

Original Article

MiR-151 promotes ovarian cancer through activation of akt/mTOR signaling pathway by decreasing RhoGDIA

Yan Lv^{1,2*}, Feng-Ling Li^{3*}, Pei-Shu Liu¹

¹Department of Obstetrics and Gynaecology, Qilu Hospital of Shandong University, Jinan, China; ²Department of Obstetrics and Gynaecology, Zhongxin Hospital of Huizhou, China; ³Department of Obstetrics and Gynaecology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, Shandong Province, P. R. China. *Equal contributors.

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Abstract: Ovarian cancer with increasing incidence and unsatisfactory prognosis is the fifth lethal gynecological malignancy for women. But surgical remove is still the major therapy for ovarian cancer. So it is urgent to develop new drugs or strategies to fight against ovarian cancer. MiR-151 played an oncogenic role in a series of cancers. But its role in ovarian cancer was not previously reported. Here we demonstrated that miR-151 was expressed highly in both ovarian cancer tissues and ovarian cancer cell lines. And high expression of miR-151 was clinically correlated with tumor grade (P=0.028) and stage (P=0.002). Moreover, stronger miR-151 expression indicated poorer 5-year survival rate in ovarian cancer patients. When miR-151 was inhibited, proliferation ability of SKOV3 cells was disrupted. The proliferation rate decreased to 38% compared to control. Further, the migration and invasion capacity of SKOV3 cells was also suppressed by miR-151 downregulation. The SKOV3 cells transferred across matrigel monolayer in test group was about 29% of that in control group. In addition, RhoGDIA level was increased when SKOV3 cells was treated with anti-miR-151 inhibitor while it was decreased after miR-151 mimics treatment. In accordance, phosphorylated level of akt and mTOR were downregulated in anti-miR-151 inhibitor-treated SKOV3 cells but upregulated in miR-151 mimics-treated SKOV3 cells. Conclusively, our data suggest that miR-151 played an oncogenic role in carcinogenesis and progression of ovarian cancer by activating akt/mTOR signaling pathway through RhoGDIA. This study provides a new promising target for patients with ovarian cancer.

Keywords: Ovarian cancer, miR-151, mTOR, akt, RhoGDIA

Introduction

Ovarian cancer is the fifth lethal gynecological malignancy for woman and the incidence rate as well as the mobility rate has been increasing in the world in recent years [1, 2]. It is estimated that about 22,280 new cases would occur in 2016 in the US and the estimated death is 14,240 [2]. Further, 5-year survival rate of patients with ovarian cancer was still less than 50% regardless of the great advancement in surgery and chemotherapy drugs [3, 4]. The implicit symptoms of ovarian cancer patients caused by heterogeneity made it difficult to diagnose at early stage. Dysregulated signaling pathways including Wnt, MAPK, and mTOR/akt were reported to accelerate the progression of ovarian cancer [5-7]. But the exact molecular mechanism underpinning ovarian cancer was rarely reported.

Increasing studies have demonstrated that microRNAs played critical roles in tumorigenesis [8]. MicroRNAs are a class of endogenous non-coding small RNA and regulate the biological processes of cancer cells via post-transcriptional regulation. To date, more than 2500 microRNAs have been discovered and their abnormal expression was associated with proliferation, differentiation, apoptosis and metastasis of tumors [9]. By microarray analysis, a series of microRNAs were detected with abnormal expression in ovarian cancer tissues and cells [10]. For example, microRNA like miR-200, miR-21, miR-29, miR-17, and miR-141 were upregulated while others like miR-145, miR-155, miR-181, miR-125, and miR-30 were downregulated in ovarian cancer [11, 12].

