

## Original Article

# MiRNA-335-5p inhibits cell proliferation and invasion through regulating DKK1 in epithelial ovarian carcinoma

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**Abstract:** The function of miRNA-335-5p (miR-335-5p) has been reported in many human cancers. However, till now, the expression and role of miR-335-5p in epithelial ovarian carcinoma was unclear. In this study, we aimed to investigate the expression and molecular mechanism of miR-335-5p in EOC development. The expression level of miR-335-5p in EOC tissues and cell lines was measured by using quantitative Real-time PCR. MiR-335-5p mimic and inhibitor were respectively transfected into EOC cell line for further investigation. CKK8 assay was used to detect cell proliferation. Transwell assay was performed to detect cell invasive ability. Dual-luciferase report assay, quantitative real-time PCR, western blot and immunocytochemical analysis were performed to explore the mechanism of miR-335-5p in EOC development by regulating DKK1 protein. Our results showed that miR-335-5p was down-expressed in EOC tissues and negatively correlated with tumor stage ( $P < 0.05$ ). MiR-335-5p mimic could inhibit cell proliferation and invasion, while miR-335-5p inhibitor promoted cell proliferation and invasion ( $P < 0.05$ ). DKK1 was identified as a target of miR-335-5p. The expression of DKK1 protein was inhibited by miR-335-5p mimic while promoted by miR-335-5p inhibitor ( $P < 0.05$ ). Our results showed miR-335-5p played an important role in suppressing the proliferation and invasion of EOC cells by directly targeting DKK1.

**Keywords:** MiR-335-5p, DKK1, EOC, proliferation, invasion

## Introduction

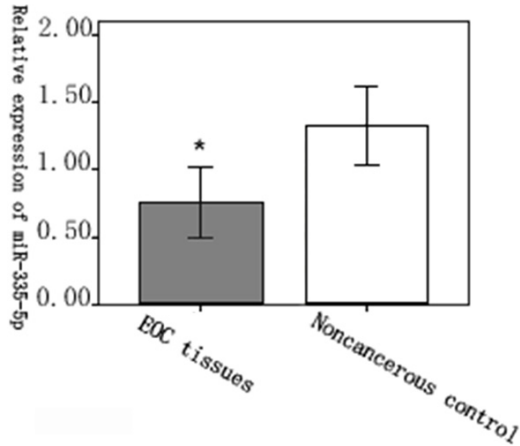
Ovarian cancer is one of the most malignant gynecological cancers. Epithelial ovarian carcinoma (EOC) is one of the most common subtypes of ovarian carcinoma [1, 2]. The early clinical symptoms of EOC patients are not obvious and the proliferative and invasive ability of EOC cells are quickly and severe. Consequently, its prognosis is usually poor [3-5]. Till now, the molecular mechanism of EOC was unclear. Therefore, it is necessary for us to investigate the molecular mechanism of EOC and develop new targeted therapies.

MicroRNAs (miRNAs), 18-25 nucleotides in length, are small non-coding RNAs. miRNAs, translationally inhibiting or directly degrading target mRNA, inhibit target protein expression by binding to complementary sequences in the 3' untranslated region (3'UTR) of the target mRNA [6, 7]. Nowadays, miRNAs play an important role in EOC cell progression [8, 9]. For

instance, miRNA-200 family [10] and miRNA-429 [11] play key roles in EOC proliferation and invasion by down-regulating target genes. Moreover, miR-200 family are associated with EOC clinical pathological data, which shown the significance of miRNAs as clinical biomarkers of EOC [12]. Consequently, it is necessary for us to explore the functions and the targets of miRNAs in EOC carcinogenesis and progression. However, the role of miRNA-335-5p in EOC remains unclear.

In the present study, we examined the expression levels of miRNA-335-5p in EOC tissues and its relationship with EOC clinicopathological features. Furthermore, overexpression of miR-335-5p inhibited cell proliferation and invasion, while down-regulated expression of miR-335-5p increased inhibited cell proliferation and invasion in EOC cells. Luciferase reporter assay showed that miRNA-335-5p targeted the 3'-UTR of DKK1 mRNA. These findings suggested that

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**Figure 1.** Reduced miR-335-5p expression in EOC samples. Fifty-eight pairs of EOC tissues and benign epithelial ovarian cysts were obtained. The data presented the mean  $\pm$  SD with  $P=0.004$ .

miRNA-335-5p could be investigated as a targeted therapy for EOC.

