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Original Article

miR-26a inhibits the proliferation of ovarian cancer cells via regulating CDC6 expression

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Abstract: MicroRNAs (miRNA) play important regulatory roles in the occurrence and development of cancers. This study aimed to investigate the effects of miR-26a on the proliferation and apoptosis of ovarian cancer cells and explore the potential mechanism. qRT-PCR was performed to measure the miR-26a expression in 46 ovarian cancer tissues, and results showed miR-26a expression reduced significantly when compared with normal ovarian tissues ($P<0.05$). Moreover, miR-26a expression was related to the extent of cell differentiation and clinical stage of ovarian cancer ($P<0.05$). miR-26a mimic was transfected into SKOV3 cells and ES2 cells, and CCK8 assay, colony formation assay and flow cytometry showed miR-26a over-expression could significantly inhibit the proliferation of ovarian cancer cells and induce their apoptosis. Bioinformatics analysis revealed Cdc6 was a target gene of miR-26a. dual-luciferase assay and validation assay showed miR-26a could act on the 3'UTR of Cdc6 to regulate Cdc6 expression. These findings suggest that miR-26a may act on the 3'UTR of Cdc6 to regulate Cdc6 expression, which then inhibit the proliferation of ovarian cancer cells and induce their apoptosis. Our findings provide a new target for the diagnosis and therapy of ovarian cancer.

Keywords: Ovarian cancer, miR-26a, cell division cycle 6, proliferation

Introduction

Ovarian cancer (OC) is one of the most common malignancies of the reproductive system and the leading cause of cancer related death in women [1]. It has been regarded the silent killer in women. Patients with early OC usually have no any symptom and thus the diagnosis of early OC is often difficult. About 70% of OC patients are diagnosed with stage III or IV OC at initial hospital visit [2]. The occurrence and development of ovarian cancer are related to the abnormalities in a variety of genes. To date, no specific parameter has been developed for the diagnosis, treatment and prognostic prediction of OC.

microRNAs (miRNA) are a group of endogenous, non-encoding small RNAs which are widely distributed in the eukaryotic cells. They are about 22 nucleotides in length. miRNA may bind to the 3'UTR of a target gene for the post-transcriptional regulation, which is important for a large amount of biological processes. To date, studies have shown that more than 30% of proteins are regulated by miRNA [3-5]. There is evidence showing that miRNAs are closely associated with the occurrence and development of cancers, and the relationship between miRNAs and cancers has been one of focuses in studies [6, 7]. A variety of studies reveal that miRNAs are closely related to the occurrence and development of OC, the expression of some miRNAs is down-regulated or up-regulated, which may affect the development of cancers via regulating their target genes [8-10].

Recent studies indicate that miRNA-26a expression is down-regulated in a lot of cancers including liver cancer [11], prostate cancer [12], gastric cancer [13] and breast cancer [14]. miRNA-26a mediated post-transcriptional inhibition of target genes is involved in a large amount of pathological processes including proliferation, differentiation and apoptosis of cells. Studies have indicated that miR-26a expression is down-regulated in the serum of OC patients [15]. To further investigated the role of miR-26a

in the occurrence and development of OC, bioinformatics (TargetScan, miRanda database of target gene prediction) analysis was conducted to predict the target gene of miR-26a, and results showed Cdc6 was a target gene of miR-26a. There is evidence showing that Cdc6 is closely associated with some biological processes (such as proliferation and apoptosis) of cancer cells [16]. In the present study, the expressions of miR-26a and Cdc6 were measured in OC and their relationship was evaluated, aiming to explore the role of miR-26a in the occurrence and development of OC as well as the potential mechanism, which may provide a new target for the diagnosis and therapy of OC.

Materials and methods

Sample collection

A total of 46 OC tissues and 13 normal ovarian tissues were collected from the patients hospitalized in the People's Hospital of Henan Province between September 2013 and September 2014. These patients did not receive radiotherapy, chemotherapy and immune therapy, and OC was pathologically proven. Of the OC tissues, moderately to well differentiated OC was found in 11 patients, moderately differentiated OC in 16 and poorly differentiated OC in 19. According to the clinical staging criteria for ovarian cancer developed by the Federation International of Gynecology and Obstetrics (FIGO), stage I OC was found in 6 patients, stage II in 8, stage III in 17 and stage IV in 15. In addition, serous cystadenocarcinoma was noted in 31 patients and mucous cystoadenocarcinoma in 15. There were 19 patients younger than 50 years and 27 patients were 50 years old or older. Informed consent was obtained before study and the whole study protocol was approved by the Ethics Committee of our hospital.