MiR-151, localized on chromosome 8q24.3, was found to be highly expressed in a several

kinds of human cancers and played an oncogenic role in tumorigenesis [13-16]. For example, miR-151 was reported to be strongly expressed in hepatocellular cancers and facilitated the migration and invasion of cancer cells [17]. MiR-151 was also up-regulated in prostate cancers and high level of miR-151 was adversely correlated with survival rate [18]. It was known that microRNA could bind to 3-UTR domain of target gene and caused its degradation at mRNA level. In a previous study in hepatocellular cancers, RhoGDIA gene was confirmed as the target gene of miR-151. It was shown that miR-151 activated Rac1, Cdc42 and Rho GTPase by directly targeting the 3-UTR of RhoGDIA [17]. However, the role of miR-151 in ovarian cancer and the mechanism remains unknown.

In this study, we investigated the role of miR-151 in carcinogenesis and progression of ovarian cancer. We demonstrated that miR-151 was highly expressed in both ovarian cancer tissues and cell lines. And high expression of miR-151 was correlated with poor prognosis in ovarian cancer patients. Moreover, miR-151 was shown to promote proliferation and invasion of ovarian cancer cells in vitro through activation of mTOR/akt signaling by targeting RhoGDIA.

Materials and methods

Cell culture and tumor tissues

Human ovarian cancer cell lines SKOV3, HO8910, A2780 and OVCAR-3 were purchased from Cell Bank Type Culture Collection of Chinese Academy of Sciences (CBTCCAS, Shanghai, China) and cultured in DMEM (Gibco, USA) with 10% FBS (Hyclone, USA) in an atmosphere of 5% CO₂ at 37°C. Anti-miR-151 inhibitor, miR-151 mimics and the negative control were chemically synthesized by Ribobio (Guangzhou, China). Before transfection, cells were allowed to grow to 60%-70% confluence for 24 h without antibiotics. Anti-miR-151 inhibitor, miR-151 mimics and the negative control were transferred into cells at a final concentration 50 nM with Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions.

About 41 patients diagnosed with ovarian cancer from 2004 to 2014 were chosen from Department of Obstetrics and Gynecology in

Qilu Hospital of Shandong University. The development and pathogenic progression of ovarian cancer were diagnosed and classified by histological examination according to the WHO criteria. Written informed consents were obtained from all subjects. The experimental protocols were observed and approved by the Ethics Committee of Qilu Hospital of Shandong University. All experiments comply with the current laws in China.

Quantitative real-time PCR (qRT-PCR)

Total RNA were extracted from ovarian cancer tissues or ovarian cancer cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ1RNase-free DNase (Promega). cDNA was synthesized from 1 µg of total RNA by SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the instructions. 2 µl of the synthesized cDNA was used for qRT-PCR with a SYBR-green-containing PCR kit by a Mini-Opticon™ Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). U6 snRNA was used as internal control. Data were analyzed using ABI 7500 V2.0.6 software based on $\Delta\Delta C_t$ method [19]. All samples were examined in triplicates.

MTT assay

Cell proliferation was determined by MTT assay. Anti-miR-151 inhibitor or mimics-treated SKOV3 cells were seeded into 96-well plates at 2×10^3 cells/well in 100 µl DMEM (Gibco, USA). After transfection for 24 h, 100 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for another 4 h at 37°C followed by removal of culture medium and addition of 100 µl dimethyl sulfoxide (Sigma, USA). Then the absorbance value at 570 nm was measured with 630 nm as the reference wavelength. The growth of SKOV3 cells was observed for consecutive five days. All experiments were performed in triplicates.

Wound healing assay

Anti-miR-151 inhibitor-treated cells were seeded in 6-well plates at 3×10^5 cells/well. After 24 h, a lesion was created by a 10 µl pipette tip and cells were washed gently with PBS to remove cell debris. Then cells were maintained in serum-free DMEM for another 24 h. At designed time points, five randomly selected

