Original Article

MiR-143-3p suppresses the progression of ovarian cancer

Haijuan Shi1,*, Huimin Shen2,*, Juan Xu3, Shanshan Zhao2, Shuzhong Yao2, Nan Jiang2

1The Second Affiliated Hospital of Guangxi Medical University, Guangxi, P. R. China; 2The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong, P. R. China; 3Jinhua Municipal Central Hospital, Jinhua Hospital of Zhejiang University, Jinhua 321000, Zhejiang, P. R. China. *Equal contributors and co-first authors.

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Abstract: MicroRNAs (miRNAs) are a class of naturally occurring, small, non-coding RNAs that target protein-coding mRNAs at the post-transcriptional level and participate in various biological processes. Our previous studies suggested that miR-143-3p functions as a tumor suppressor and has a role in the progression of ovarian cancer, in part through the regulation of the tumor promoter. In this study, we found that the mRNA expression level of miR-143-3p was significantly decreased in ovarian cancer tissues, in comparison with normal ovarian tissues by high-throughput miRNA profiling and quantitative RT-PCR. Secondly, we indicated that the up-regulation of miR-143-3p in the ovarian cancer cell lines SKOV3, ES2, and OVCAR3 significantly reduced their proliferation, migration, and invasion. Furthermore, miR-143-3p inhibited the growth of ovarian tumors in vivo in a xenograft experiment. In addition, miR-143-3p down-regulated the expression of transforming growth factor (TGF)-β-activated kinase 1 (TAK1) in human ovarian cancer cells. Therefore, our study indicates that miR-143-3p inhibited the proliferation, migration, and invasion of ovarian cancer cells in vitro, as well as ovarian tumorigenesis in vivo. This inhibitory effect may target TAK1, suggesting a potential application of the miR-143-3p-TAK1 pathway in the clinical diagnosis and treatment of ovarian cancer.

Keywords: MiR-143-3p, ovarian cancer, proliferation, migration, xenograft, TAK1

Introduction

Ovarian cancer is one of the most common cancers in women. Approximately 238,700 women worldwide are annually diagnosed with this disease, with an estimated 151,900 associated deaths [1]. Most patients present with advanced disease and have a poor prognosis with the current therapies. In addition, disease recurrence continues to be a major problem for patients with advanced ovarian cancer [2, 3]. The widely-used therapies include maximum surgical debulking of the tumor mass followed by platinum/taxane chemotherapy, in which initial response rates may reach up to 80% [4]. Nevertheless, tumor recurrence is seen in almost all of these patients at a median of 15 months from diagnosis [3]. Therefore, the elucidation of the functionally relevant molecular drivers of ovarian cancer progression is urgent for improving survival outcomes in terminal patients.

MiRNAs are non-coding, single-stranded RNAs of ~22 nucleotides, which act by modulating the expression of target genes through sequence complementarity between the miRNA and the target messenger RNA (mRNA). This binding decreases the expression of the target protein by reducing the stability of the mRNA and inhibiting its translation [5]. Many studies showed that miRNAs account for only 1%-3% of human genome, but regulate approximately 30% of the protein-encoding genes in humans [6-9]. Various miRNAs have been reported to be related to ovarian cancer progression [10-12]. Among these, miR-143 (also named mi-143-3p), a highly conserved miRNA located on chromosome 5, position 33, in the human genome, is a potential regulator of tumor growth. Its abnormal expression has been observed in many cancers [13]. For instance, the down-regulation of miR-143 might be associated with worse prognosis in endometri-
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In human bladder and colorectal cancers, miR-143-3p was found to be a tumor suppressor by targeting ERK5 and/or Akt [15] and through the inhibition of KRAS translation [16]. In addition, miR-143 regulates cell apoptosis in lung cancer [17]. In osteosarcoma, Bcl-2 was identified as a novel direct target of miR-143-3p, and its function has been further suggested to occur through targeting Bcl-2 expression [18]. All of these reports show the direct correlation between miR-143-3p expression and tumor genesis and progression. However, its functional relevance in ovarian cancer remains elusive.

In this study, we found a differential expression level of miR-143-3p between ovarian tumor cells and normal tissue. Overexpression of miR-143-3p inhibited proliferation, invasion, and migration in ovarian cancer cells. By contrast, this inhibitory effect was offset in the presence of an miR-143-3p inhibitor. More importantly, we also observed the adverse effect of miR-143-3p overexpression on ovarian tumor growth *in vivo* through xenografting. The regulation of miR-143-3p in ovarian cancer cells could alter the expression level of kinase transforming growth factor (TGF)-β-activated kinase 1 (TAK1), which belongs to the family of mitogen-activated protein kinase kinases (MAPKK). Therefore, this work demonstrated that miR-143-3p plays a critical role in the proliferation and invasion of ovarian cancer cells *in vitro*, as well as ovarian tumorigenesis *in vivo*, providing a potential clinical target in the treatment of ovarian cancer.