### Materials and methods

#### Patients

Fifty-eight paired epithelial ovarian tissue specimens from patients with epithelial ovarian cancer and patients with benign epithelial ovarian cysts were collected at Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University. The including and excluding method was followed the criteria as previously reported [13]. The obtained fresh tissues were stored immediately in  $-80^{\circ}\text{C}$ . All patients signed their written informed consent. The experimentation was approved by the Ethics Committee of China Medical University.

#### Cell culture

Human EOC cell line SKOV-3 was purchased from the American Type Culture Collection (ATCC, USA). Cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$ .

#### Real-time qRT-PCR

MicroRNA was extracted by the mirVana miRNA Isolation Kit (Ambion). Total RNA was extracted by Trizol (Invitrogen). Quantitative PCR (qRT-PCR) of miR-335-5p was conducted using a stem-loop primer-based method as

mentioned by Chen et al [14]. qRT-PCR of DKK1 was conducted using the method as mentioned previously [15]. Experiments were performed using the SYBR Green PCR Master Mix (Solarbio, Beijing, China) in the Exicycler 96 Real-Time Thermal Block (Bioneer, Daejeon, Korea). The expression of miR-335-5p and DKK1 gene was normalized by  $\beta$ -actin and U6, respectively. The relative expression level was calculated by normalization using  $2^{-\Delta\Delta\text{Ct}}$  method. Primer sequences were designed by Primer 6.0 and synthesized by Invitrogen (Shanghai, China).

#### Cell transfection

MiR-335-5p mimic, miR-335-5p inhibitor and were chemically synthesized by Sangon Biotech (Shanghai, China). Scrambled sequences were used as control.  $15 \times 10^4$  (6-well) SKOV-3 cells were incubated in RPMI DMEM medium without antibiotics. After 24 h, miRNA 520b mimic, miRNA inhibitor were transiently transfected into SKOV-3 cell respectively with Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested for western blot or qRT-PCR.

#### Cell proliferation assays

To evaluate cell proliferation,  $3 \times 10^3$  SKOV-3 cells per well were incubated into 96-well plates and transfected with miR-335-5p mimic, miR-335-5p inhibitor or scrambled sequences. After 24, 48, 72 and 96 h, cell proliferative ability was measured using CKK8 assay solution and incubated for 2 h (Dojindo, Japan), following the manufacture's protocol.

#### Matrigel invasion analysis

Invasion chamber system (BD) with Matrigel membrane (8.01  $\mu\text{m}$  pore) was used for analysis (Corning, NY). Matrigel gel was used to precoat the membrane for invasion assay. The  $2 \times 10^4$  cells were incubated in the upper chamber with 1% FBS medium, and the bottom of the chamber was covered with the medium containing 20% FBS. The cells migrated for 24 h, the cells were fixed, stained with HE and counted under a microscope.

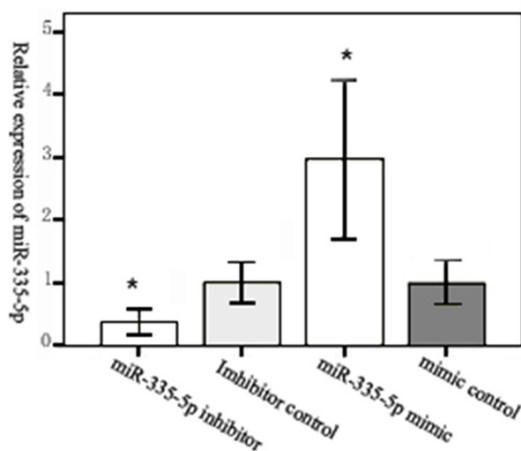
#### Luciferase reporter assay

Luciferase reporter plasmid containing miR-335-5p binding sites or mutant miR-335-5p binding sites of DKK1 3'UTR mRNA sequences was cloned into the pmirGLO Dual-Luciferase

