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MiRNA-8073 targets ZnT1 to inhibit malignant progression of ovarian cancer

L. ZHANG, Y.-H. WANG, L. WANG

Department of Obstetrics and Gynecology, The Central Hospital of Wuhan, Wuhan, China

Li Zhang and Yanhong Wang contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to investigate the effect of microRNA-8073 on the malignant progression of ovarian cancer (OC) and whether the underlying mechanism is through the regulation of ZnT1 expression.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of microRNA-8073 in 50 tumor tissues and adjacent tissues of OC patients, and the relationship between microRNA-8073 expression and clinical indicators of OC was analyzed. Negative control group (NC) and microRNA-8073 overexpression group (microRNA-8073 mimics) were set in OC cell lines, and the transfection efficiency was further verified by qRT-PCR. In OC cell lines including SKOV3 and OVCAR3, the effects of microRNA-8073 on cell proliferation and apoptosis were analyzed by cell counting kit-8 (CCK-8), cell clone formation assay, and flow cytometry. Finally, the regulatory mechanism of microRNA-8073 on the downstream gene ZnT1 was explored by a recovery experiment.

RESULTS: QRT-PCR results revealed that microRNA-8073 expression in cancer tissue specimens of OC patients was significantly lower than that in corresponding normal tissues, and the difference was statistically significant. Compared with patients with high expression of microRNA-8073, NC group had low expression of microRNA-8073 and had a higher pathological stage and lower overall survival rate. In the OC cell lines including SKOV3 and OVCAR3, compared with the NC group, the cell proliferation ability of the microRNA-8073 mimics group was significantly decreased, while the apoptotic ability was significantly increased. Also, ZnT1 had high expression in OC cell lines and tissues and was confirmed negatively correlated with microRNA-8073 level. Meanwhile, the recovery experiment revealed that overexpression of ZnT1 can counteract the effect of microRNA-8073 mimics on OC cell proliferation and apoptosis so as to affect the malignant progression of OC.

CONCLUSIONS: We demonstrated that microRNA-8073 was significantly associated with the pathological stage and poor prognosis of OC. In addition, microRNA-8073 might inhibit malignant progression of OC by regulating ZnT1 expression.

Key Words:
MicroRNA-8073, ZnT1, OC, Proliferation.

Introduction

Ovarian cancer (OC) is one of the three malignant tumors in women and is the highest among female reproductive tumors[1-3]. However, although the surgical satisfaction and chemotherapy regimen has been gradually improved in recent years, the prognosis of patients with OC is still unsatisfied[4]. The 5-year survival rate for patients with OC is only 46%, and the 5-year survival rates for patients with advanced OC are 32% (FIGO III) and 18% (FIGO IV)[5,6]. In recent years, the mortality rate of OC in China has also increased[7,8]. However, the mechanism of the onset and progression of OC still remains elusive. The role of epigenetics in tumorigenesis has become a hot topic of research, which has played an extremely important role in the occurrence of malignant tumors[9]. Without changing the gene sequence, epigenetics can control gene expression at various levels through histone modification, deoxyribonucleic acid (DNA) methylation, non-coding ribonucleic acid (RNA), and regulation of chromatin remodeling[10,11]. In the progression of cancer, chromatin remodeling is to reposition nucleosomes, thereby inhibiting tumor suppressor genes or activating oncogenes, and participating in the occurrence and development of cancer[12,13]. Also, non-coding RNA is a hotspot of current research. It is a kind of RNA that does not encode protein but can lead to the degradation of related target gene mRNA, causing changes in chromatin structure or other ways to regulate the expression of tumor suppressor genes and proto-oncogenes[13].

