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CircRNA_MYLK promotes malignant progression of ovarian cancer through regulating microRNA-652

Y. ZHAO[1], Y. HU[1], Q. SHEN[1], Q. CHEN[1], X.-J. ZHU[1], S.-S. JIANG[2], Q. ZHANG[1]

[1]Department of Obstetrics and Gynecology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, China
[2]Department of Gynecology, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

Yu Zhao and Yue Hu contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to investigate circRNA_MYLK level in ovarian cancer (OC), and to further investigate whether it could promote the malignant progression of OC via regulating microRNA-652.

PATIENTS AND METHODS: quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine circRNA_MYLK level in 46 tumor tissue specimens and paracancerous normal ones collected from OC patients, and the interplay between circRNA_MYLK expression and clinical indicators of OC and patient prognosis was analyzed. Meanwhile, qPCR was also used to further verify circRNA_MYLK level in OC cell lines. In addition, circRNA_MYLK knockdown model was constructed using lentivirus in OC cell lines including A2780 and CAOV3, and the impacts of circRNA_MYLK on the biological functions of OC cells was evaluated using Cell Counting Kit-8 (CCK-8) and cloning experiments. Finally, Luciferase reporting assay and recovery experiment were performed to investigate the regulatory interplay between circRNA_MYLK and microRNA-652.

RESULTS: qPCR results indicated that circRNA_MYLK level in OC patients was remarkably higher than that in adjacent ones, and the difference was statistically significant. Compared with patients with low expression of circRNA_MYLK, patients with high expression of circRNA_MYLK had a higher pathological staging and a lower overall survival rate. Compared with the control group (sh-NC), the OC cell proliferation ability was remarkably attenuated in the circRNA_MYLK knockdown group (sh-circRNA). In addition, qPCR verification revealed that the expression levels of microRNA-652 and circRNA_MYLK were negatively correlated in OC tissues. At the same time, bioinformatics analysis and Luciferase reporter gene assay results confirmed that circRNA_MYLK can be targeted by microRNA-652. Finally, it was found that simultaneous knockdown of circRNA_MYKK and microRNA-652 could reverse the enhanced OC cell proliferative capacity induced by downregulation of circRNA_MYLK alone.

CONCLUSIONS: CircRNA_MYLK may promote the malignant progression of OC via regulating microRNA-652, and its expression was remarkably associated with pathological staging and poor prognosis in patients with OC.

Key Words:
CircRNA_MYLK, MicroRNA-652, OC, Malignant progression.

Introduction

Ovarian cancer (OC) is one of the three major malignancies of the female reproductive system, ranking second in the incidence of gynecological malignancies[1-3]. Due to the insidious early symptoms, lack of specific clinical manifestations and early diagnosis methods, about 70% of the patients were diagnosed as stage III and IV clinicopathology, and the lack of effective treatment in the late stage, the mortality of OC continues to top the list of gynecological malignancies[4-6]. Among them, epithelial OC (EOC) accounts for 85-90% of ovarian malignant tumors, which is characterized by rapid progression, easy invasion, diffusion, and distant metastasis[7,8]. Because of this, it is difficult to completely remove tumor lesions with simple surgical treatment[9,10]. According to statistics, about 90% of cancer patients die from

Corresponding Authors: Shanshan Jiang, MM; e-mail: 617674846@163.com
Qiong Zhang, MD; e-mail: joanzhang2002@126.com

tumor metastasis clinically, and survival analysis has confirmed that invasion and metastasis are important factors affecting the prognosis of OC patients[11,12]. Therefore, it is essential to illuminate the process and related regulatory mechanism of OC invasion and metastasis from the molecular level, which could promote the development of new targeted therapy drugs to prolong the survival or reduce the mortality of patients[12-14].

Circular RNA (circRNA) is a recently discovered non-coding RNA. Different from traditional linear RNA, circRNA molecule has a closed circular structure and is not affected by RNA exonuclease, and its expression is more stable and difficult to degrade[15,16]. CircRNA molecules are rich in microRNA (miRNA) binding sites and act as a miRNA sponge in cells, thereby removing the inhibition effect of miRNA on its target genes and increasing the expression level of target genes. This mechanism is known as the competitive endogenous RNA (ceRNA) mechanism[17,18]. It has been reported that abnormal circRNA expression affects the prognosis of breast cancer, OC, and non-small cell lung cancer, and plays a part in the occurrence of many diseases[19,20]. MicroRNA-652 is a member of the family of small molecular RNA (miRNA), which is a kind of highly conserved non-coding single-stranded small molecular RNA with a length of about 22 nucleotides, which can degrade or inhibit the translation of target genes into proteins by completely or incompletely binding to the 3'-terminal non-translation region of the target gene[21,22]. MiRNA has been found to be engaged in various biological processes, such as proliferation, invasion, and metastasis by modulating gene expression[23,24]. In this study, circRNA_MYLK level and microRNA-652 in OC was detected to analyze the correlation between circRNA_MYLK and microRNA-652 and the clinicopathological characteristics of OC, and to explore the correlation between circRNA_MYLK and microRNA-652 expression, so as to provide certain theoretical basis for further investigation of circRNA_MYLK and microRNA-652 in the diagnosis, treatment, and prognosis of OC.