Cell lines and materials

SKOV3 cells and ES2 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 (FBS; Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 1000 U/ml penicillin and 100 mg/ml streptomycin in an environment with 5% CO_2 at 37°C.

RNA synthesis and transfection

miR-26a mimic and negative control (miR-NC) were synthesized in Shanghai GenePharma Co. Ltd. Cells were seeded into 6-well plates at a density of 2×10^5 cells/well and maintained in medium without antibiotics for 24 h. When the cell confluence reached 80%, cell transfection was conducted with Lipofectamine™ 2000 kit. Cells were divided into 3 groups: miR-21 mimic group, miR-NC group and blank group. Transfection was conducted for 6 h in the presence of Lipofectamine, and the medium was refreshed with complete medium, followed by incubation for another 24 h. Cells were harvested for further detections.

RNA extraction and RT-qPCR

Real time fluorescence quantitative PCR (RT-qPCR) was employed to detect the mRNA expressions of miR-26a and Cdc6 in the OC tissues and normal ovarian tissues. Total RNA was extracted with RNA extraction kit (Qiagen, Venlo, the Netherlands) and the absorbance was measured with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) for the detection of RNA concentration and purity. Total RNA was used for reverse transcription into cDNA with MMLV RTase cDNA Synthesis Kit (TaKaRa, Dalian, China). qPCR was conducted to amplify the target fragments with ABI Power SYBR-Green PCR Master Mix. The copy number of U6 was used for correction, and cycle threshold (CT) value was determined, and miR-26a and Cdc expressions were calculated with $2^{-\Delta CT}$ method. β-actin served as an internal reference.

Western blot assay

Total protein was extracted and protein concentration was determined with BCA method. Proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membrane which was subsequently blocked. After incubation with primary antibody (1:500; Cdc6; Abcam) at 4°C over night, the membrane was washed in TBST thrice (15 min for each), and then treated with secondary antibody (1:1000; Horseradish peroxidase conjugated IgG; Abcam). Visualization was conducted with chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). β-actin (Santa Cruz) served as an internal