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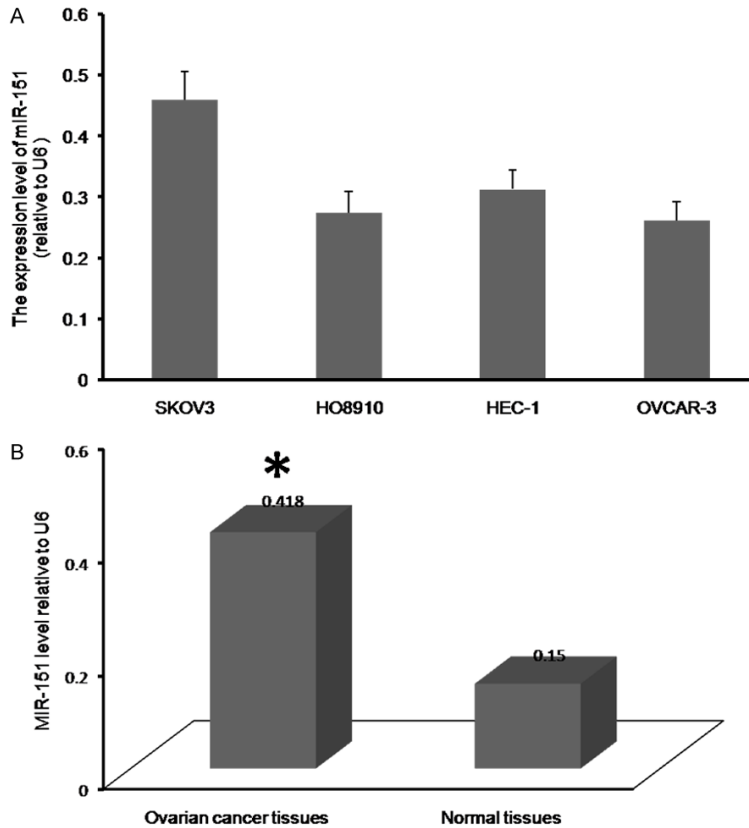


Figure 1. qRT-PCR analysis of miR-151 in ovarian cancer. A. MiR-151 was expressed abundantly in four ovarian cancer cell lines: SKOV3, HO8910, HEC-1 and OVCAR-3. B. The level of miR-151 in tumor tissues was higher compared to that in normal tissues.

fields along the lesion were observed and the width were recorded. The migration rate was calculated as following:

$$\text{Migration rate} = (S_{2h} - S_{24h}) / S_{2h} \times 100\%$$

in which S_{2h} stands for the width of the lesion at 2 h while S_{24h} stands for the width at 24 h. Each experiment was performed in triplicates.

Cell invasion assay

About 5×10^4 cells were seeded into upper chamber (8.0 mm pore size, Millipore, USA) pre-coated with 1 mg/ml Matrigel Matrix (BD Biosciences, China). The chambers were suspended in the 24-well culture plates. And 500 μ l fresh medium with 20% FBS was added into the lower chamber as chemoattractant. Then the cells in upper chamber were induced to invade toward the lower chamber in the CO_2 incubator at 37°C for 24 h and 48 h. The non-invading cells were removed and the migrated

cells were stained with dye solution containing 0.1% crystal violet and 20% methanol. Then five fields were randomly selected under a microscope (Olympus, Japan) after washing the chambers thoroughly in distilled water and cell numbers were averaged. Each experiment was performed in triplicates.

BrdU incorporation assay

The transfected cells were spread to 96-well plates at 2×10^3 cells/well. 10 μ l 1 \times bromodeoxyuridine (BrdU) reagents were added from 2 h to 24 h. Then 100 μ l fixing solution was added into the cells for 30 min at 24 h and 72 h respectively, followed by washing with wash buffer. After incubation with 50 μ l 1 \times BrdU antibody for 1 h, 50 μ l 1 \times Goat anti-mouse IgG and 50 μ l TMB substrate solutions were added sequentially. Then stop solution was added after 30 min and absorbance value at 450 nm was measured.

Western blotting analysis

After transfection for 48 h, cells were harvested and total protein was extracted using RIPA lysing buffer supplemented with 1/100 PMSF (phenylmethanesulfonyl fluoride) at 4°C. Then 20 μ g proteins was separated by 8% SDS-PAGE, transferred onto PVDF membrane and stained for the following protein: p-AKT, p-mTOR (Santa Cruz, USA), RhoGDIA (Cell Signaling Technology, USA) and β -actin (Abcam, USA). The secondary antibodies included horseradish peroxidase-conjugated sheep anti-mice IgG and anti-rabbit IgG (R&D Systems China, China). Then proteins were detected by ECL reagents and analyzed using Image J software.