### Materials and methods

#### Cell culture

The ovarian cancer cell lines SKOV3, ES2, and OVCAR3 were purchased from ATCC (USA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO₂. The cells were passaged every 3-5 days.

#### MiR-143-3p transfection

The miR-143-3p mimics and inhibitors used in this study were purchased from Ambion. Ovarian cancer cells were transfected with 50 nM miR-143-3p mimic (Cat #4464066, ID MC-10883, Applied Biosystems, California, USA) with or without the addition of 50 nM miR-143-3p inhibitor (Cat #4464084, ID: MH108-83, Applied Biosystems, California, USA). The construct was transfected into ovarian cancer cells through Lipofectamine 2000 (Thermo Fisher Scientific). Further analysis was conducted 48 hours after transfection.

#### MTT assay

MTT was used to detect the effect of miR-143-3p expression on ovarian cancer cell viability at different time points (24, 48 and 72 hours after miR-143-3p regulation). Briefly, MTT was incubated with cells for at least 4 hours to produce formazan. When formazan was completely dissolved by SDS-HCl, the absorbance at 570 nm was measured with a Universal Microplate Reader (Bio-Tek instruments), and the OD (NSC-CM-treated group)/OD (blank control group) was calculated.

#### EdU analysis

The EdU reagent was added to the ovarian cancer cells and incubated for 24 hours. Cells were then fixed in 4% paraformaldehyde for 20 min and then stained with crystal violet for 15 min. After washing three times with PBS, the number of colonies larger than 1 mm was counted for each group.

#### Xenograft tumor models

The inoculation area of the mice was cleaned and sterilized with ethanol and iodine solutions. Wild-type, miR-143-3p-overexpressed ES2 cells with or without the miR-143-3p inhibitor (2 × 10⁶ cells in 200 μL PBS) were subcutane-
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Previously injected into the unilateral flanks of 6-week-old BALB/c nude mice. An obvious tumor was observed 4 weeks after cell injection. The implanted mice were observed daily for 30 days. Tumor volume (V) was calculated using the following equation: \( V = \frac{a^2 \times b}{2} \), where \( a \) is the width of the tumor (small diameter) and \( b \) is the length (large diameter) (mm).

**RNA extraction and qRT-PCR**

Total RNA extraction was performed using TRizol reagent (Life Technologies) according to the manufacturer’s instruction. Two microgram of total RNA extracted from the cells was subjected to reverse transcription (RT). The cDNA synthesis was performed using a one-step RT-PCR kit from Takara. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed with an ABI 7300 real-time PCR thermal cycle instrument (ABI, USA), according to the supplied protocol. The relative gene expression was calculated with the ΔΔCt method. Primers used were as follows: TAK1, 5’-CAAAGCTAAGTG-GAGAGCAAAAGA-3’ and 5’-GATAACTGCCGAAGCTCTACAATAA-3’. The relative expression levels were normalized to the expression of endogenous GAPDH (5’-GGTATCGTGGAAGGACTCAT-GAC-3’ and 5’-ATGCCAGTGAGCTTCCCGTTCA-GC-3’).

**Scratch wound assay**

Cells were seeded onto 60 cm² tissue-culture plastic dishes at 80% cell confluence. A scratch wound was performed using a sterile 200 µl pipette tip. Phase-contrast images were taken at different time points, including the starting time point (0 h) and at 24 and 48 hours after the scratch.

**Invasion assay**

The invasion assay was performed using BD BioCoatmatrigel invasion chambers. In brief, 5 × 10⁶ cells were seeded on top of a matrigel-coated transwell in 100 µL of serum-free medium. The transwells were embedded into the medium and cells were allowed to invade for 48 h. At the end of the assay, the cells (invading) on the bottom side of the well were stained with Giemsa, photographed, and counted under a microscope. This experiment was independently repeated in triplicate.

**Western blotting**

2 µg cell lysates were loaded on each lane of 10% polyacrylamide gel, and then blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with PBST containing 5% nonfat dry milk, the membrane was incubated with antibodies against TAK1 and GAPDH (Cell Signaling Technologies, USA). Peroxidase-linked anti-rabbit IgG (Life Technologies) was used as the secondary antibody. These proteins were visualized with an ECL western blotting detection kit (Amersham Biosciences).

