MiR-26a Promotes Ovarian Cancer Proliferation and Tumorigenesis

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Abstract

MicroRNAs (miRNAs) important for posttranscriptional gene expression are involved in the initiation and progression of human cancer. In this study, we reported that miR-26a was over-expressed in human EOC specimens and the expression level of extracellular miR-26a in plasma can distinguish patients from healthy controls in EOC. Ectopic expression of miR-26a in ovarian cancer (OC) cells increased cell proliferation and clonal formation. This growth promoting effect of OC cell growth was mediated by miR-26a inhibition of the posttranscription of ERα. Furthermore, inhibition of miR-26a suppressed the tumor formation generated by injecting OC cells in nude mice. Our results suggest that aberrantly expressed miR-26a may contribute to OC development.

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Introduction

OC is the fifth most common cancer in women and the leading cause of cancer deaths from gynecological malignancy in western countries [1]. In 2012, there are 22,280 new cases and 15,500 deaths from OC in the United States according to the national cancer statistics. EOC accounts for 85%–90% of ovarian cancer. Unfortunately, the overall prognosis is poor and the molecular events that lead to the development of this disease are still little-known.

miRNAs represent a large family of endogenous noncoding RNAs and posttranscriptionally regulate gene expression [2]. Recent studies have revealed critical functions of miRNAs in essential processes, including proliferation, differentiation and cell death [3]. Altered expression or mutation of miRNAs has been reported in cancer, such as lung cancer, breast cancer, leukemia and other carcinomas [4]. In lung cancer cells, over-expression of let-7 inhibited their growth by targeting Ras [5,6]. Furthermore, miR-21 directly targets the tumor suppressor PTEN in hepatocellular cancer [7]. Moreover, several studies showed that miRNAs can function either as tumor suppressors (as is the case for the miR-15a–miR-16-1 cluster [9]) or oncogenes (as is the case for miR-21 [9]).

Genome-wide miRNA expression profiling showed miR-26a dysregulation in diverse cancers[10]. In this study, we found that miR-26a is over-expressed in human EOC. We demonstrate that inhibition of miR-26a decreased proliferation of human EOC cells, and suppressed growth of EOC cells in nude mice. In addition, ERα was down-regulated by miR-26a in EOC cells. Furthermore, we found that extracellular miR-26a levels in plasma can distinguish patients from healthy controls in EOC. Our study suggests that aberrant expression of miR-26a is critical for the development of human EOC and measurement of circulating miR-26a may be a good approach to EOC diagnosis.

Materials and Methods

Patients

Clinical specimens (including tissue and plasma samples) were collected from patients registered at The First Affiliated Hospital of China Medical University (Shenyang, China). The patients’ information is summarized in Table 1, Table S1 and Table S2.

Materials

Antibody against ERα was from Santa Cruz (Santa Cruz, CA, USA), antibody against α-tubulin from Sigma (St Louis, MO, USA). All other reagents were from Sigma. Anti-miR-26a and nonsense anti-miR were from GenePharma (Shanghai, PR China). Cel-miR-39 mimics were from IBS (Shanghai, PR China).

Quantitative RT-PCR (qRT-PCR, Quantitative Reverse Transcriptase-polymerase Chain Reaction)

Total RNA isolated from clinical specimens or cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) were reverse-transcribed into cDNA according to the previous report [11]. Isolation of RNA from plasma and quantification of miRNA were carried out.
out as described in [12]. In brief, 25 fmol of Cel-miR-39 mimics was added to 400 ul plasma. Real-time PCR was carried out using SYBR green PCR master mix (TaKaRa, Otus, Shiga, Japan). Amplification and detection were performed using ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Cel-miR-39 was taken as reference gene for plasma samples. Primers used were listed in Table S3.

**Cell Culture**

The human OC cell lines, SKOV-3, ES2 (Cellbank, Shanghai, PR China) were maintained in McCoy’s 5a supplemented with 10% (v/v) fetal calf serum (Invitrogen, Carlsbad, CA, USA). These cells were incubated at 37°C with 5% CO2.