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**Table 1.** Relationships between miR-335-5p and clinicopathological factors in 58 cases of ovarian cancers

	No.	miR-335-5p folds			
		Mean	SD	T/F	P
<b>Age</b>					
<45	17	0.83	0.84	0.405	0.687
≥45	41	0.72	1.07		
<b>FIGO stage</b>					
I~II	39	0.94	1.15	-2.871	0.006
III~IV	19	0.35	0.41		
<b>Differentiation</b>					
G1~G2	32	0.74	1.10	-0.103	0.918
G3	26	0.77	0.90		
<b>Lymph metastasis</b>					
No	38	0.77	1.03	0.207	0.837
Yes	20	0.71	0.99		
<b>Histology</b>					
Serous	32	0.76	1.08	0.39	0.679
Mucous	23	0.80	0.96		
Others	3	0.25	0.27		



**Figure 2.** The expression of miR-335-5p in SKOV-3 transfected cells. The expression of miR-335-5p was overexpressed in miR-335-5p mimic transfecting cells as compared with control mimic transfecting cells with  $P=0.002$ . MiR-335-5p was downexpressed in miR-335-5p inhibitor transfecting cells as compared with control inhibitor transfecting cells with  $P=0.003$ .

miRNAT target Expression Vector (Promega, Madison, WI, USA), respectively. MiR-335-5p modified ( $7 \times 10^5$ /well) SKOV-3 cells were seeded in 24-well plates for 24 h, after which they were transfected with  $1 \mu\text{g}$  of Luciferase reporter plasmids per well. Luciferase activities were

detected by the dual-luciferase reporter gene assay kit (Promega) and normalized by renilla luciferase activity.

### Western blot

Cell lysates were prepared in NP-40 lysis Buffer. Protein concentration was measured using BCA kit, analyzed by 10% SDS-PAGE and transferred onto nitrocellulose membrane. The primary antibodies were incubated as follows: anti-DKK1 1:500 (Abcam) at  $4^\circ\text{C}$  over night. Then the membrane was incubated with secondary antibody for 30 min. Protein were visualized by using an enhanced chemiluminescence visualization analysis.

### Immunocytochemical analysis

After 48 hours of transfection,  $2 \times 10^4$  SKOV-3 cells on cover glass were fixed with 4% paraformaldehyde at  $4^\circ\text{C}$  for 2 h, seeded with 0.1% Triton X-100 for 15 min and were blocked for 30 min with blocking buffer (5% FBS in PBS) at room temperature. The cells were incubated with primary antibodies: anti-DKK1 1:400 (Abcam, USA) at  $4^\circ\text{C}$  overnight then washed with washing buffer (0.1% BSA, 0.5% FBS in PBS) for 3 times. Then the cells were incubated with secondary antibodies containing FITC conjugated anti-IgG 1:200 (Beyotime, China), for 1 h. All images were analyzed with a laser scanning confocal microscope (Olympus, Japan).

### Statistical analysis

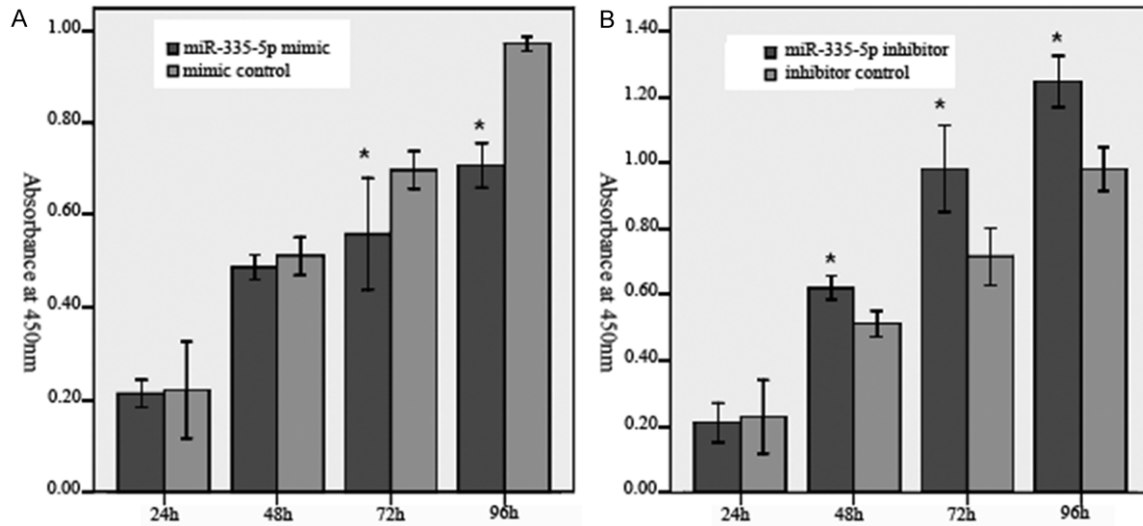
Statistical analyses were performed by SPSS 13.0 for windows. Student's t-test and ANOVA were used for comparison between groups. Statistical significance was defined as  $P < 0.05$ .