MiRNAs are non-coding RNAs composed of approximately 19-25 nucleotides that play important roles primarily at post-transcriptional or translational levels[14,15]. However, scientists in various fields have

Corresponding Author: Li Wang, MM; e-mail: 521yagai@163.com

discovered thousands of miRNAs found in different biological species such as fruit flies, plants, and humans, and gradually confirmed that these miRNAs are not only involved in biological processes such as cell growth and development, but also played an important role in tumor differentiation[16,17]. MiRNAs are the largest class of gene expression regulators. It is estimated[18,19] that more than one thousand miRNAs are present in the human genome, and the expression of about 1/3 of the protein-coding genes is regulated by these miRNAs. Some studies[19] have found that miRNA aberrant expression and mutations are associated with the occurrence and progression of human tumors, and can act as a tumor suppressor or oncogenic molecule to regulate the expression of many tumor-associated genes.

In this work, bioinformatics and molecular biology were performed to clarify the association between microRNA-8073 and ZnT1 and the value of ZnT1 in OC tissues and cells. By using multiple bioinformatics databases, microRNA-8073 was further identified as a specific microRNA capable of regulating the expression of OC, and its ability to proliferate in OC cells was also showed.

Patients and Methods

Patients and PCa Samples

The tumor tissue and corresponding adjacent normal tissues of 42 OC patients who underwent surgical resection were collected and hematoxylin-eosin staining (HE) staining was performed. All specimens were frozen and stored in a -80°C refrigerator for subsequent RNA extraction. According to the 8th edition of UICC/AJCC OC TNM staging criteria, all patients were diagnosed as OC by postoperative pathological analysis, and no anti-tumor treatment such as radiotherapy or chemotherapy was performed before the surgery. The study was approved by the Ethics Committee of the Central Hospital of Wuhan and all patients signed the informed consent.

Cell Lines and Reagents

Four human OC cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and one normal human ovarian surface epithelial cells (HOSEPiCs) were purchased from ATCC, USA, while Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from American Life Technologies. Cells were cultured in DMEM containing 10% FBS and placed in an incubator at 37°C with 5% CO_2.

Transfection

Negative control (NC) and microRNA-8073 overexpression sequence (microRNA-8073 mimics) were purchased from Shanghai Jima Company. Cells were plated in 6-well plates (Corning, Corning, NY, USA) and grown to a cell density of 70%, and then, the transfection was performed using Lipofectamine 3000 (Invitrogen, CA, USA). After 48 h, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell functional experiments.

Cell Proliferation Assay

After 48 h of transfection, the cells were harvested and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h, cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well of the culture plate. And 2 hours later, the optical density (OD) value of each well was measured with a microplate reader at 490 nm of absorption wavelength.

Colony Formation Assay

The cells after 48 h of transfection were collected, and 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed after one week and then twice a week. The medium should not be replaced as much as possible in the previous week to prevent the cells from adhering to the wall. After 2 weeks, the cells were cloned, washed twice with PBS, and fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, cells were stained with 0.1% crystal violet staining solution for 20 min. After washed 3 times with phosphate-buffered saline (PBS), cells were photographed and counted under a light-selective environment.

Flow Cytometry Analysis of the Cell Apoptosis

Cell apoptosis was detected by flow cytometry based on the method of binding with Annexin V-FITC (Merck, USA) and PI. The procedure was as follows. First, the cell density was adjusted to about 1×10^6 cells/mL; after washed twice with PBS, cells were gently resuspended with 0.5 mL of pre-cooled 1× binding buffer, and 1.25 uL of Annexin V-FITC was added to incubate the cells for 15 mins at room temperature in the dark. Subsequently, centrifugation was performed at 1000 g for 5 min at room temperature, with the supernatant removed. After the cells were resuspended with 0.5 mL of pre-cooled l × binding buffer, 10 μL

of PI was added in. At last, the sample was placed on ice and stored in the dark, and immediately analyzed by flow cytometry (BD, USA).