**Vector Construction**

Full-length human miR-26a was amplified from human genomic DNA and cloned into the pcDNA3.1 at KpnI and XhoI sites according to the previous report [13] and [14]. The human wild-type ERα was generated by PCR from cDNA and the PCR products were inserted into pcDNA3.1 as described in [15].

**Cell Growth Assay**

Cell growth was estimated by determination of the cell number and the colony formation. The cells were transfected with miR-26a or anti-miR-26a using FuGene HD (Roche, Indianapolis, IN) according to the manufacturer’s protocol. After culture for 24 hours the cells were seeded at an initial density of 1x10⁵ per 35 mm-dish. The cells were then harvested at the indicated times and the numbers were counted using the COULTERTM (Beckman, Fullerton, CA, USA).

Cells transfected with miR-26a were seeded into 96 well plates at a concentration of 2.5x10³ cells, and measured after 48 h using a WST-1 assay (Boster, Wuhan, PR China), performed according to the manufacturer’s protocol.

A total of 500 cells transfected with miR-26a or empty vector (Ctrl) were seeded in 35 mm dishes separately in triplicate. Three weeks later, the colonies were fixed with 4% paraformaldehyde, permeated with 20% methanol and stained with crystal violet. The stained cells were eluted by 10% glacial acetic acid and the

**Luciferase Reporter Assay**

1.5x10⁵ SKOV3 stable cells in 35 mm dishes were co-transfected with pGL3-ERα-WT (1 µg) or pGL3-ERα-Mut (1 µg) and pRL-TK (1 µg) using FuGENE® HD Transfection Reagent(Roche, Diagnostics Corp., Indianapolis, IN) following the manufacturer’s protocol. Forty-eight hours after transfection, luciferase activity was measured using a dual luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity.

**Western Blotting**

Proteins were separated on SDS-8%PAGE, transferred to PVDF membranes (Amersham, Buckinghamshire, UK) and probed with primary antibodies and secondary antibodies conjugated with horseradish peroxidase. The protein bands were visualized by the Amersham ECL system and scanned. Their densities were determined by ImageQuant 5.2 software (Amersham).

**In vivo Tumorigenesis**

SKOV3 cells transfected with miR-26a or anti-miR-26a separately. Each nude mouse was subcutaneously injected with

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**Table 1. clinicopathologic data of ovarian cancer patients.**

| Total number | 26 |
|-------------|----|
| Pathological tumor stage | |
| I | 3 |
| II | 4 |
| III | 17 |
| IV | 2 |
| Grade | |
| G1 | 10 |
| G2 | 9 |
| G3 | 7 |
| Histological type | |
| Serous cystadenocarcinoma | 15 |
| Mucinous cystadenocarcinoma | 5 |
| Endometrioid carcinoma | 3 |
| Clear cell carcinoma | 3 |

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**Figure 1. The expression of miR-26a was increased in EOC patients.** (A)Quantitative analysis of the expression levels of miR-26a in EOC samples normalized to those of 18s rRNA by qRT-PCR. Data for each dot were mean value of one sample repeated in three independent experiments (normal, n = 19; tumor, n = 26). **P<0.01 vs Normal. (B)Quantitative analysis of the expression levels of plasma miR-26a by qRT-PCR. Data for each dot were mean value of one sample repeated in three independent experiments (normal, n = 13; tumor, n = 17). doi:10.1371/journal.pone.0086871.g001

OD595 values were measured by spectrophotometer as the indicator of cell number.
2 x 10^6 transfected SKOV3 cells. Thirty or thirty-five days after injection the mice were killed and the tumors were taken. The longest and shortest diameter of the tumor was noted as d1 and d2. The tumor volume was calculated using the equation: V = d1 x d2 x d2 / 2. Tumor weight was then measured.

Statistics
Multiple comparisons were assessed by SPSS statistical software for statistical analysis. Wilcoxon Test and Kruskal Wallis H Test were used in statistical analysis of patient samples. Other comparisons between groups for statistical significance were performed with Student’s t test and analysis of variance (ANOVA). Results were considered significant difference at *P < 0.05; **P < 0.01.

Ethics Statement
The research meets all requirements for the ethics of experiment. Clinical specimens were collected with consent of patients and healthy volunteers and approval of Medical Research Ethics Committee of the First Affiliated Hospital of China Medical
University. All participants have provided their written informed consent to participate in this study. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of China Medical University.