Patients and Methods

Patients and OC Samples

The tumor tissue samples, and corresponding adjacent ones were collected from of 46 OC patients underwent surgical resection, and then, analyzed by routine hematoxylin and eosin (HE) staining. All specimens were frozen and stored in a -80°C refrigerator for subsequent RNA extraction. According to the 8th edition of UICC/AJCC OC tumor node metastasis (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 surgery. The study was approved by the Ethics Committee of the hospital and all patients had signed informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cell Lines and Reagents

Human OC cells (SKOV3, OVCAR3, PEO1, 3AO, A2780, CAOV3) and a normal human ovarian surface epithelial cells (HOSEPiCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). OC cell lines were cultured with DMEM medium containing 10% FBS in a 37°C, 5% CO_2 incubator.

Transfection

The control group (sh-NC) and the lentivirus (sh-circRNA_MYLK) containing the circRNA_MYLK knockdown sequence were purchased from Shanghai Jima Company (Shanghai, China). The cells were plated in 6-well plates and grown to a cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After 48 h, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qPCR) analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

The proliferation of cells was examined using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). After 48 h of transfection, the cells were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 6 h, 24 h, 48 h, and 72 h respectively, and then, added with 10 μg of CCK-8 reagent. After incubation for one hour, the optical density (OD) value of each well was measured in the microplate reader at 450 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, the cells were collected, and 200 cells were seeded in each well of a

6-well plate and cultured with 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 avoid cell adhesion. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution for 20 minutes, washed 3 times with phosphate-buffered saline (PBS), photographed and counted under a light-selective environment.

QPCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and the 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, and Real Time-PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qPCR reaction: circRNA_MYLK: F: 5'-CAGTGCATGCTGTTTGTTCA-3', R: 5'-TCGGAGCTTGACTTCCAG-3'; microRNA-652: F: 5'-AACCCUAGGAGAGGGUGCCAUUCA-3', R: 5'-UGAAUGGCACCCUCUCCUAGGGUU-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'; U6: F: 5'-ATTGGAACGATACAGAGAAGATT-3', R: 5'-GGAACGCTTCACGAATTTG-3'. Three replicate wells were repeated for each sample and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The GAPDH and U6 genes were used as internal parameters, and the gene expression was calculated using the $^{2-\Delta\Delta Ct}$ method.

Dual-Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the Luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter plasmid into the A278 and CAOV3 cell lines. If the transcription factor activates the target promoter, the Luciferase gene is expressed, and the amount of Luciferase expression is directly proportional to the intensity of the transcription factor. After the specific Luciferase substrate is added, Luciferase can react with the substrate to generate fluorescence. By measuring the intensity of the fluorescence, the activity of the Luciferase can be used to determine whether the transcription factor can interact with the target promoter fragment.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA). The *t*-test was used to compare the measurement data, and the categorical variables were analyzed by χ^2 test or Fisher's exact probability method. Survival analysis was performed using the Kaplan-Meier method and survival curves were plotted. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$), and $p<0.05$ was considered to be statistically significant.

Results

High Expression of CircRNA_MYLK in OC

46 pairs of tumor tissue samples and adjacent ones of OC patients were collected to determine circRNA_MYLK level by qPCR, and the results revealed that circRNA_MYLK expression was higher in OC tumor tissues than in the adjacent ones (Figure 1A). In addition, circRNA_MYLK was remarkably higher in OC cells than in normal cell lines (Figure 1B), suggesting that circRNA_MYLK may play a role in promoting cancer in OC.

CircRNA_ MYLK Expression was Correlated with Pathological Staging and Overall Survival in OC Patients

According to the mRNA expression of circRNA_MYLK in 46 tumor tissue specimens and paracancerous normal ones of OC patients, the above tissue samples were divided into high expression and low expression group, and the interplay between circRNA_MYLK level and the age, pathological stage, lymph node, or distant metastasis was analyzed. As shown in Table I, high expression of circRNA_MYLK was posi-