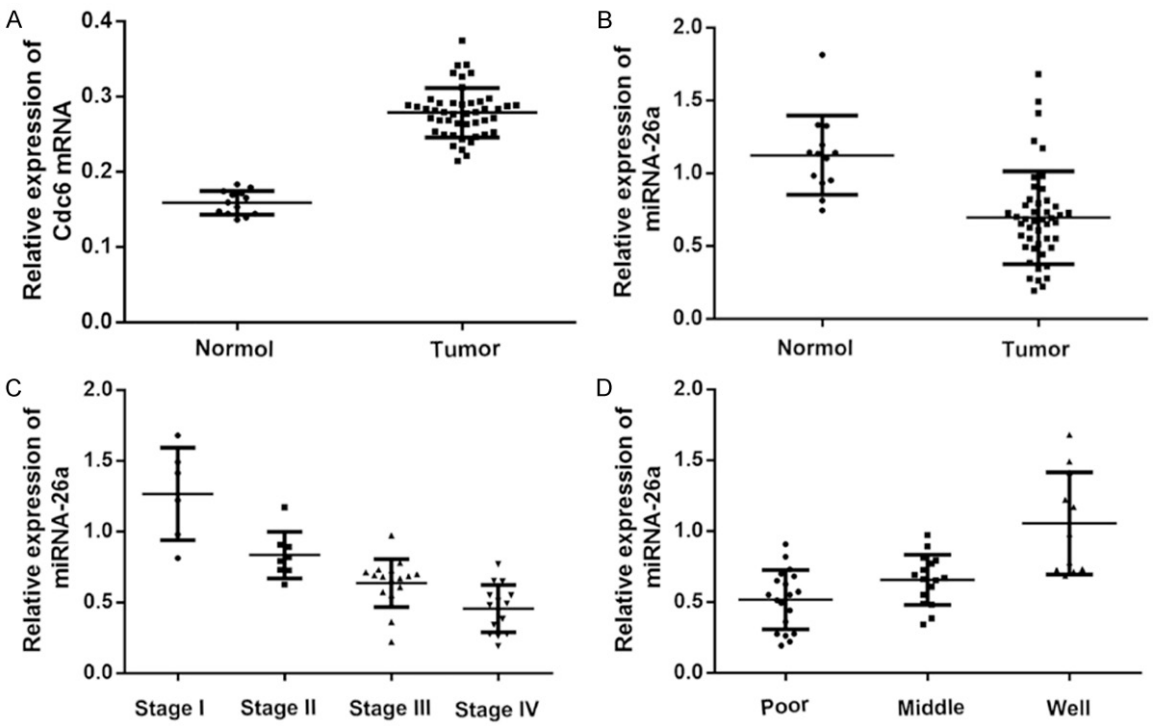

Figure 1. Detection of miR-26a and Cdc6 mRNA expressions in the OC tissues by qRT-PCR. A. Cdc6 mRNA expression in OC tissues was significantly higher than in normal ovarian tissues ($P<0.05$). (Tumor: OC tissues; Normal: normal ovarian tissues). B. miR-26a expression in OC tissues reduced significantly as compared to normal ovarian tissues ($P<0.05$) (Tumor: OC tissues; Normal: normal ovarian tissues). C. The higher the FIGO stage of OC, the lower the miR-26a expression was ($P<0.05$). D. miR-26a expression in well differentiated OC was significantly higher than in poorly and moderately differentiated OC ($P<0.05$).

reference, and the relative expression of target gene was calculated.

Detection of cell proliferation with CCK-8 assay

CCK-8 assay (Dojindo Laboratories, Japan) was performed to detect cell viability. Cells in logarithmic growth phase were seeded into 96-well plates at a density of 2×10^3 cells/well. After incubation for 24 h, 48 h, 72 h and 96 h, 10 μL of CCK-8 solution was added to each well. The optical density (OD) was measured at 450 nm and the cell proliferation was determined.

Colony formation assay

The RPMI 1640 containing 0.6% agarose gel and 10% FBS was added to 6-well plates, and incubated at room temperature until solidification. Cells in logarithmic growth phase were harvested and suspended in RPMI 1640 containing 0.3% agarose gel and 10% FBS (low melting point). Then, the cell suspension was added to above 6-well plates, followed by incubation for 12 days in an environment with 5% CO_2 at 37°C. After crystal violet staining, colonies were counted. A colony contained more than 50 cells. The mean number of colonies reflects the colony formation ability.

Detection of cell apoptosis by flow cytometry

Annexin V-FITC/PI cell apoptosis detection kit (BestBio, Shanghai, China) was used to detect apoptotic cells. Cells in logarithmic growth phase were harvested and cell suspension at 1×10^6 cells/ml was prepared. After addition of propidium iodide (PI) and FITC Annexin V, the apoptotic cells were detected by flow cytometry within 30 min. Detection was conducted in triplicate and data were analyzed with Cell Quest.

Dual luciferase reporter gene assay

Genome was extracted from healthy subjects and the 3'UTR of wild type Cdc6 with miR-26a binding site was amplified. After recycling and purification, it was inserted into the multiple

Table 1. Correlation of miR-26a and Cdc6 mRNA expressions with clinicopathological factors

Clinicopathological Characteristics	n	miRNA-26a expression (median ± SD)	*P* value	Cdc6 expression (median ± SD)	*P* value
Age (yr)			0.700		0.880
<50	19	0.7155±0.38206		0.2794±0.02970	
≥50	27	0.6781±0.27384		0.2779±0.03555	
Histological type			0.570		0.125
Serous cystadenocarcinoma	31	0.6746±0.33396		0.2733±0.0344	
Mucous cystoadenocarcinoma	15	0.7326±0.29432		0.2892±0.3614	
Extent of differentiation			<0.001*		0.004*
Well	11	1.0536±0.36007		0.2961±0.03463	
Moderately	16	0.6559±0.17594		0.2714±0.02862	
Poorly	19	0.5168±0.20959		0.2961±0.03463	
Clinical stage			<0.001*		<0.001*
I-II	14	1.0187±0.32381		0.2520±0.02044	
III-IV	32	0.5513±0.18848		0.29001±0.03064	
Lymph node metastasis			0.117		0.120
No	18	0.7858±0.33032		0.2691±0.02669	
Yes	28	0.6342±0.30344		0.2845±0.03544	

Note: *$P<0.05$ between two groups.

cloning site of pmirGLO which was then named pmirGLO-Cdc6-wt. overlap PCR was employed to mutate the 3'UTR of CDC6, achieving mutant CDC6 3'UTR, which was then inserted into the multiple cloning site of pmirGLO (named pmirGLO-CDC6-mut). Recombinant vector and miR-26a mimics or miR-NC were co-transfected into SKOV3 cells. At 24 h after transfection, dual luciferase signals were measured according to the manufacturer's instructions.