QRT-PCR Assay

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, real time-PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) kit instructions. The following primers were used: microRNA-8073: F: 5'-TTAGCTCAGGATCATCATCATTTACATAGATAGGG-3'; R: 5'-AACACTCGAGTGAGAGAAGAGAGTGCCTAGA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'- AACGCTTCACGAATTTGCGT-3'; ZnT1: F: 5'-TACCATCAACTCCAACGG-3', R: 5'-GAACCCAAGGCATCTCCA-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Bio-Rad PCR instrument (Hercules, CA, USA) was used to analyze the data. Data was processed with software iQ5 2.0. The β-actin and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analysis was performed using SPSS 22. 0 software (SPSS Inc., Chicago, IL, USA). The univariate analysis was performed with the χ^2-test and the exact probability Fisher test. COX regression analysis was performed to process multivariate data. Patient survival was analyzed by the Kaplan-Meier method, and intergroup curves were compared using the Log-rank test. Data were expressed as mean ± standard deviation (x±s), and $p<0.05$ was considered to be statistically significant.

Results

Downregulated MicroRNA-8073 in Primary OC Tissues, and Reduced Expression of MicroRNA-8073 Connected With Advanced Clinical Procedure and Poor Patient Prognosis

The results of qRT-PCR showed that the expression level of microRNA-8073 was decreased in cancer tissues of OC patients compared with paracancerous tissues, and the difference was statistically significant (Figure 1A, 1B). In addition, microRNA-8073 was found markedly lower in OC cells than in HOSEPiCs, especially in the SKOV3 and OVCAR3 cell lines (Figure 1C). According to the expression of microRNA-8073 in 50 pairs of OC tumor tissues and paracancerous tissues, they were divided into high expression group and low expression group to explore the relationship between the expression of microRNA-8073 and the prognosis of patients with OC. As a result, Kaplan-Meier survival curves revealed that lowly-expressed microRNA-8073 was strikingly associated with poor prognosis of OC ($p<0.05$; Figure 1D).

Subsequently, we further explored the relationship between microRNA-8073 level and age, pathological stage, lymph node or distant metastasis of OC patients. As shown in Table I, the lowly-expressed microRNA-8073 was positively related to the pathological stage of OC, but not with age, lymph node metastasis, and distant metastasis. Therefore, the above results suggested that microRNA-8073 might be a new biological indicator for predicting the malignant progression of OC.

Upregulation of MicroRNA-8073 Inhibited Cell Proliferation and Promoted Cell Apoptosis

To explore the effect of microRNA-8073 on OC cell function, we first successfully constructed the microRNA-8073 overexpression model and verified it by qRT-PCR (Figure 2A). We then performed CCK-8 assay and flow cytometry in the SKOV3 and OVCAR3 cell lines. It was found that the proliferation of cells in the microRNA-8073 mimics group was notably lower than that of NC group (Figure 2B). Subsequently, cell colony assay demonstrated that the number of tumor cell proliferation was reduced in the microRNA-8073 mimics group compared with NC group, suggesting that cell proliferative capacity was inhibited (Figure 2C). In addition, flow cytometry result revealed that cell apoptosis of the microRNA-8073 mimics group was conspicuously higher than that of the NC group (Figure 2D).

ZnT1 Was Highly Expressed in OC Tissues and Cell Lines

In the OC cell lines, the mRNA level of ZnT1 was found conspicuously reduced after overexpression of microRNA-8073 (Figure 4A). In addition, we found through qPCR assay that