Statistical analysis

All statistical analysis was conducted with the SPSS version 16.0 software. The difference between groups was carried out with a two-tailed Student t test. The association of miR-

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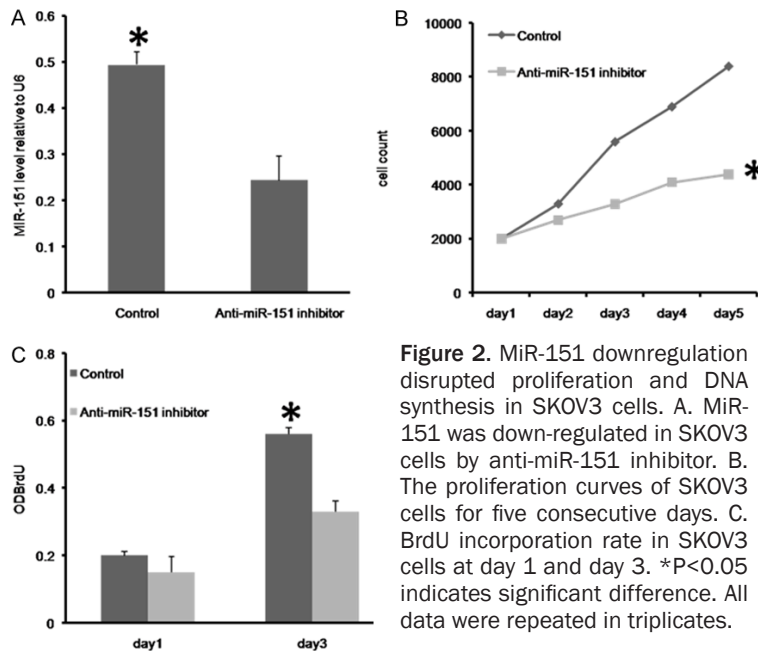


Figure 2. MiR-151 downregulation disrupted proliferation and DNA synthesis in SKOV3 cells. A. MiR-151 was down-regulated in SKOV3 cells by anti-miR-151 inhibitor. B. The proliferation curves of SKOV3 cells for five consecutive days. C. BrdU incorporation rate in SKOV3 cells at day 1 and day 3. *P<0.05 indicates significant difference. All data were repeated in triplicates.

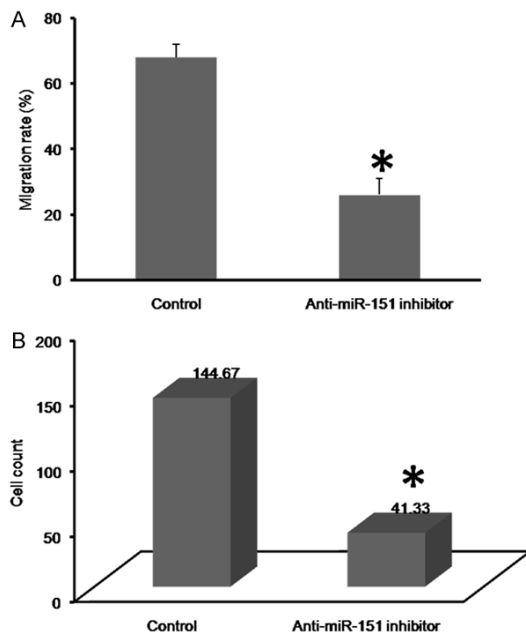


Figure 3. Downregulated miR-151 inhibited migration and invasion of SKOV3 cells. A. The migration rate of SKOV3 cells after 24 hours in wound-healing assay. B. The cells transferred through the monolayer matrigel in transwell assay. *P<0.05 indicates significant difference. All data were obtained from three independent experiments.