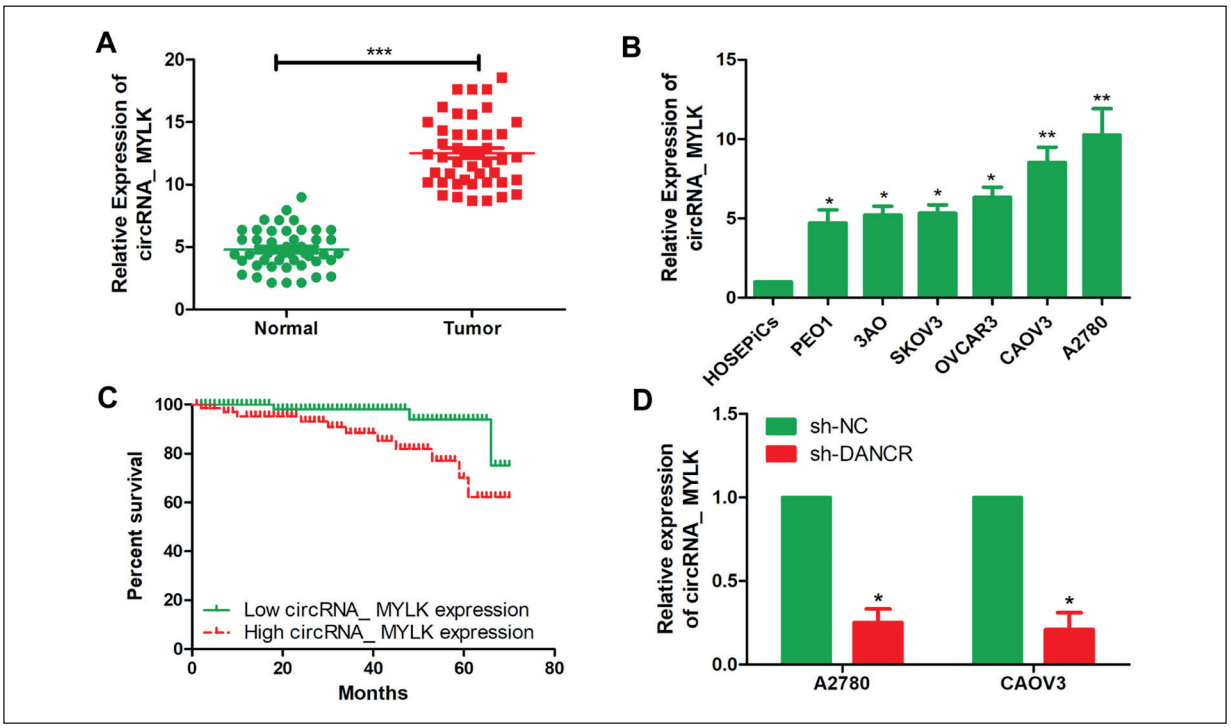

Figure 1. High expression of circRNA_MYLK in ovarian cancer tissues and cell lines. **A,** qRT-PCR was used to detect the difference in expression of circRNA_MYLK in ovarian cancer tumor tissues and adjacent tissues. **B,** qRT-PCR was used to detect the expression level of circRNA_MYLK in ovarian cancer cell lines. **C,** The Kaplan Meier survival curve of ovarian cancer patients based on circRNA_MYLK expression showed that the prognosis of patients with high expression was significantly worse than that of the bottom expression group. **D,** qRT-PCR verified the interference efficiency of circRNA_MYLK after transfection of circRNA_MYLK knockdown vector in A2780 and CAOV3 cell lines. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

tively correlated with pathological stage of OC, but not with the other indicators. In addition, in order to figure out the interplay between circRNA_MYLK level and the prognosis of OC patients, Kaplan-Meier survival curve revealed that high expression of circRNA_MYLK was closely relevant to the OC prognosis, namely, the higher circRNA_MYLK level, the worse the prognosis (p<0.05) (Figure 1C). These results showed that circRNA_MYLK expression was correlated with pathological staging and overall survival in OC patients.

Table I. Association of circRNA_ MYLK expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	circRNA_ MYLK expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.938
< 60	21	12	9	
≥ 60	25	14	11	
T stage				0.047
T1-T2	26	18	8	
T3-T4	20	8	12	
Lymph node metastasis				0.655
No	27	16	11	
Yes	19	10	9	
Distance metastasis				0.348
No	31	19	12	
Yes	15	7	8	

CircRNA_MYLK Upregulated Cell Proliferation in OC

To investigate the cellular functional role of circRNA_MYLK in OC, a lentiviral vector was constructed and knocked down circRNA_MYLK. After transfection of the circRNA_MYLK lentiviral knockdown vector in A2780 and CAOV3 OC cell lines, qPCR experiments were performed to verify the interference efficiency (Figure 1D). In the A2780 and CAOV3 OC cell lines, CCK-8 experiments revealed that compared with the control group sh-NC, the proliferation rate of OC cells was remarkably attenuated after knocking down circRNA_MYLK (Figure 2A). Plate cloning experiments revealed that the proliferation of OC cells was remarkably decreased after knocking down circRNA_MYLK (Figure 2B). The above research shows that circRNA_MYLK can promote the cell proliferation ability of OC, thereby aggravating the malignant progression of OC.