**Statistical analysis**

The statistical analysis was performed with the SPSS software package (version 18.0, SPSS). Data are presented as mean ± SEM. Analyses of multiple groups were assessed using one-way ANOVA followed by Tukey’s post-hoc test. A p value less than 0.05 was considered significantly different. Three biological replicates of each assay were performed for this study.

**Results**

The relatively lower expression level of miR-143-3p in ovarian tumors

Through high-throughput miRNA profiling, we found that the expression level of miR-143-3p...
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was significantly decreased in ovarian tumors. In order to validate this observation, we determined the expression level of miR-143-3p in normal ovarian tissue and compared it to that of ovarian tumor tissue with quantitative RT-PCR. Compared with the normal tissues, the expression level of miR-143-3p was significantly reduced in the ovarian tumor and cancer cells (\(P<0.001\), Figure 1). This data demonstrates the potentially critical role of miR-143-3p in ovarian tumors.

**Up-regulation of miR-143-3p in ovarian cancer cells impaired their proliferation**

Next, we determined whether or not the regulation of miR-143-3p could affect the proliferation of ovarian cancer cells. We used three ovarian cancer cell lines, SKOV3, ES2, and OVCAR3. The MTT assay indicated that miR-143-3p overexpression in ovarian cells led to impaired proliferation, compared with either wild type controls or miR-143-3p-overexpr-
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Figure 2A-C. The effect of miR-143-3p expression on the proliferation of ovarian cancer cells was also confirmed by EdU analysis, which demonstrated that the ratio of EdU-positive cells was reduced in the miR-143-3p-overexpressed cells compared with the wild type control, while the miR-143-3p-overexpressed cells stimulated with an anti-miR-143 inhibitor showed a robust increase in the EdU+ cell ratio in contrast to the cells without miRNA inhibitor treatment (Figure 2D-F). Thirdly, we found that the colony-forming efficiency was drastically decreased in the three types of ovarian cancer cells with miR-143-3p overexpression, while the miR-143-3p-overexpressed cells treated with the inhibitor showed a strong recovery in colony-forming efficiency (Figure 2G). Collectively, all these data demonstrate the inhibitory effect of miR-143-3p overexpression on the proliferation of ovarian cancer cells in vitro.

Figure 3. Overexpression of miR-143-3p affected the migration and invasion of ovarian cancer cells. (A) The scratch wound assay was used to detect cell migration in SKOV3 and ES2 cells transduced with NC, miR-143-3p, and miR143-3p+anti-miR143-3p (B) Cell migration and invasion assessed with a transwell assay in SKOV3, ES2, and OVCAR3 cell lines transduced with NC, miR-143-3p and miR143-3p+anti-miR143-3p. (C-E) Statistical analysis of the transwell assay. The significance was determined by one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.
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Overexpression of miR-143-3p inhibited ovarian tumor growth in vivo

To elucidate the role of miR-143-3p expression in ovarian tumor growth in vivo, the size of the xenografted tumors was measured. We found that the size of tumors originating from miR-143-3p-overexpressed ES2 cells was significantly smaller than that of the untreated control (Figure 4). However, the application of the miR-143-3p inhibitor offset this effect in ovarian cancer cells (Figure 4). In contrast, the tumor size did not show any significant difference between tumors formed by miR-143-3p-overexpressed cells treated with miR-143-3p inhibitor and tumors formed by wild type ES2 cells. These data provide evidence to support the conclusion that the overexpression of miR-143-3p weakens ovarian tumor malignancy in vivo.

MiR-143-3p overexpression inhibited migration and invasion in ovarian cancer cells

Next, we determined whether or not miR-143-3p overexpression affected the migration and/or invasion of ovarian cancer cells. The scratch wound assay showed that the width of the scratch line in miR-143-3p-overexpressed cells was significantly larger than that of the wild type controls (Figure 3A). In contrast, the cells overexpressing miR-143-3p that were treated with an inhibitor showed a drastic decrease in the width of the scratch line (Figure 3A), demonstrating that the overexpression of miR-143-3p inhibited the migration of ovarian cancer cells. In order to further validate the role of miR-143-3p expression in the invasion of ovarian cancer cells, we performed a transwell assay. The results indicated that either the number of invaded or migrated cells decreased when miR-143-3p expression increased in ovarian cancer cells, while application of the miR-143-3p inhibitor counteracted this increase in migrated and invaded cells (Figure 3B-E). Collectively, these data demonstrate that the overexpression of miR-143-3p inhibited the migration and invasion of ovarian cancer cells.