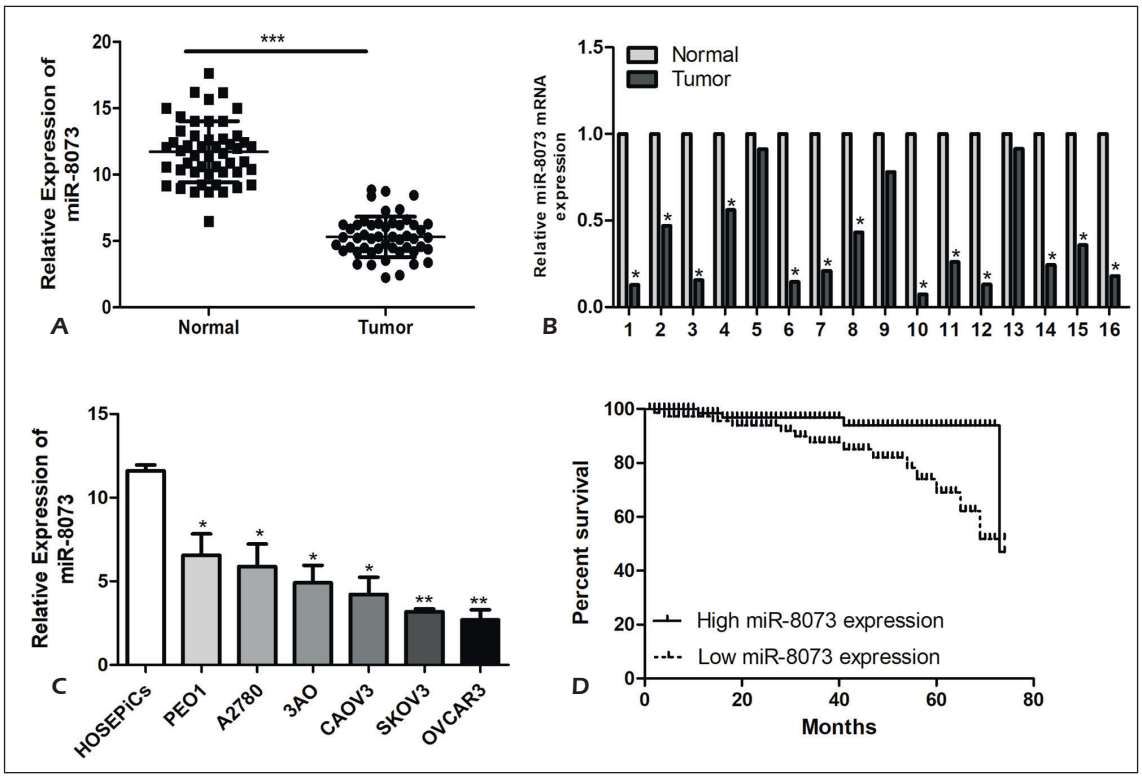

Figure 1. MiR-8073 is underexpressed in ovarian cancer tissues and cell lines. ***A-B***, qRT-PCR detection of miR-8073 expression in ovarian cancer tumor tissues and adjacent normal tissue. ***C***, qRT-PCR detection of miR-8073 expression level in ovarian cancer cell lines. ***D***, Kaplan-Meier survival curve for ovarian cancer patients based on miR -8073 expression level. Data are shown as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

ZnT1 expression was enhanced in OC tumor tissues compared with adjacent tissues (Figure 4B). Meanwhile, ZnT1 was conspicuously higher in OC cells than in HOSEPiCs, and the difference was statistically significant (Figure 4C). Therefore, we examined the mRNA expression of microRNA-8073 and ZnT1 in OC tissues and the results showed that microRNA-8073 and ZnT1 were significantly negatively correlated in tumor tissues of OC patients (Figure 4D).

Table I. Association of miR-8073 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	miR-8073 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.405
<60	20	11	9	
≥60	30	20	10	
T stage				0.043
T1-T2	30	22	8	
T3-T4	20	9	11	
Lymph node metastasis				0.923
No	32	20	12	
Yes	18	11	7	
Distance metastasis				0.481
No	37	24	13	
Yes	13	7	6	