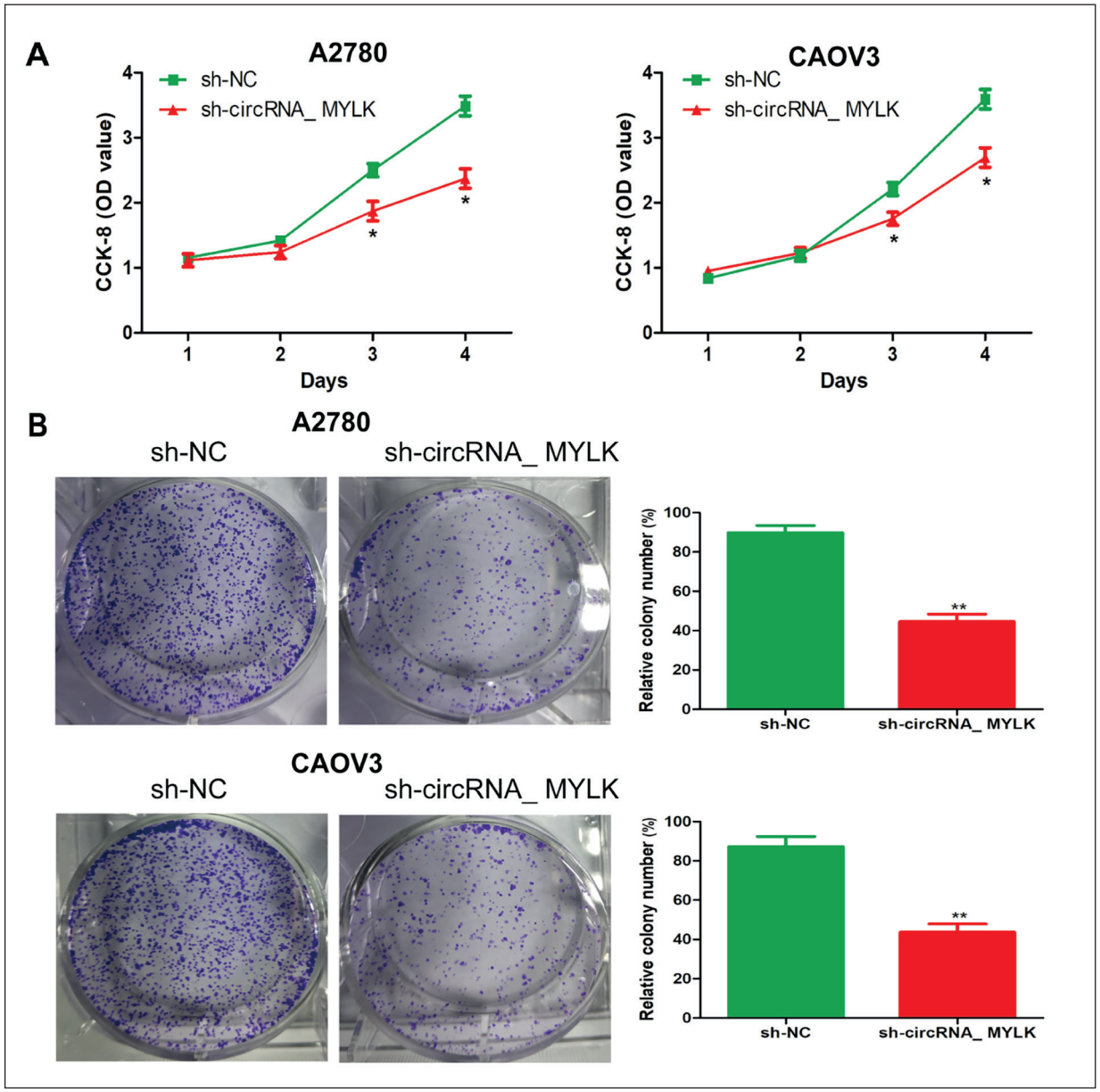

Figure 2. Silencing circRNA_ MYLK inhibits the proliferation of ovarian cancer cells. **A,** The CCK-8 assay detected the effect of silencing circRNA_MYLK on the proliferation rate of ovarian cancer cells in A2780 and CAOV3 cell lines. **B,** Cloning assays verified the effect of silencing circRNA_MYLK on proliferation of ovarian cancer cells in A2780 and CAOV3 cell lines (Magnification: 10×). Data are mean ± SD, *p<0.05, **p<0.01.

CircRNA_MYLK Bound to MicroRNA-652

In order to further explore how circRNA_MYLK plays a role in OC, bioinformatics was used to predict the possible target of circRNA_MYLK, which suggested that circRNA_MYLK could bind to microRNA-652. As shown in Figure 3A and 3B, in order to further verify the targeting of CRC-MYLK by microRNA-652, a Luciferase reporter assay was performed. The results revealed that overexpression of microRNA-652 remarkably attenuated the Luciferase activity of the wild-type circRNA_MYKK vec-