151 expression with clinicopathological characteristics was analyzed by chi-square test. Survival curves were plotted with the Kaplan-

Meier method and analyzed by the log-rank test.

All our experiments were repeated at least three times and P<0.05 was considered statistically significant.

Results

Expression pattern of miR-151 in ovarian cancer tissues and cells

To investigate the role of miR-151 in ovarian cancer, we determined the expression of miR-151 in four typical ovarian cancer cell lines including SKOV3, HO8910, HEC-1 and OVCAR-3 by qRT-PCR assay. We observed that miR-151 was expressed abundantly in

these ovarian cancer cell lines, particularly in SKOV3 cells (**Figure 1A**). Further, we detected the level of miR-151 in 41 ovarian cancer tissues from patients registered in our hospital between 2004 and 2014. As shown in **Figure 1B**, miR-151 was significantly higher in ovarian cancer tissues compared to normal adjacent tissues. These data suggest the implicit role of miR-151 in carcinogenesis of ovarian cancer.

Knockdown of miR-151 inhibited proliferation and DNA synthesis in SKOV3 cells

To examine the function of miR-151 in ovarian cancer, we performed loss-of-function assay by transfection of anti-miR-151 inhibitor into SKOV3 cells. As shown in **Figure 2A**, miR-151 was efficiently down-regulated in SKOV3 cells. Then after transfection for 48 h, we demonstrated by MTT assay that the proliferation rate of SKOV3 cells dropped dramatically in miR-151 inhibitor-treated group (**Figure 2B**). Moreover, the DNA synthesis in SKOV3 cells was also disrupted by knockdown of miR-151. In BrdU incorporation assay, the cells treated with anti-miR-151 inhibitor displayed significant DNA synthesis defect with a reduction of 41% at 48 h compared to the control group (**Figure 2C**). Therefore, knockdown of miR-151 impaired the proliferation as well as the DNA synthesis in ovarian cancer cells.

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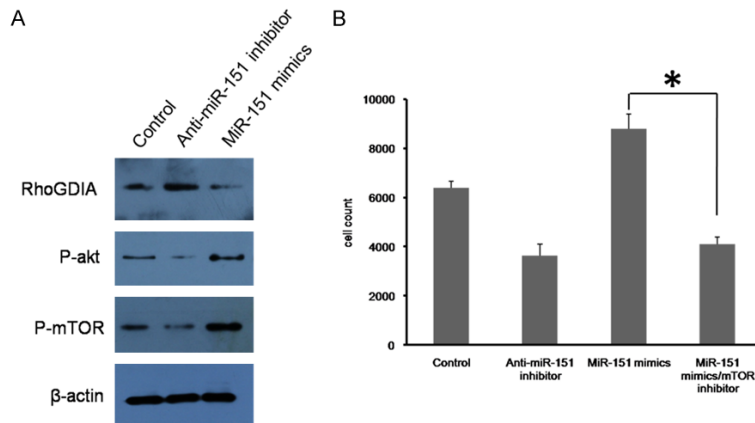


Figure 4. MiR-151 activated akt/mTOR signaling by targeting RhoGDIA. A. The western blot analysis of akt/mTOR and RhoGDIA in SKOV3 cells treated with anti-miR-151 inhibitor or mimics. B. The growth of SKOV3 cells was affected by mTOR inhibitor at day 4. All data were repeated three times.

as well as invasion of SKOV3 cells.

MiR-151 activated akt/mTOR signaling through decreasing RhoGDIA

In previous report, miR-151 was proved to directly bind to the 3-UTR region of RhoGDIA in both HCC and prostate cancer. In this study, we showed RhoGDIA was significantly increased in miR-151 inhibitor-treated SKOV3 cells while it was decreased in miR-151 mimics-treated SKOV3 cells (**Figure 4A**). Further, we found that the level of phosphorylated akt (P-akt) and phosphorylated mTOR (P-mTOR) was much lower in anti-miR-151 inhibitor-transfected-SKOV3 cells than that in control group. But miR-151 mimics promoted P-akt and P-mTOR (**Figure 4A**). It is widely known that mTOR/akt signal pathway is vital to proliferation and survival in a series of tumors. When specific inhibitor against mTOR was used in combination with miR-151 mimics, aberrant proliferation was seen in SKOV3 cells (**Figure 4B**). Therefore, these data support the deduction that miR-151 prompts the akt/mTOR signaling by targeting RhoGDIA, which provided a new way to understand the mechanism of carcinogenesis and progression of ovarian cancer.