Reverse mutation assay

Cdc6 gene without 3'UTR was amplified by PCR and then inserted into eukaryotic expression vector pcDNA3.1 (named pcDNA3.1-Cdc6). pcDNA3.1-Cdc6 was transfected into SKOV3 cells. At the same time, miR-26a mimic and miR-NC was used to transfected SKOV3 cells. After 48-h transfection, Cdc6 protein expression was detected by western blot assay, and cell apoptosis was detected by flow cytometry.

Statistical analysis

Statistical analysis was performed with SPSS version 18.0. Quantitative data are expressed as mean ± standard deviation (SD), and comparisons were conducted with one way analysis of variance (ANOVA) among groups. A value of $P<0.05$ was considered statistically significant. Pearson correlation analysis was employed to evaluate the correlation among groups.

Results

Expressions of miR-26a and Cdc6 in the OC tissues

qRT-PCR was employed to detect the mRNA expressions of miR-26a and Cdc6 in the OC tissues. When compared with normal ovarian tissues, the miR-26a expression reduced significantly in the OC tissues ($P<0.05$; **Figure 1B**), but Cdc6 expression in the OC tissues was significantly higher than in normal ovarian tissues ($P<0.05$; **Figure 1A**). This indicates miR-26a expression was negatively associated with Cdc6 expression in OC tissues. In addition, we further evaluated the correlation of miR-26a expression and Cdc6 expression with clinicopathological characteristics of 46 patients. Results showed miR-26a expression was associated with the differentiation of cancer cells and the clinical stage ($P<0.05$; **Table 1**; **Figure 1C** and **1D**), but not with age, pathological type and lymph node metastasis (P>0.05; **Table 1**). Cdc6 expression was related to the differentiation of cancer cells and clinical stage ($P<0.05$; **Table 1**), but not with age, pathological type and lymph node metastasis ($P>0.05$; **Table 1**).

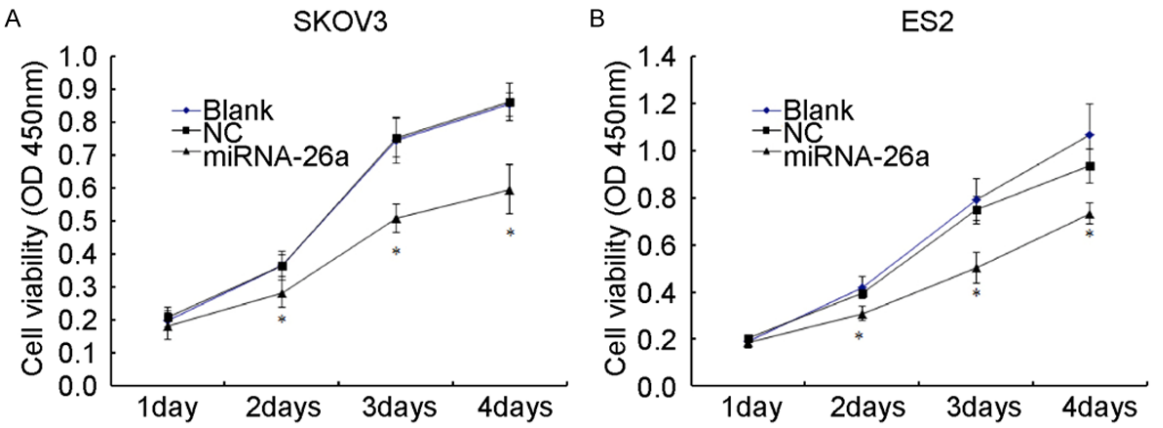

Figure 2. miRNA-26a over-expression inhibits the proliferation of OC cells. miRNA-26a mimic was transfected into OC cells and cell proliferation was detected by CCK-8 assay. Results showed the proliferation rate of SKOV3 cells (A) and ES2 cells (B) reduced significantly at 2, 3, and 4 days when compared with NC group and blank group (P<0.05). **P*<0.05.