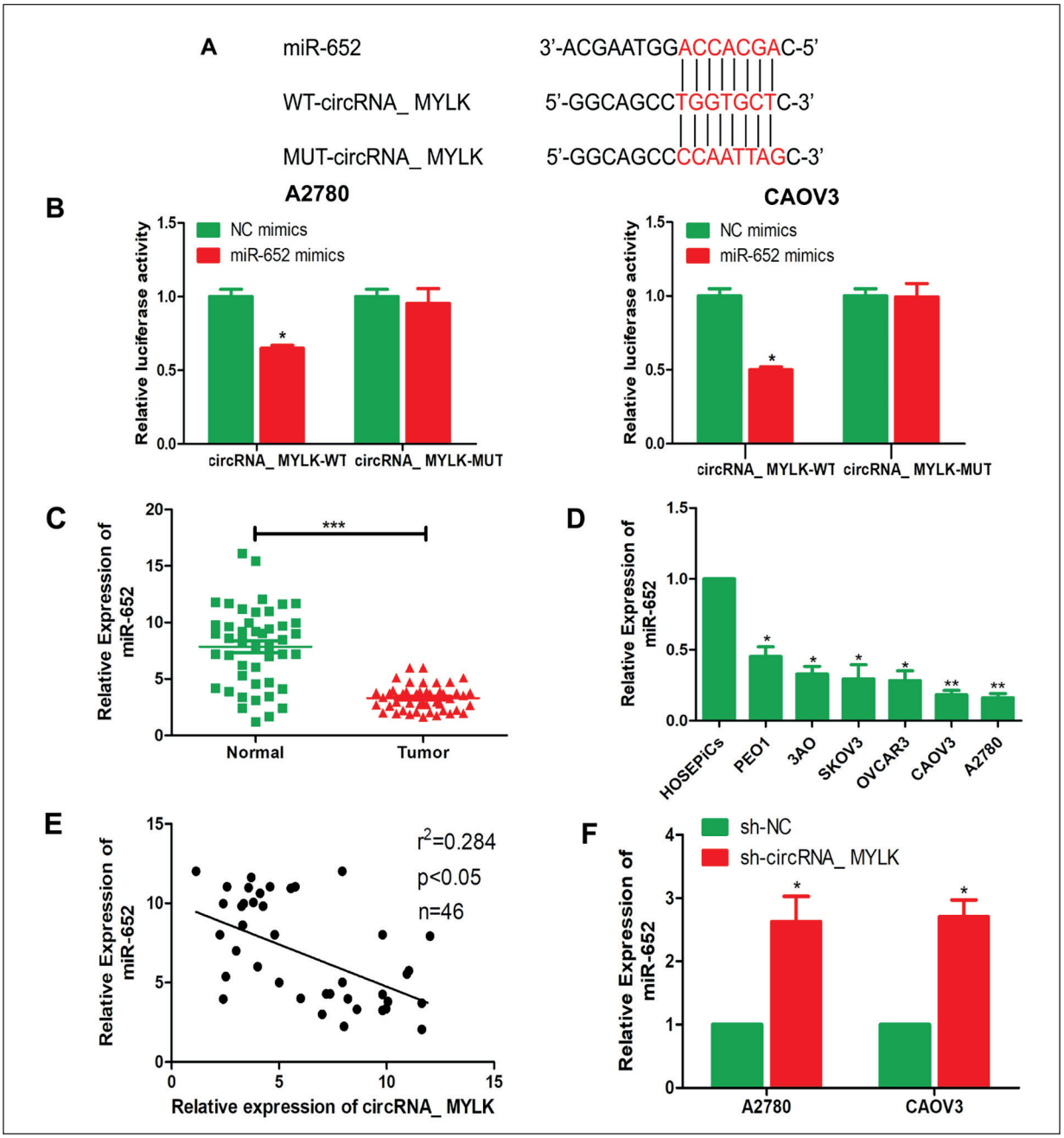

Figure 3. Interaction of circRNA_MYLK with miR-652. **A,** Schematic diagram of the specific binding sequence of circRNA_MYLK and miR-652. **B,** The luciferase reporter gene suggested that circRNA_MYLK can specifically bind to miR-652. **C,** qRT-PCR was used to detect the differential expression of miR-652 in ovarian cancer tumor tissues and adjacent tissues. **D,** qRT-PCR was used to detect the expression level of miR-652 in ovarian cancer cell lines. **E,** There was a significant negative correlation between the expression levels of circRNA_MYLK and miR-652 in ovarian cancer tissues. **F,** Silencing circRNA_MYLK expression significantly increased miR-652 expression levels. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

tor ($p<0.05$) without attenuating the Luciferase activity of the mutant vector ($p>0.05$) or the empty vector ($p>0.05$), further demonstrating that circRNA_MYLK can target microRNA-652.

Therefore, qPCR assay was performed to detect the expression of microRNA-652 in 46 pairs of OC patients' tumor tissues and their corresponding adjacent tissues, as well as OC cell lines. The results showed that the expression level of miR -652 was remarkably decreased in OC tissues compared with adjacent tissues, and the difference was statistically significant (Figure 3C). Using qPCR, it was found that microRNA-652 was remarkably expressed in OC cells in OC cell lines (Figure 3D). Besides, the results revealed that the gene expressions of circRNA_MYLK and microRNA-652 was negatively correlated in OC tissues (Figure 3E). After constructing the circRNA_MYLK knockdown lentiviral vector in the A2780 and CAOV3 OC cell lines, the expression level of microRNA-652 in the circRNA_MYLK-silencing group was remarkably increased, and the difference was statistically significant (Figure 3F). These results recommended that circRNA_MYLK could bound to microRNA-652.