## Results

### MiR-335-5p was downregulated in human EOC tissues and correlated with tumor stage

We compared the expression of miR-520b in human EOC tissues and paired noncancerous tissues by qRT-PCR. We found that the expression of miR-335-5p was significantly downregulated in EOC tissues than that in noncancerous tissues ( $P=0.004$ ; **Figure 1**). We also evaluated the associations of miR-335-5p with clinicopathological features. As shown in **Table 1**, miR-335-5p in patients with late FIGO stage

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**Figure 3.** MiR-335-5p could inhibit cell proliferation in vitro. A. CCK8 assay showed that miR-335-5p could inhibit SKOV-3 cell proliferation after 72 h transfection (all  $P < 0.05$ ). B. miR-335-5p inhibitor could promote SKOV-3 cell proliferation after 48 h transfection (all  $P < 0.05$ ).

were lower than those in patients with early FIGO stage ( $P = 0.006$ ). The relative levels of miR-335-5p were not associated with age, differentiation, lymphatic metastasis and histology in patients with EOC (all  $P > 0.05$ ). These results indicated that deregulated expression of miR-335-5p may play a role in the development of EOC.

### *MiR-335-5p expression in SKOV-3 cells*

Our results found that miR-335-5p, transfected into SKOV-3 cells, resulted in the upregulation of miR-335-5p as compared with negative control cells with  $P = 0.002$ . MiR-335-5p inhibitors, transfected into SKOV-3 cells, resulted in the down-regulation of miR-335-5p as compared with negative control cells with  $P = 0.003$  (Figure 2).

### *MiR-335-5p could inhibit ovarian cancer cell proliferation in vitro*

We detected the invasion ability by CCK8 assay. Our results found that miR-335-5p could inhibit SKOV-3 cell proliferation as compared with control group after 72 h (Figure 3A). MiR-335-5p inhibitor could promote SKOV-3 cell proliferation as compared with control group after 48 h (Figure 3B). These results indicated that miR-335-5p inhibited proliferative ability of EOC in vitro.