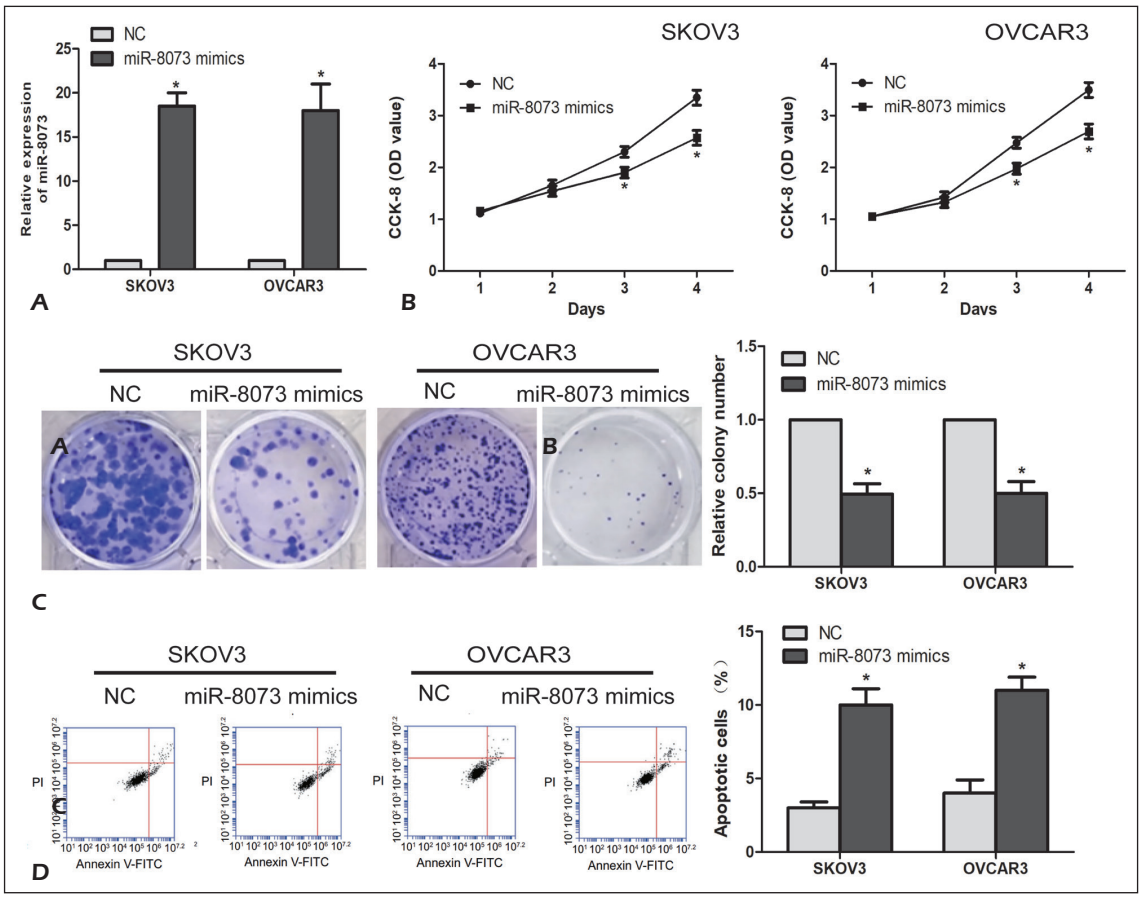

Figure 2. MiR-8073 mimics inhibits the proliferation of ovarian cancer cells and promotes cell apoptosis. ***A***, qRT-PCR validated the transfection efficiency after transfection of NC and miR-8073 mimics in SKOV3 and OVCAR3 cell lines. ***B***, CCK-8 assay was performed to detect the proliferation ability of SKOV3 and OVCAR3 cell lines. ***C***, Clonal formation assay was used to detect the number of ovarian cancer-positive proliferating cells (magnification: 20X). ***D***, Flow cytometry assay revealed the apoptosis of SKOV3 and OVCAR3 cell lines. Data are shown as mean ± SD, *$p<0.05$.

MicroRNA-8073 Exactly Inhibited ZnT1 Gene Expression

To further explore the ways in which microRNA-8073 inhibits the malignant progression of OC, we overexpressed ZnT1 in the OC cell lines which had been transfected with NC and microRNA-8073 mimics and verified the transfection efficiency also through qRT-PCR (Figure 5A). Subsequently, through CCK-8, cell colony formation, and flow cytometry assay, we demonstrated that the overexpression of ZnT1 could counteract the effect of microRNA-8073 mimics on proliferation and apoptosis of OC cells (Figure 5B, 5C, 5D).

