breast cancer cells, the mechanism of which involves a negative regulation of Wnt/ β -catenin [11]. In the present study, we focused on the previously unreported role of miR-494 in ovary cancers. We showed for the first time that miR-494 expression was decreased either in ovarian cancer tissues or in SKOV3 cell line. More importantly, ectopic expression of miR-494 not only inhibited the proliferation but also suppressed the migration activity in SKOV3 cells, and anti-miR-494 was able to reverse its inhibitory effects, suggesting the anti-tumor role of miR-494 in ovary cancers. Our results are in consistent with the previous report in pancreatic cancers [17] and therefore indicated the potential application of miR-494 in ovary cancer treatment.

MicroRNAs functions to repress downstream gene expression by binding to the 3'UTR of

mRNA, and a microRNA can have multiple targets and thus exerts various biological functions in different context. In searching for the possible mechanism by which miR-494 regulates the proliferation and migration of SKOV3 cells, we found a free energy favorable 8mer binding site within the 3'UTR of SIRT1. As a nicotinamide adenine dinucleotide dependent Histone deacetylase, SIRT1 has been reported to regulate numerous basic biological processes such as inflammation, circadian rhythms, hypoxia response, oxidative stress, cell metabolism and cell survival [18-21]. Despite the fact that SIRT1 has been recently shown play important tumor suppressive roles in the development and progression of multiple cancers by negatively regulating β -catenin and survivin [21], SIRT1 is believed to facilitate cancer progression in a number of cancer types [22, 23]. One of the important substrates of SIRT1 is p53, by deacetylating p53, SIRT1 is able to inhibit the activity of p53 [24]. Previous investigation has shown that miR-34a is involved in the SIRT1/p53 pathway and inhibited migration and invasion in human colorectal cancer cells [25]. In our present work, we showed that miR-494 can directly target SIRT1 by luciferase assay. Moreover, overexpression of miR-494 significantly inhibited SIRT1 expression in SKOV3 cells, whereas anti-miR-494 increased it. Our data supports the conclusion that SIRT1 is a direct target of miR-494 for the first time. Given the previously well documented function of SIRT1, it is conceivable that inhibition of SIRT1 is sufficient for the tumor suppressive effect of miR-494 in ovary cancers. However, as previously noted, other possible mecha-

nisms may not be excluded due to the complexity of microRNA-mRNA interactions.

In summary, our present work demonstrated for the first time that miR-494 inhibited the proliferative and migration activity of ovarian cancer cells. Our work not only established a novel mechanism in which SIRT1 function as a target of miR-494, but also laid a foundation for the development of new treatment strategies for ovarian cancers.

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Disclosure of conflict of interest

None.

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