MicroRNA-652 Downregulated Cell Proliferation in OC

To investigate the cellular functional role of microRNA-652 in OC, a microRNA-652 knockdown vector was constructed. qPCR experiments were performed in A2780 and CAOV3 OC cell lines to verify microRNA-652 interference efficiency (Figure 4A). In the A2780 and CAOV3 OC cell lines, microRNA-652 was knocked down, and CCK-8 and plate cloning experiments were conducted to detect cell proliferation. The CCK-8 experiment revealed that compared with the NC inhibitor group, OC cell proliferation ability was remarkably enhanced after knocking down microRNA-652 (Figure 4B). Plate cloning experiments revealed that the proliferation of OC cells was remarkably upregulated after knocking down microRNA-652 compared to the control NC inhibitor (Figure 4C). The above experiments showed that microRNA-652 can inhibit the cell proliferation ability of OC, thereby alleviating the malignant progression of OC.

CircRNA_MYLK Modulated MicroRNA-652 in OC

To further explore the ways in which circRNA_MYLK promoted malignant progression of OC, a possible relationship between circRNA_MYLK and microRNA-652 was found by relevant bioinformatics analysis and Luciferase reporter gene expression. In addition, in order to further explore the interaction between circRNA_MYLK and microRNA-652 in OC cells, microRNA-652 was silenced in a cell line silencing circRNA_MYLK in OC cells to investigate their role in OC, and qPCR experiments were performed to examine the transfection efficiency of circRNA_MYLK after co-transfection (Figure 5A). Subsequently, through CCK-8 assay and plate cloning experiments, it was found that silencing of microRNA-652 counteracted the effect of knockdown of circRNA_MYLK on OC cell proliferation (Figure 5B and 5C).

Discussion

As an important reproductive organ of women, ovaries play a significant role in maintaining women's secondary sexual characteristics and fertility[1,2]. Ovarian cancer is one of the most fatal reproductive system malignancies in women, and its mortality is the highest among gynecological malignancies[2-4]. Because of its insidious early symptoms, only 20% of OCs are detected in early detection. Many patients had abdominal metastasis before the onset of symptoms, and most patients were detected in the advanced stage. The 5-year survival rate of patients with advanced OC is only 10-20%, while the 5-year survival rate of patients with early diagnosis is as high as 85-90%[5-8]. Early detection is difficult, the prognosis is poor, and the incidence of diseases has been increasing in recent years, which has attracted wide attention from the academic community[8,9]. The most important prognostic factor is whether metastasis has occurred, so it is of great significance to detect lesions early and to determine whether metastasis has occurred[23,24].

Circular RNAs (circRNAs) are closed circular RNAs that are formed by covalent bonds and are abundant in the transcriptome of eukaryotic cells, which are different from traditional linear RNAs. They usually include exon sequences and are spliced at classical splicing sites[15,16]. CircRNA is mainly produced by exons or exon sequences, and reverse complement or RNA-binding proteins are necessary for the production of circRNA[18-20]. Most circRNAs are conserved in species. They are stable and resis-