### *MiR-335-5p could inhibit ovarian cancer cell invasion in vitro*

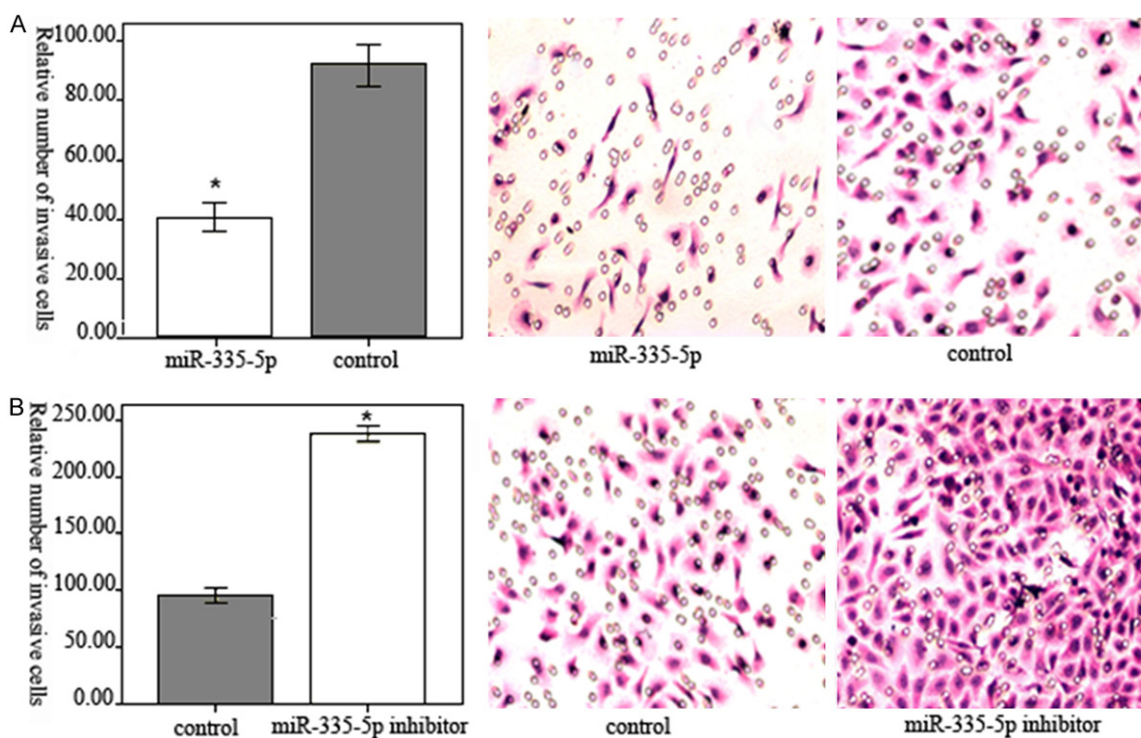
We detected the invasion ability by invasion chamber system. Our results found that miR-335-5p ( $40.42 \pm 7.66$ ) could inhibit SKOV-3 cell invasion as compared with control group ( $91.17 \pm 10.94$ ) with  $P < 0.0001$  (Figure 4A). MiR-335-5p inhibitor ( $238.33 \pm 11.5$ ) could promote SKOV-3 cell invasion as compared with control group ( $95.83 \pm 7.97$ ) with  $P < 0.0001$  (Figure 4B). These results indicated that miR-335-5p inhibited invasive ability of EOC in vitro.

### *DKK1, negatively regulated by miR-335-5p, was the target gene of miR-335-5p*

DKK1 has been confirmed to be a direct target gene of miR-335-5p in mouse mesenchymal stem cells or in mouse osteoblast [16, 17] with dual luciferase reporter assay. However, the role and mechanism of one miRNA could be species specific. Our previously study reported DKK1 overexpression could promote EOC cell growth and invasion [15]. Thus we hypothesized that miR-335-5p might regulate EOC progression through targeting DKK1 protein. Consequently, we repeated the dual luciferase reporter assay on SKOV-3 cells to testify it. Luciferase activities showed that miR-335-5p was significantly inhibits the activity of DKK1 wild-type 3'-UTR but not DKK1 mutant 3'-UTR in SKOV-3 cells (Figure 5A,  $P < 0.05$ ). Then, we



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**Figure 4.** MiR-335-5p could inhibit SKOV-3 cell invasion. A. The invading cells in miR-335-5p mimic group was decreased as compared with that in control mimic group ( $P < 0.0001$ ). B. The invading cells in miR-335-5p inhibitor group was decreased as compared with that in control inhibitor group ( $P < 0.0001$ ).

transfected miR-335-5p mimic, miR-335-5p inhibitor and controls into SKOV-3 cells. Our results found that the expression of DKK1 mRNA was unchanged (Figure 5B,  $P > 0.05$ ). Western bolts results showed that the expression of DKK1 protein in miR-335-5p mimic group ( $0.11 \pm 0.06$ ) showed significantly decreased, as compared with that in control group ( $0.33 \pm 0.01$ ) with (Figure 5C,  $P < 0.0001$ ). While, the expression of DKK1 protein in miR-335-5p inhibitor group ( $0.20 \pm 0.02$ ) showed significantly increased, as compared with that in control group ( $0.47 \pm 0.04$ ) (Figure 5C,  $P = 0.001$ ). Immunocytochemical analysis showed that the expression of DKK1 protein in miR-335-5p inhibitor group was strong as compared with control groups, while weak in miR-335-5p mimic group as compared with control groups (Figure 5D). All of these results indicated that miR-335-5p could down-regulate DKK1 protein by directly targeting DKK1 gene binding sites.