Discussion

Epigenetics is a hot topic in current research which refer to the expression of the gene that has undergone heritable changes when the DNA sequence in the nucleus does not change[10-12]. Genetic data provides information on the synthesis of various proteins, including epigenetic modifications, while epigenetic information regulates a set of expressed genes and the extent of their expression[11,12]. The correct expression of genes is not only controlled by DNA sequences, but also by epigenetics[11]. Post-transcriptional regulation and gene transcriptional regulation are two major components of epigenetic research; the former mainly includes ncRNA (non-coding RNA), antisense RNA, etc., while the latter mainly includes DNA methyl chemotherapy, histone modification, and chromatin remodeling[10-13]. Among them, epigenetic regulation studies[12,13] are characterized by histone modification, non-coding RNA, and DNA methylation, which are involved in various biological processes *in vivo*, including

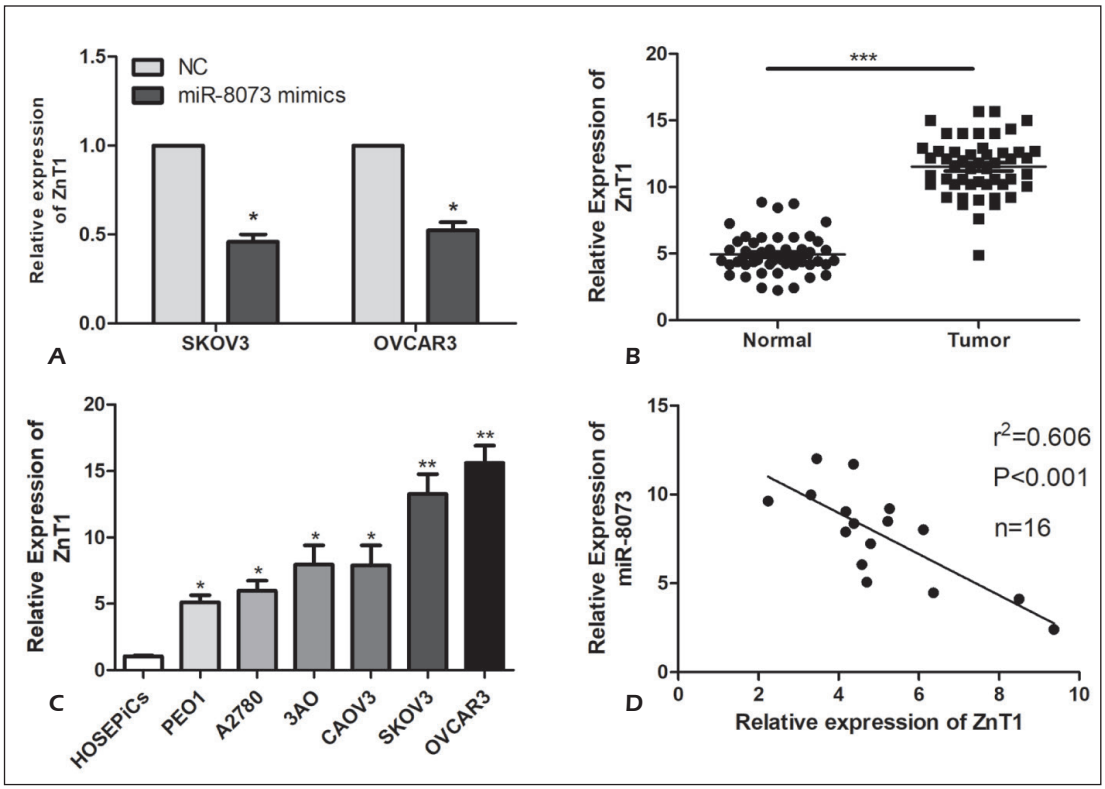

Figure 3. ZnT1 has high expression in ovarian cancer tissues and cell lines. ***A***, qRT-PCR indicated the expression level of ZnT1 after transfection of miR-8073 mimics in SKOV3 and OVCAR3 cell lines. ***B***, qRT-PCR was used to detect the ZnT1 expression in ovarian cancer tumor tissues and adjacent tissues. ***C***, qRT-PCR was used to detect the expression level of ZnT1 in ovarian cancer cell lines. ***D***, There was a significant negative correlation between miR-8073 and ZnT1 expression in ovarian cancer tissues. Data are shown as mean ± SD, *p<0.05, *p<0.01, ***p<0.001.

the regulation of cell proliferation and differentiation. Previous studies[12] have shown that many tumors are closely related to abnormalities of epigenetic regulation.