Results

Increased Expression of miR-26a in Specimens and Plasma in Human EOC

It has been observed that the expression level of miR-26a was decreased or increased in human malignancies, such as breast carcinoma [16], nasopharyngeal carcinoma [17,18], and glioblastoma [19], indicating a complicated role of miR-26a in progression of the malignancies. To investigate the possible role of miR-26a in EOC development, we first examined the expression of miR-26a in specimens and plasma in EOC by SYBR-Green stem-loop qRT-PCR [20]. We examined the expression of miR-26a in 26 tumor samples and 19 normal ovaries. As shown in Figure 1A, the expression levels of miR-26a in tumor samples were much higher than those in normal ovary samples. Similarly, the concentrations of miR-26a were higher in plasma from EOC patients (n = 17) than in that from healthy controls (n = 13) (Figure 1B). Together, these results provide us initial evidence that miR-26a may play a role in the development of human EOC.

Over-expressing miR-26a Promoted EOC Cell Growth and Inhibiting miR-26a Suppressed EOC Cell Proliferation

We next examined whether miR-26a affects EOC cell growth using SKOV3 and ES2 cells as models. As shown in Figure 2A, the growth ability of SKOV3 and ES2 cells was increased by over-expression of miR-26a. On the contrary, the proliferation of the cells transfected with anti-miR-26a was markedly decreased compared with that of the cells transfected with nonsense (Figure 2B). WST-1 assay showed similar results in cell proliferation (Figure 2C). These results suggest that miR-26a indeed affected EOC cell growth.

The notion was further tested in the clonal formation assay. The number and size of the colonies formed were markedly increased in miR-26a transfected cells (Figure 2D). Together, these results suggest that miR-26a was indeed involved in the regulation of EOC cell growth.

ERα was a Target Gene of miR-26a in EOC Cells

We then investigated the mechanisms by which miR-26a promote EOC cell proliferation. In patients with hepatocellular carcinoma, the expression of miR-26a was higher in women than in men [21]. Moreover, miR-26a regulated liver tumor cell growth by targeting ERα [22]. As shown in Figure 3A, the luciferase activity of pGL3-ERα-WT in SKOV3-stable cells (S1) was much lower than in control cells. The luciferase activity of pGL3-ERα-Mut was rescued in Stable1 cells. We next examined whether miR-
miR-26a could regulate endogenous ERα expression in EOC cells. Compared with control, endogenous ERα mRNA levels (Figure 3B) and protein levels (Figure 3C) were down-regulated when cells were transfected with miR-26a.

MiR-26a Controlled the Proliferation of EOC Cells through ERα

Given the fact that miR-26a was involved in proliferation of EOC cells and ERα was a target of miR-26a, we next tested whether over-expression of ERα could rescue promotion of proliferation by miR-26a in EOC cells. Moreover, stable expression of miR-26a in two SKOV3 cell clones (S1 or S2) increased the growth of the cells about 1.58 or 1.37 fold, respectively (Figure 3D). QRT-PCR analysis showed that miR-26a expression level was greatly increased in S1 and S2 cells (Figure S1). As shown in Figure 3E, whereas growth of S1 cells transfected with ERα, were not different from that of control cells. These results indicate that miR-26a controlled proliferation of EOC cells through regulation of ERα expression.

MiR-26a Promoted the Development of Tumor in Nude Mice

To provide direct evidence that miR-26a was responsible for EOC development, SKOV3 cells transfected with either miR-26a or anti-miR-26a were injected into the flank of nude mice as described. A week after implantation, xenografted tumors could be seen. After thirty or thirty-five days, all nude mice were killed and the tumors were taken out and weighed. At the macroscopic observation, the differences in tumor size and volume among the two groups were indicated. The tumor volume generated from empty vector was 0.435±0.042 (cm³, P<0.01), that in miR-26a group was 0.829±0.172 (cm³, P<0.01) (Figure 4A), that in negative control group was 0.941±0.163 (cm³, P<0.01), and that in anti-miR-26a was 0.248±0.05 (cm³, P<0.01) (Figure 4B). The average weight of the tumor generated from empty vector was 0.433±0.07, whereas the average weight of the tumor generated from miR-26a was 0.821±0.02 (gram, P<0.01). The average weight of the tumor generated from nonsense was 1.062±0.169, whereas the average weight of the tumor generated from anti-miR-26a was 0.473±0.05 (gram, P<0.01), respectively.
results mentioned above suggest that miR-26a was critical for the development of EOC in vivo.