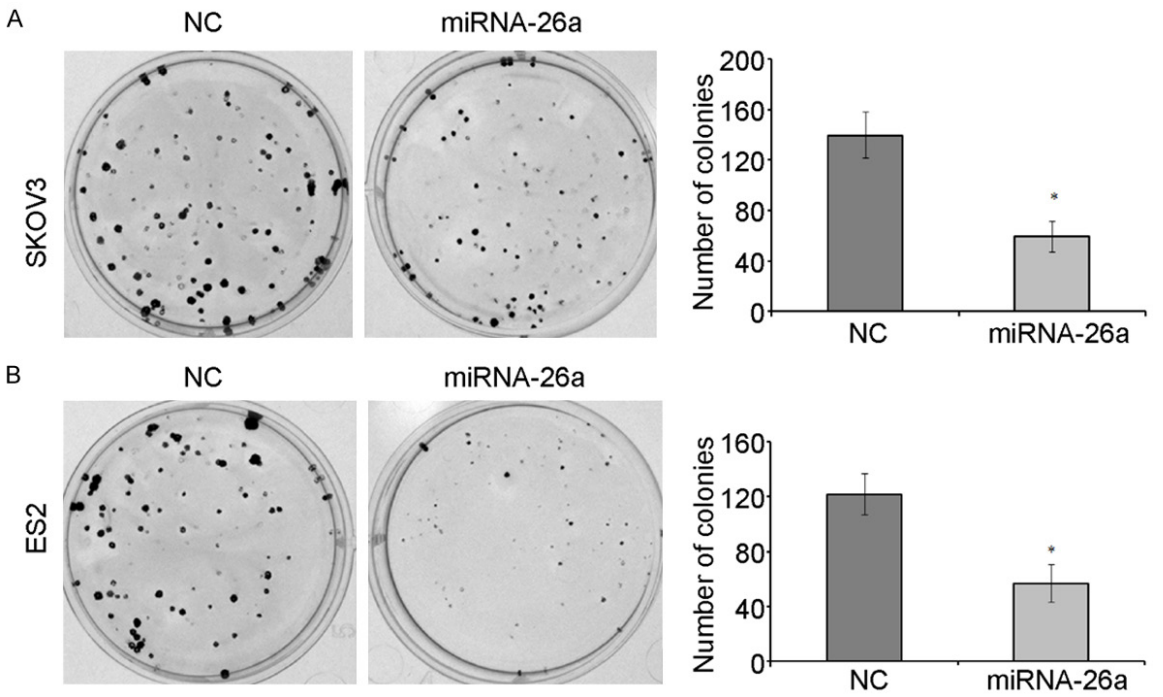

Figure 3. miRNA-26a over-expression reduces the number of colonies of OC cells. miRNA-26a mimic was transfected into OC cells, and the number of colonies was determined. Results showed the number of colonies formed by SKOV3 cells (A) and ES2 cells (B) reduced markedly when compared with NC group (*P*<0.05). **P*<0.05.

Effect of miR-26a on SKOV3 cell proliferation

CCK-8 assay and clony formation assay were employed to evaluate the effect of miRNA-26a on the proliferation of SKOV3 cell proliferation. CCK-8 assay showed, at 48 h after transfection with miRNA-26a mimics, the proliferation rate of SKOV3 cells and ES2 cells increased significantly as compared to blank group and NC group (P<0.05), but there was no marked difference between blank group and NC group at any time point (P>0.05) (**Figure 2**). Colony formation assay revealed that the number of colonies reduced dramatically after transfection with miRNA-26a mimics (P<0.05) (**Figure 3**). These findings indicate that miRNA-26a over-expres-