The binding of miRNAs to target genes is usually performed in a way that is not completely complementary. Therefore, there is still a big problem in how to accurately identify the target mRNA regulated by miRNAs[14]. Although there are still no sensitive miRNA cloning methods and high-throughput experimental methods for identifying miRNA target genes, some software or programs are currently used to predict them due to the regularity of the interaction between miRNA and target mRNA[15-17]. However, since the miRNA molecule is small, only about 19-25 nt, and the binding of the miRNA to the target gene is not completely complementary, the target gene complementary to the miRNA is theoretically abundant in the entire genome[20,21]. Therefore, this study used bioinformatics analysis tools to predict downstream mRNAs that might be targeted bound by miRNA. The Targetscan online data (http://www.targetscan.org) was used to predict that ZnT1 may be a possible target gene for microRNA-8073. MicroRNA-8073 has been shown to be lowly expressed in various cancer tissues such as colorectal cancer, and its expression level is closely related to clinicopathological features and clinical prognosis, suggesting that this microRNA is involved in many malignant tumors including OC. To explore the role of microRNA-8073 in the development and progression of OC, qRT-PCR was used to detect the expression of microRNA-8073 in 50 OC tissues and adjacent tissues, which was found significantly down-regulated and correlated with tumor clinical stage, and thus we believe that microRNA-8073 may play a role in tumor suppression in OC. Subsequently, in order to further explore the effect of microRNA-8073 on the biological function of OC, we constructed the microRNA-8073 mimics model. And the results of CCK-8, cell clone formation, and flow cytometry showed that microRNA-8073 can inhibit the OC cell proliferation of OC while can promote cell apoptosis.