MiR-143-3p regulated TAK1 expression in ovarian cancer cells

We then detected which genes miR-143-3p could target when its expression level was regulated in ovarian cancer cells. The western blot data indicated that the overexpression of miR-143-3p could decrease the TAK1 expression level in three types of ovarian cancer cells compared to that of the wild type cells (Figure 5A-D). Contrarily, the TAK1 expression level increased when the miR-143-3p inhibitor was applied, making it comparable with the TAK1 expression level in the untreated control groups (Figure 5A-D). Similar results were obtained with quantitative real time PCR. The level of TAK1 mRNA decreased in miR-143-3p-overexpressed ES2, SKOV3, and OVCAR3 cells compared to the wild type controls (Figure 5E-G). Nevertheless, the expression level of TAK1 mRNA drastically increased when the three types of ovarian cancer cells overexpressing miR-143-3p were treated with the miR-143-3p inhibitor (Figure 5E-G). These data show that the regulation of miR-143-3p expression in ovarian cancer cells could subsequently modulate TAK1 expression.

Discussion

In this report, we demonstrated the potential clinical application of miR-143-3p in ovarian tumor therapy. We found that miR-143-3p overexpression could inhibit the proliferation, migration, and invasion of ovarian cancer cells in vitro. Meanwhile, the xenograft experiment showed that miR-143-3p suppressed...
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Figure 5. MiR-143-3p regulated TAK1 expression in ovarian cancer cells. A-D. Western blot was used to detect the protein levels of TAK1 in SKOV3, ES2, and OVCAR3 cells transduced with NC, miR-143-3p mimic, and miR-143-3p mimic+inhibitor. E-G. Quantitative real time PCR was performed to measure the TAK1 mRNA level in ES2, SKOV3, and OVCAR3 cells. GAPDH was used as a loading control. The significance was determined by one-way ANOVA. ***P<0.001.

ovarian tumor growth in vivo. This process may be mediated through TAK1 signaling. Therefore, our study provides direct evidence to support the potential therapeutic value of miR-143-3p in ovarian cancer treatment.

A large body of evidences has shown that miRNAs play essential roles in the progression of ovarian cancer. For example, miR-762 down-regulates the expression of menin to promote the development of ovarian cancer [19]. Also, miR-490-3p sensitizes ovarian cancer cells to cisplatin [20]. In addition, miR-28-5p promotes the development and progression of ovarian cancer [21], and the overexpression of miRNA-221 promotes cell proliferation, leading to a poor prognosis [22]. Consistent with previously described findings, we demonstrated that miR-143-3p is aberrantly down-regulated in ovarian tumors. The ectopic expression of miR-143-3p in ovarian cancer cells significantly suppressed cellular proliferation, invasion, and migration, as well as inhibited the growth of xenografted tumors in nude mice. This indicates that miR-143-3p acts as a tumor suppressor, providing a potential therapeutic target in ovarian cancer treatment.

We also investigated the mechanisms underlying the miR-143-3p induced inhibitory effect in ovarian cancer cell proliferation and invasion. We found that miR-143-3p regulated the expression of TAK1 in ovarian cancer cells, implying that TAK1 may function as a target gene of miR-143-3p. TAK1 participates in several signaling pathways and is involved in controlling the activation of p38MAPK and JNK in various cellular systems [23]. According to a previous report, the deletion of TAK1 resulted in the inactivation of both JNK and NF-kappaB signaling, both of which play critical roles in cell survival and proliferation [24]. In cancers, TAK1 inhibition has been reported to promote apoptosis in cervical cancer cells [25]. Furthermore, a direct correlation between miR-143 and TAK1 was elucidated in pancreatic ductal adenocarcinoma through the NF-kappaB pathway [26]. Based on these findings, we firstly showed the role of miR-143-3p in ovarian cancer and its potential correlation with TAK1. The expression of TAK1 was down-regulated in miR-143-3p-overexpressed ovarian cancer cells (Figure 5), which weakened their capacity for proliferation and invasion. This data is consistent with what was reported for cervical cancer cells [25]. Therefore, this work strengthens and broadens the potential therapeutic application of miR-143-3p or its target gene in tumor treatment. Our future work will focus on a deeper investigation of the molecular mechanisms and critical signaling pathways underlying the effects of miR-143-3p in cancer therapy.

In summary, through in vitro and in vivo studies, this work proposes a potentially effective target for the treatment of ovarian cancer by regulating the expression level of miR-143-3p and
facilitates the understanding of the critical role of microRNAs in cancer therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Shuzhong Yao and Nan Jiang, The First Affiliated Hospital, Sun Yat-sen University, No. 58 Zhongshan II Road, Guangzhou 510080, Guangdong, P. R. China. E-mail: qo63lig@163.com (SZY); lgo990@163.com (NJ)

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