Table 1. Correlation of miR-151 expression with clinicopathological characteristics in ovarian cancer

Characteristics	Total	MiR-151 expression level		P value
		≤2 fold	>2 fold	
All cases	41	15	26	
Age (y)				0.923
<50	25	9	16	
≥50	16	6	10	
Grade				0.028
G1/G2	30	14	16	
G3/G4	11	1	10	
TNM Stage				0.002
Early (I/II)	17	11	6	
Late (III/IV)	24	4	20	

Knockdown of miR-151 suppressed migration and invasion of SKOV3 cells

To explore the effect of miR-151 on metastasis of ovarian cancer, we performed wound healing assay and transwell assay to study the motility and invasion of SKOV3 cells. As shown in **Figure 3A**, the migrated distance was much shorter in anti-miR-151 inhibitor-treated SKOV3 cells than that treated with negative control. In consistent, the cells invaded through the matrigel monolayer in miR-151 inhibitor-treated group were much fewer than that in the control. From the data in **Figure 3B**, the cells in test group was only 29% of that in control group. Thereafter, miR-151 was favorable for the migration

as well as invasion of SKOV3 cells.

MiR-151 expression was associated with clinical features of ovarian cancer

In the above text, we demonstrated that miR-151 was highly expressed in both ovarian cancer tissue and cell lines. But what's the relationship between miR-151 expression and the clinical features of ovarian cancer? Statistical analysis revealed that miR-151 expression was significant correlated with tumor grade (P=0.028) and tumor stage (P=0.002) (**Table 1**). Moreover, Kaplan-Meier survival curves and the log-rank test survival analysis indicated that the 5-year survival rate of patients with high levels of miR-151 was much poorer than that with low levels of miR-151 (23% vs 40%, P=0.035) (**Figure 5**). These data further support that miR-151 may be a promising marker for diagnosis or prognosis of ovarian cancer.

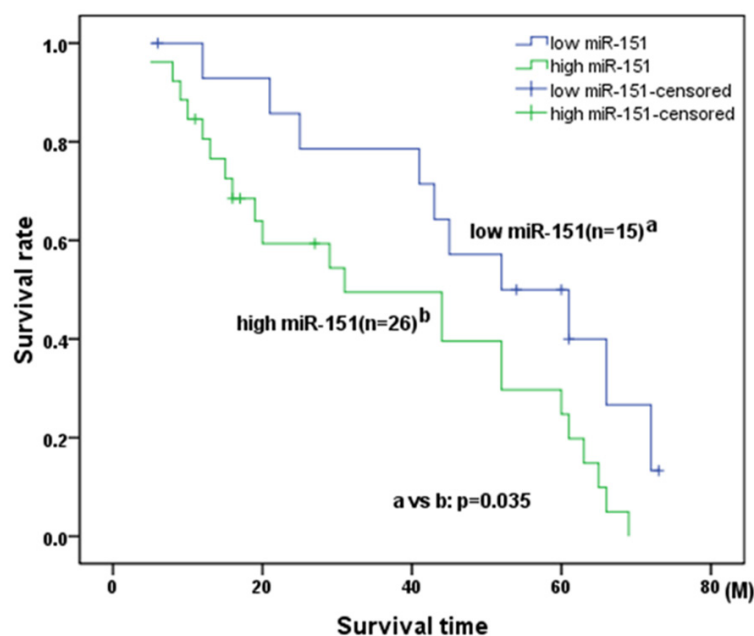


Figure 5. Survival analysis of ovarian cancer patients based on miR-151 expression. The survival rate of patients with low miR-151 was better than that with high miR-151 (P=0.035).