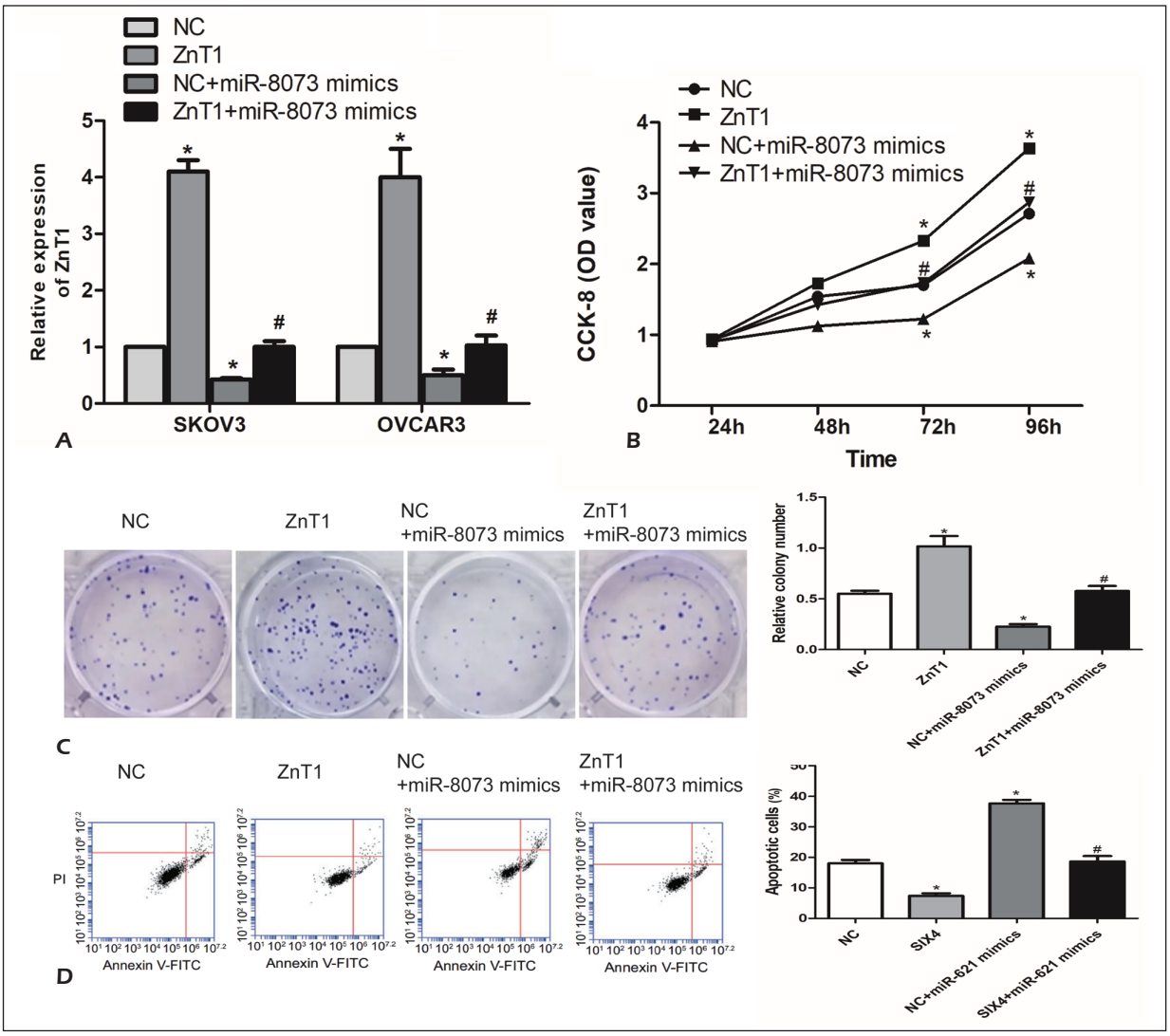

Figure 4. MiR-8073 can regulate ZnT1 expression to inhibit ovarian cancer development. ***A***, ZnT1 expression level in OC cell lines co-transfected with miR-8073 and ZnT1 was detected by qRT-PCR; ***B***, CCK-8 assay was used to detect the proliferation of ovarian cancer cells after co-transfection of miR-8073 and ZnT1; ***C***, Cloning assay was used to detect the effect of co-transfection of miR-8073 and ZnT1 on the number of ovarian cancer-positive proliferating cells (magnification: 20X); ***D***, Flow cytometry assay was used to detect the effect of co-transfection of miR-8073 and ZnT1 on the OC cell apoptosis. Data are shown as average ± SD, *#p<0.05.

We further confirmed the structural and functional validation of the microRNA-8073/ZnT1 interaction. By transfecting microRNA-8073 mimic, the effect of altering the expression level of microRNA-8073 in cells was achieved. Subsequently, qRT-PCR confirmed that the expression levels of microRNA-8073 and ZnT1 were negatively correlated in OC tissues, suggesting that microRNA-8073 may directly target ZnT1 and play a negative regulatory role, and the regulation of microRNA-8073 on ZnT1 was further verified by dual luciferase reporting assay. The results of this study revealed that microRNA-8073 can target the 3'UTR of ZnT1 to inhibit its expression. Subsequently, we examined ZnT1 expression in OC tumor tissues and adjacent tissues, and we found that ZnT1 was significantly increased in OC tissues. In addition, when microRNA-8073 was overexpressed in OC cells, the expression level of ZnT1 was significantly decreased, and the malignant phenotypes such as cell proliferation and invasion of OC cells were also decreased, which achieved similar effects to the downregulation of ZnT1, suggesting that the microRNA-8073/ZnT1 regulatory axis may play a vital role in the progression of OC.

Conclusions

We showed that microRNA-8073 was significantly associated with pathological stage and poor prognosis of OC patients. In addition, microRNA-8073 may inhibit malignant progression of OC by regulating ZnT1 expression.

Conflict of Interests

The authors declare that they have no conflict of interest.

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