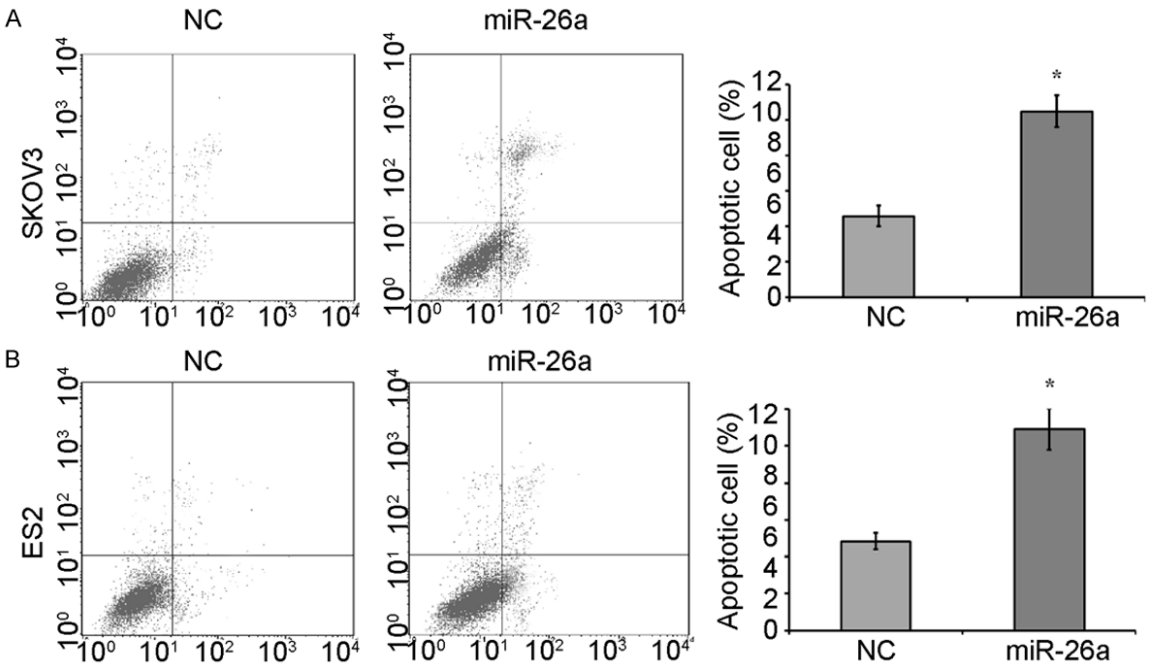

Figure 4. miRNA-26a over-expression induces apoptosis of OC cells. miRNA-26a mimic was transfected into OC cells, and results showed the apoptosis rate of SKOV3 cells (A) and ES2 cells (B) increased significantly when compared with NC group (P<0.05). **P*<0.05.

sion inhibits the proliferation of OC SKOV3 cells and ES2 cells.

Effect of miR-26a on the apoptosis of SKOV3 cells and ES2 cells

Flow cytometry was performed to detect the apoptotic OC cells. After transfection with miR-26a mimic, the apoptotic SKOV3 cells and ES2 cells increased significantly when compared with NC group (*P*<0.05; **Figure 4**). This suggests that miRNA-26a over-expression promotes the apoptosis of OC SKOV3 cells and ES2 cells.

miR-21 acts on the 3'UTR of Cdc6 mRNA to inhibit Cdc6 expression

Bioinformatics analysis with TargetScan and miRanda databases revealed that Cdc6 was a target gene of miR-26a (**Figure 5A**). Western blot assay showed Cdc6 protein expression reduced significantly in SKOV cells after transfection with miR-26a mimic (**Figure 5B**). To confirm Cdc6 was a target gene of miR-26a, the 3'UTR of wide type Cdc6 gene and mut-type Cdc6 gene was inserted into dual luciferase reporter vector pmirGLO. Then, pmirGLO and miR-26a mimic or miR-NC were transfected into OC SKOV cells, and the luciferase signals were measured. Results showed, after transfection with miR-26a mimic and pmirGLO-Cdc6-wt, the luciferase activity was significantly inhibited as compared to miR-NC and pmirGLO-Cdc6-wt groups (*P*<0.05, **Figure 5C**). After co-transfection with miR-26a mimic and pmirGLO-Cdc6-mut, the luciferase activity was similar to that in control groups (miR-NC and pmirGLO-Cdc6-mut co-transfection group) (P>0.05, **Figure 5C**). These findings indicate that miR-26a is able to bind to the 3'UTR of Cdc6 mRNA to negatively regulate Cdc6 expression (P>0.05, **Figure 5C**).

Cdc6 over-expression is able to reverse its pro-apoptotic effect on OC cells

To further investigate the target of miR-26a, eukaryotic expression vector pcDNA3.1-Cdc6 without 3'UTR was constructed and co-transfected with miR-26a mimic or miR-NC into OC SKOV3 cells. In addition, miR-26a mimic or miR-NC alone was transfected into SKOV3 cells. Western blot assay showed pcDNA3.1-Cdc6 and miR-26a mimic co-transfection significantly increased Cdc6 protein expression, indicating that pcDNA3.1-Cdc6 is able to reverse the inhibitory effect of miR-26a on Cdc6 protein expression (**Figure 6A**). Detection of