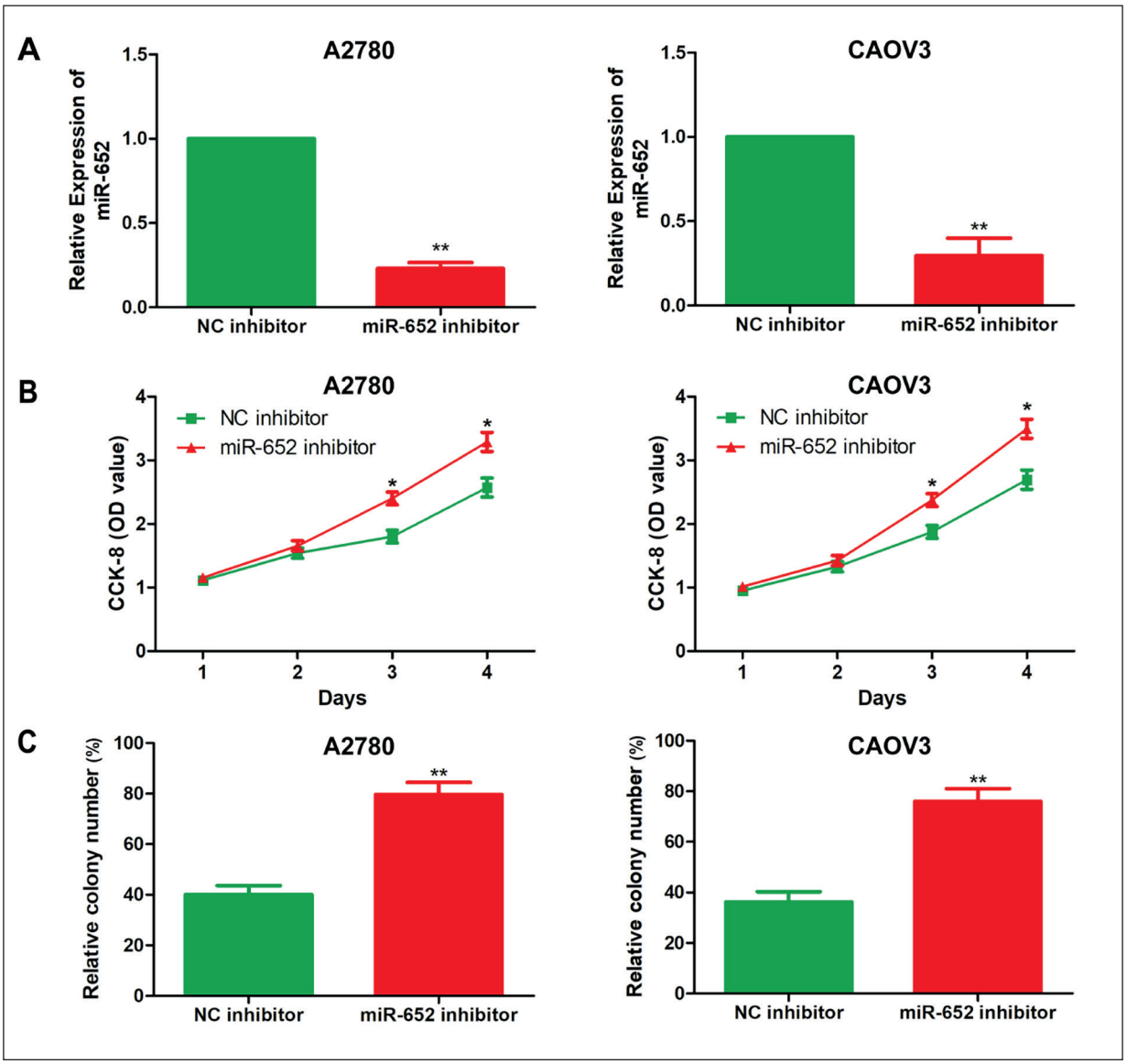

Figure 4. Silencing miR-652 promotes ovarian cancer cell proliferation. **A,** qRT-PCR verified the interference efficiency of miR-652 after transfection of the miR-652 knockdown vector in the A2780 and CAOV3 cell lines. **B,** The CCK-8 assay detected the effect of silencing miR-652 on the proliferation rate of ovarian cancer cells in A2780 and CAOV3 cell lines. **C,** Cloning formation experiments verified the effect of silencing miR-652 on proliferation of ovarian cancer cells in A2780 and CAOV3 cell lines. Data are mean ± SD, *p<0.05, **p<0.01.

tant to RNases, and often exhibit tissue-specific or developmental stage-specific expression[20]. Although circRNA research has not started for a long time, many studies have proved that circRNA is involved in many biological behaviors, such as proliferation, apoptosis, and invasion of tumor cells, and is related to the occurrence and development of many diseases, such as cancer[19,20]. Different circRNA play different functions, and some circRNA play a role in promoting tumor growth[20]. This study revealed that circRNA_MYLK level was upregulated in OC tissues. In addition, biological function analysis revealed that the silencing of circRNA_MYLK could regulate the proliferation behavior of OC cells to a certain extent. This study indicated that circRNA_MYLK was negatively correlated with the expression level of microRNA-652 in OC. In addition, high expression of circRNA_MYLK can promote the malignant progression of OC and serve as a biomarker for the prognosis detection of OC patients.