Discussion

In this study, we have shown that expression level of miR-26a was greatly increased in human EOC samples, and blood-based miR-26a level can distinguish patients from healthy controls in EOC. Our results also displayed that expression level of miR-26a affected EOC cell growth in culture. Moreover, ERα was a target for miR-26a in EOC cells. Furthermore, expression level of miR-26a affected tumor formation in nude mice. Therefore, our results are consistent with an idea that miR-26a is critical for the development of human EOC.

MicroRNAs are known to regulate the expression of genes involved in several biological processes such as development, proliferation, apoptosis and stress response [23]. Recent studies showed a direct link between miRNAs and human cancers [4,24,25,26]. MiRNAs can be oncogetic miRNA (oncomirs) or tumor suppressor relevant to cancer. As previously shown, loss of oncogenic miR-21, acting to repress RHOB, is associated with an elevation of RHOB in hepatocellular carcinoma and breast cancer cells [13]. The importance of other miRNAs, such as let-7, miR-16, miR-126 and miR-125b has also been demonstrated [27]. Recent microarray profile data shows miR-26a dysregulation in diverse cancer [4]. In liver cancer, miR-26a protects normal liver tissue from hepatocellular carcinoma-promoting inflammation[21], and appears to antagonize human breast cancer [16] and rhabdomyosarcoma [28]. On the contrary, miR-26a facilitates glioblastoma formation as an oncogene [19]. Our results obtained from gain-of-function and loss-of-function approaches indicated that miR-26a promoted proliferation and inhibition of miR-26a suppressed EOC cell proliferation.

In ovarian cancer, ERα expression has been studied to correlate to chlino-pathological parameters and prognosis [29,30]. Previous study showed that it did not reveal any correlations with histologic type of tumors and ovarian cancer grading [30]. Univariate survival analysis revealed that patients with positive-ERα status had a significant better progression-free survival compared with the patients with no expression[31]. In our results, the expression of miR-26a in specimens and plasma in EOC were much higher than those in normal ovary samples, and miR-26a promote EOC cell proliferation by targeting ERα. It means that miR-26a might be an important factor in the survival of ovarian cancer.

Circulating biomarkers are used to diagnostic disease, monitor therapeutic effect and predict recurrence in clinical applications. The discovery of circulating miRNAs in cancer patients indicates their potential application as powerful biomarkers in cancer diagnostics[32]. Recent studies revealed that cancer-related miRNAs could stably detectable in plasma and serum which originate from cancer tissues [4,33]. Our results investigate the possibility of miR-26a applied as a biomarker in clinical setting through examining the expression of miR-26a in plasma of EOC patients.

In conclusion, inhibition of miR-26a decreased EOC cell growth in culture and in nude mice. MiR-26a affected proliferation of EOC cells by targeting ERα. An important implication of current study is that miR-26a might be a potential target for therapeutic intervention to human EOC.

Supporting Information

Figure S1 MiR-26a expression level was greatly increased in S1 and S2 cells. QRT-PCR determined the expression levels of miR-26a in S1 and S2 cells. Data were mean±s.e. of three independent experiments in triplicate.*P<0.01 vs Ctrl. (DOC)

Table S1 clinicopathologic data and miR-26a expression level of control. (DOC)

Table S2 clinicopathologic data and miR-26a expression level of ovarian cancer patients. (DOC)

Table S3 Primers used in Quantitative RT-PCR. (DOC)

Author Contributions

Conceived and designed the experiments: WS MS. Performed the experiments: WS MS JL GQ TL YH HL. Analyzed the data: WS MS HL. Contributed reagents/materials/analysis tools: WS MS JL GQ TL YH HL. Wrote the paper: WS.

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