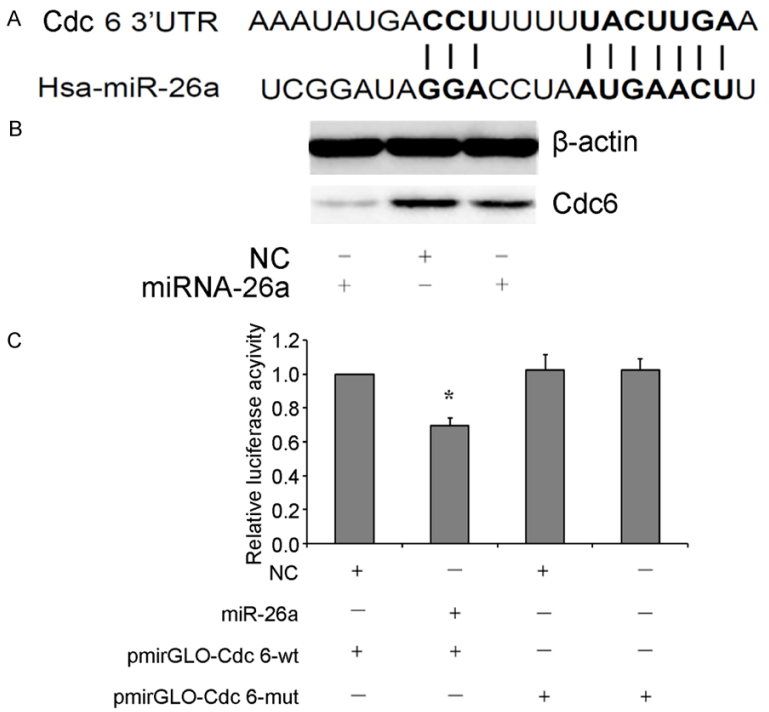

Figure 5. miR-26a acts on the 3'UTR of Cdc6 gene to regulate Cdc6 expression. A. miR-26a acted on the 3'UTR of Cdc6 mRNA; B. Western blot assay showed miR-26a over-expression inhibited the Cdc6 protein expression in OC SKOV cells. C. Dual-luciferase reporter gene assay showed the luciferase activity reduced significantly in SKOV3 cells after co-transfection with miR-26a mimic and pmirGLO-Cdc6-wt when compared with miR-NC group and pmirGLO-Cdc6-wt group (P<0.05). After co-transfection with miR-26a mimic and pmirGLO-Cdc6-mut, the luciferase activity in SKOV cells was similar to that in control groups (miR-NC and pmirGLO-Cdc6-mut co-transfection) (P>0.05). **P*<0.05.

cell apoptosis indicated that the apoptosis rate increased significantly after transfection with miR-26a mimic (*P*<0.05; **Figure 6B**). After co-transfection of miR-26a mimic with pcDNA3.1-Cdc6 or pcDNA3.1-Cdc6, the apoptosis rate reduced dramatically (*P*<0.05; **Figure 6B**). This further indicates that miR-26a is able to act on the 3'UTR of Cdc6 gene to regulate Cdc6 expression, exerting pro-apoptotic effect of Cdc6.

Discussion

In recent years, studies reveal that there is abnormal expression in some miRNAs in OC, which is involved in the occurrence, development, metastasis and resistance of OC. Ioria et al [17] investigated the miRNA expression profile in OC and found 4 miRNA (miR-141, miR-200a, miR-200b, and miR-200c) had a high expression and 25 miRNAs (miR-199a, miR-140, miR-145, miR-125b-1, et al) displayed a low expression. In a study, the miRNA expression was compared between 18 epithelial ovarian cancers (EOC) and 4 immortalized ovarian surface epithelium (IOSE) and results showed 35 differentially expressed miRNA: the expressions of 31 miRNAs in EOC was significantly lower than in IOSE [18]. Kan et al [19] found that the expressions of miR-200a, miR-200b, and miR-200c in the serum of OC patients increased dramatically as compared to healthy controls and might serve as new markers in the diagnosis of OC.

The mature miR-26a includes miR-26a-1 and miR-26a-2 which have the same sequence but are derived from different chromosomes [20]. miR-26a acts on the "seed region" of a target gene to exert multiple biological effects. In some solid cancers, studies have confirmed the low miR-26a expression [21]. Deng et al [13] found that miR-26a expression in the gastric cancer reduced significantly when compared with adjacent normal tissues, and miR-26a expression was negatively related to the lymph node metastasis and clinical stage of gastric cancer. Song et al [22] found the miR-26a expression reduced in osteosarcoma, and the reduced miR-26a expression was negatively associated with the clinical stage and distance of metastasis of osteosarcoma. Zhang et al [14] found miR-26a expression in breast cancer was significantly lower than in adjacent normal tissues, and low miR-26a expression was related to the age and HER2 expression in the breast cancer. In OC patients, findings also reveal that serum miR-26a expression is significantly lower than in control group and miR-26a may serve as a biomarker for the diagnosis of serous ovarian cancer [15]. Our study showed the miRNA-26a expression reduced in OC tissues, and reduced