Discussion

Ovarian cancers have brought great threat to women’s life and were reported to be prone to form secondary lesions at a distance from the ovary [20]. Metastasis is common in tumor patients and is directly associated with the prognosis of patients. A list of factors was shown to influence metastasis such as gene mutation, epigenetic modification, microenvironment and aberrant microRNA expression. Accumulating evidence suggest that microRNA expression was correlated with the progression of ovarian cancer. For example, miR-30 and miR-181 were significantly high in the low grade patients [21, 22]. Decreased progression-free survival of ovarian cancer patients was partly attributed to high level of miR-200 [23]. In this study, we demonstrated that miR-151 was expressed highly in the ovarian cancer tissues compared to tumor-adjacent tissues. And miR-151 expression was significantly correlated with tumor grade and the age of patients while high miR-151 predicted poor 5-year survival rate. These data indicated that miR-151 may play an important role in ovarian cancer but studies on a larger number of samples are needed to support more precise evaluation.

The evolutionary conserved microRNAs are single-stranded non-coding RNA containing 19-21 nucleotides in length. Mature microRNAs are able to modulate gene translation at post-transcriptional level by binding 3’ untranslated region (3’-UTR) of messenger RNA and alternate biological behavior of cells. For instance, up-regulated miR-21 promoted proliferation and inhibited apoptosis in ovarian cancer cells [24]. High miR-17 expression induced metastasis and drug resistance in ovarian cancer cells [25]. Meanwhile, some microRNAs functioned as tumor suppressor in ovarian cancer. That low expression of miR-199 impaired proliferation, metastasis and chemosensitivity of ovarian cancer cells was a good example

[26]. By using anti-miR-151 inhibitor and miR-151 mimics, we found that miR-151 contributed to proliferation, migration and invasion of ovarian cancer cells, which suggest that miR-151 played a tumor suppressor role in ovarian cancer.

In this study, we found that miR-151 expression was accompanied by FAK expression. FAK was a gene encoding protein tyrosine kinase and favored cell migration and invasion in various cancers [27, 28]. Interestingly, miR-151 was derived from the intron of FAK gene and was also reported to play oncogenic role in tumorigenesis [29]. In addition, we showed that ARHGDI A, a cellular regulatory protein negatively regulating most Rho GTPases, was increased in SKOV3 cells treated with anti-miR-151 inhibitor while it was decreased by miR-151 mimics. This was consistent with the previous reports in HCC that ARHGDI A was a direct target of miR-151 [17]. Our data demonstrated miR-151 targeted ARHGDI A and regulated its expression in ovarian cancers. It was reported that loss of ARHGDI A enhanced tumor metastasis and progression in prostate cancer [18]. Also ARHGDI A was reported decreased frequently in malignant gliomas [30]. Taken together, it is conceived that miR-151 may pro-

mote tumorigenesis in ovarian cancer by targeting ARHGDI1 and down-regulating its expression. At the same time, we demonstrated the phosphorylation level of both mTOR and akt was dramatically enhanced in miR-151 mimics-treated SKOV3 cells. Activation of the mTOR/akt signaling pathway was reported in various kinds of cancers and prompted cell proliferation. For example, miR-21 mediated tumor progression through regulating the activation of mTOR/akt signal pathway [31]. Therefore, based on the above data, we deduced that miR-151 activated the mTOR/akt signaling pathway in ovarian cancer.

In summary, our data support the conclusion that miR-151 promotes tumorigenesis in ovarian cancer by targeting ARHGDI1 followed by activation of mTOR/akt signaling. And miR-151 may be considered as a potential clinical diagnostic biomarkers and predict the prognosis of ovarian cancer patients after surgery.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Pei-Shu Liu, Department of Obstetrics and Gynaecology, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan 250012, Shandong Province, P. R. China. E-mail: peishul_0001@tom.com

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