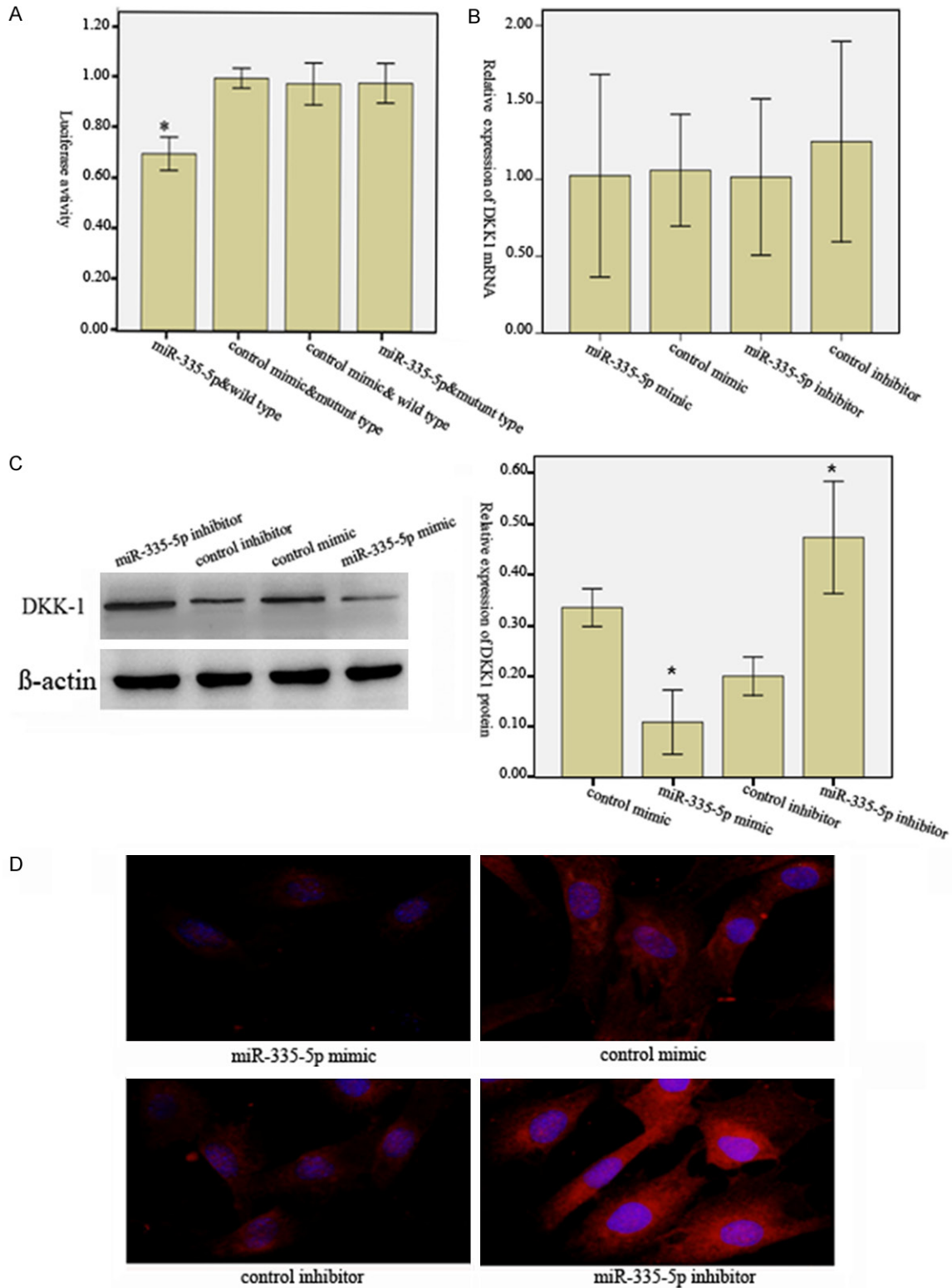
### Discussion

Nowadays, Researches which were focus on cancer-specific miRNA and their target gene