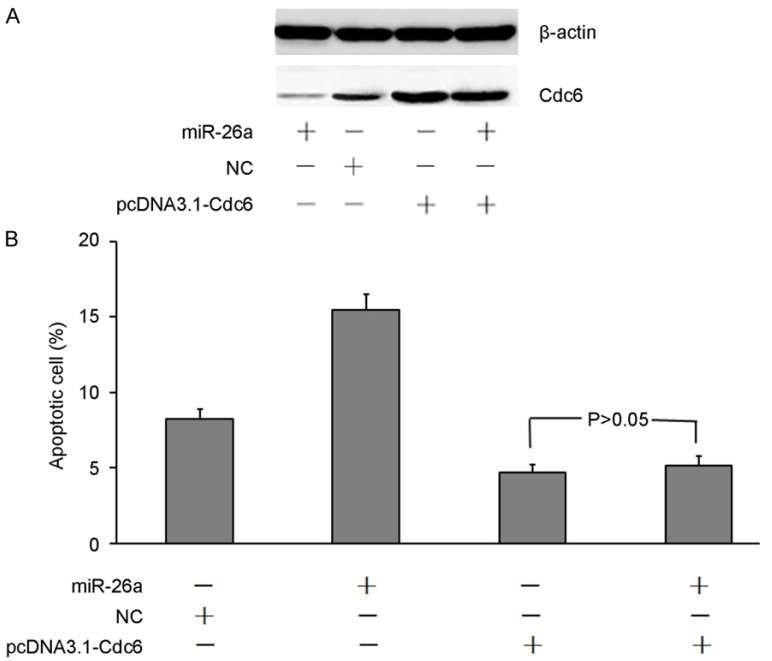

Figure 6. Cdc6 over-expression reverses the pro-apoptotic effect of miR-26a on OC cells. A. Western blot assay showed Cdc6 protein expression increased dramatically in SKOV3 cells after co-transfection with pcDNA3.1-Cdc6 without 3'UTR and miR-26a mimic. B. After co-transfection with pcDNA3.1-Cdc6 without 3'UTR and miR-26a mimic, the apoptosis rate of SKOV3 cells reduced significantly ($P<0.05$).

miRNA-26a expression was associated with the extent of differentiation and clinical tsage, suggesting that miRNA-26a is involved in the occurrence and development of OC. In OC cell lines (SKOV3 cells and ES2 cells), miR-26a over-expression was able to reduce the proliferation of OC cells and increase their apoptosis rate, indicating that miR-26a has the capability to inhibit the proliferation of OC cells.

Bioinformatics analysis showed Cdc6 was a target gene of miR-26a. Human Cdc6 gene is mapped to 17q21.3 [23] and is an important component of pre-replication complexes (pre-RC). Cdc6 is indispensible in the DNA replication, and silencing of Cdc6 may inhibit the DNA replication and arrest cells in G1/S phase resulting in unability to enter S phase [24, 25]. Studies have revealed that Cdc6 not only inhibits DNA replication, but induces cell apoptosis [26]. The abnormal Cdc6 expression may cause malignant proliferation of cells. In a lot of cancers (such as cervical cancer [27, 28], lung cancer [29], oral squamous cell carcinoma [30] and prostate cancer [31]) and cancer cell lines, Cdc6 shows a high expression, which is related to the extent of malignancy. In this study, qRT-PCR was employed to detect the Cdc6 mRNA expression in 46 OC tissues. Results showed the Cdc6 mRNA expression in OC tissues was significantly higher than in normal ovarian tissues and Cdc6 mRNA expression was associated with the extent of differentiation and clinical stage of OC. These findings indicate that Cdc6 may serve as an oncogene in the occurrence and development of OC.

To further confirm that Cdc6 was a target gene of miR-26a and investigate the binding site of Cdc6 in miR-26a, Cdc6 3'UTR was cloned into dual luciferase reporter vector pmirGLO. Results showed miR-26a was able to bind to the 3'UTR of Cdc6 to negatively regulate Cdc6 expression. Reverse mutation assay showed Cdc6 over-expression could reverse the pro-apoptotic effect of miR-26a on OC cells. This further indicates that miR-26a is able to bind to the 3'UTR of Cdc6 mRNA to regulate Cdc6 expression, which then inhibits the proliferation of OC cells and induce their apoptosis.

Conclusion

Taken together, our findings reveal the abnormal miR-26a expression is closely related to the proliferation of OC cells. In the occurrence and development of OC, miR-26a may act on the 3'UTR of Cdc6 to inhibit Cdc6 expression, exerting anti-tumor effect. To further elucidate the target gene of miR-226 and the specific mechanism underlying the bio-effects of miR-226 may render miR-226 as a new target for the diagnosis and treatment of OC.

Disclosure of conflict of interest

None.

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