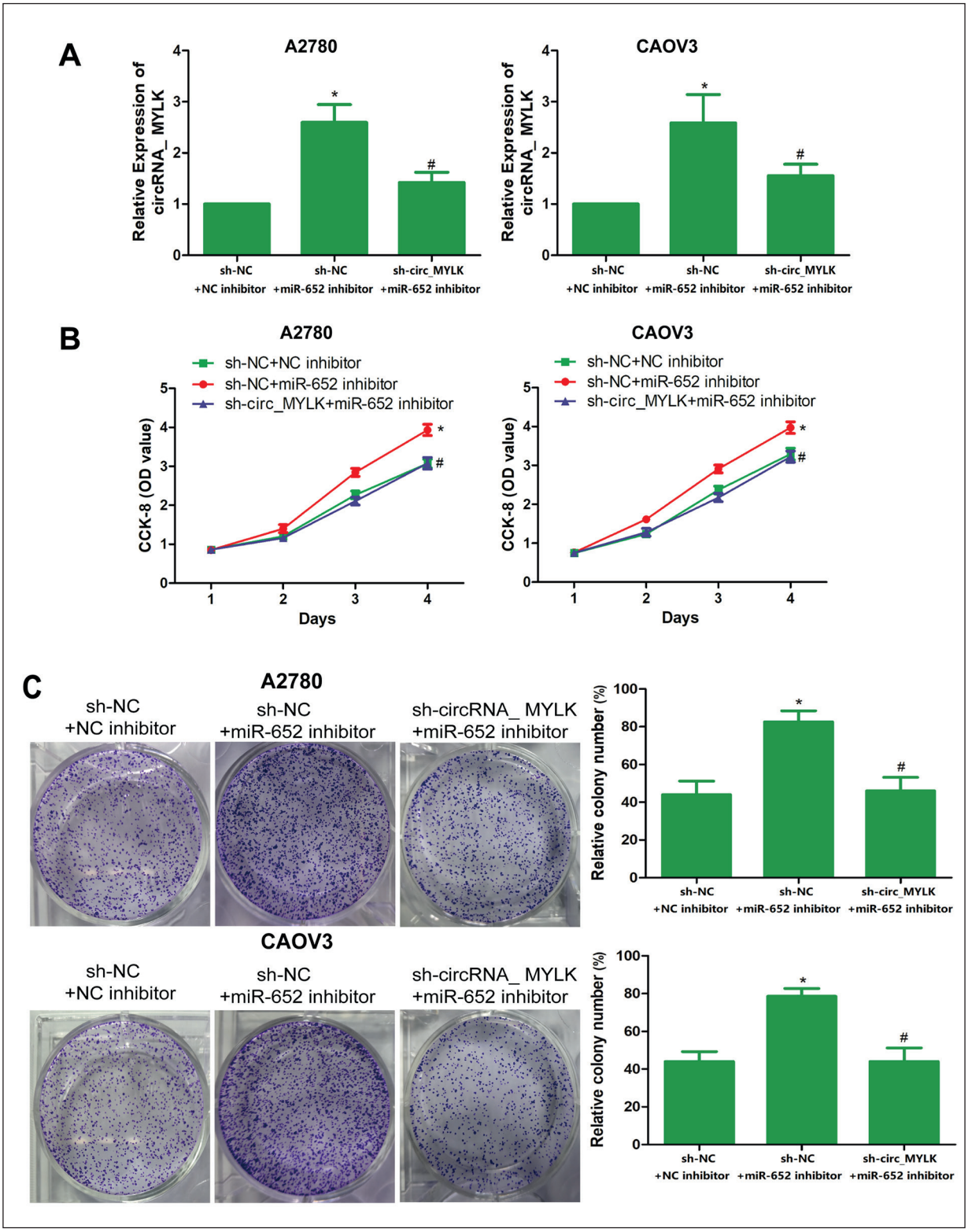

Figure 5. CircRNA_MYLK regulates the expression profile of miR-652 in ovarian cancer cells. **A,** The expression level of circRNA_MYLK in ovarian cancer cell lines co-transfected with circRNA_MYLK and miR-652 was detected by qRT-PCR. **B,** CCK-8 assay detected the effect of co-transfection of circRNA_MYLK and miR-652 on the proliferation of ovarian cancer cells. **C,** The colony formation assay examined the effect of co-transfection of circRNA_MYLK and miR-652 on the regulation of ovarian cancer cell proliferation (Magnification: 10×). Data are mean ± SD, $*^{\#}p<0.05$.

Some studies[17-19] have shown that circRNA can function as a miRNA sponge, regulate splicing and transcription, and modify parental gene expression. CircRNA do not have 5' end cap and 3' end poly real (A) tail, which is formed by reverse splicing by non-canonical splicing and is abundantly present in the cytoplasm of eukaryotic cells, but there are also a small number of intron-derived circRNA present in nucleic acids. It has considerable tissue-specificity, timing-specificity, and disease-specificity, and has a certain relationship with the occurrence and development of many diseases, and even the proliferation of malignant tumors, playing an important role in malignant tumors[18-20]. The prediction of the bioinformatics website in this study revealed that circRNA_MYLK and microRNA-652 had certain similar sequences. Subsequently, the direct binding between circRNA_MYLK and microRNA-652 was also verified by the Dual-Luciferase reporter gene, and the circRNA_MYLK vector without the microRNA-652 binding site could not enrich microRNA-652, thus further verifying the binding site between circRNA_MYLK and microRNA-652. CircRNA_MYLK may be involved in the behavior regulation of OC cells after regulating the expression of microRNA-652, and subsequent CCK-8 and colonogenic experiments verified the previous hypothesis.

In conclusion, circRNA_MYLK level in OC tissues was remarkably higher than that in adjacent tissues, suggesting that it may play a role in promoting oncogenes by affecting the proliferation of OC cells. In addition, the proliferation ability of OC cells was remarkably reduced after knocking down circRNA_MYLK. CircRNA_MYLK regulated the proliferation ability of OC cells by interacting with microRNA-652. Furthermore, the study suggested that circRNA_MYLK may attenuate OC cell proliferation ability by regulating microRNA-652, providing certain theoretical support for the early diagnosis and treatment targets of OC and contributing to the treatment and prognosis monitoring of OC.

Conclusions

CircRNA_MYLK was found to be remarkably correlated with the pathological stage and poor prognosis of OC patients and could promote the malignant progression of OC by regulating microRNA-652.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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