might be contribute to clarifying the mechanism of EOC cell proliferation and invasion [18, 19]. In this study, we firstly demonstrated that miRNA-335-5p was down-expressed in EOC tissues and negatively correlated with tumor stage. These findings indicated that miR-335-5p was identified as a cancer suppressor and played different functional role in different cancer type [20-24]. A520b played a role in the development of EOC. Next, we used the assays to further identify the invading function of miR-335-5p in EOC. It has been shown that miRNA-335-5p mimic could inhibit cell proliferation and invasion, while miRNA-335-5p inhibitor promoted cell proliferation and invasion. To further investigation, the potential downstream gene of miRNA-335-5p was identified. Dual luciferase reporter assay showed that DKK1 was identified as a target of miRNA-335-5p in EOC cells. The results of cell transfection by regulating the expression of miRNA-335-5p in EOC cell further supported it conclusion.

MiR-335-5p was identified as a cancer suppressor and played different functional role in different cancer type. In functional studies,

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**Figure 5.** DKK1 was the target gene of miR-335-5p and negatively regulated by miR-335-5p. A. Luciferase report showed miR-335-5p could directly target DKK1 3'-UTR region in SKOV-3 ( $P=0.001$ ) cells. B. MiR-335-5p failed to inhibit DKK1 mRNA expression ( $P>0.05$ ). C. Western blot showed that miR-335-5p could inhibit the expression of DKK1 protein ( $P<0.0001$ ), while miR-335-5p inhibitor could increase the expression of DKK1 protein ( $P=0.001$ ). D. Immunocytochemical assay showed that the expression of DKK1 protein in miR-335-5p inhibitor group was strong as compared with control group, while weak in miR-335-5p mimic group as compared with control mimic group.

miR-335-5p has been implicated in several human carcinomas and identified its potential roles in diagnostics, invasion prediction, as well as therapeutic target for tumors [20-24]. For example, miR-335-5p was found to be downregulated in gastric cancer and involved in tumorigenesis [21].

In addition, miR-335-5p down-regulation could induce TRIM29 over-expression, which induces proliferation, EMT and metastasis of NPC through the PTEN/AKT/mTOR signaling pathway [25]. These findings provided evidences for us to expand the tumor suppressive role of miR-335-5p in EOC.

MicroRNAs could play its role in regulating tumor progression by inhibiting target protein expression through binding to complementary sequences in the 3' untranslated region of the target mRNA. Consequently, it is important for us to identify its target genes in EOC progression. In the present study, the negative correlation between miR-335-5p and DKK1 was observed [16, 17]. DKK1 protein was firstly known as a negative antagonist that inhibited Wnt  $\beta$ -Catenin signaling pathway [26, 27]. Nowadays, other new studies have indicated that DKK1 protein was overexpressed in cancer cells and could promote cancer cell invasion [28, 29]. Our previous study also indicated that DKK1 promoted the invasion of EOC cell [13, 15]. Zhang et al [16] found that miR-335-5p could modulate mouse osteogenic differentiation by specifically downregulating Wnt Antagonist DKK1. Li et al [17] also found that miR-335-5p promoted chondrogenesis in mouse mesenchymal stem cells through targeting DKK1 to increase  $\beta$ -catenin/TCF activity. Our previously study reported DKK1 overexpression could promote EOC cell growth and invasion [15]. However, the role and mechanism of one miRNA could be species specific. Thus we hypothesized that miR-335-5p might regulate EOC progression through targeting DKK1 protein. Our results of dual luciferase reporter assay have testified it.

In summary, our results showed that miR-335-5p might inhibit cell proliferation and invasion through regulation of DKK1 in EOC progression via targeting DKK1 protein. MiR-335-5p might be a potential therapeutic target for treating EOC. However, it needed for further investigation.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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