European Review for Medical and Pharmacological Sciences 2018; 22: 2606-2613

MiR-299-5p targets cell cycle to promote cell proliferation and progression of osteosarcoma

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Abstract. – OBJECTIVE: Osteosarcoma is a common primary bone tumor with high mortality. MicroRNA (miRNA, miR) is a small RNA with 20-25 nucleotides, which could regulate diverse biological processes by targeting 3'-UTR of gene to degrade it. MiR-299-5p has been reported to participate in the progression of many diseases, but the role in osteosarcoma is still uncertain. The aim of this work was to investigate the expression of miR-299-5p in osteosarcoma and its clinical significance.

MATERIALS AND METHODS: The datasets of osteosarcoma miRNA was searched in Gene Expression Omnibus (GEO) datasets, including GSE65071, GSE39040, and GSE39055. Osteosarcoma U2 and MG-63 cells were cultured in our study. Cell proliferation level after transfection was detected by using Cell Counting Kit-8 (CCK8) and colon formation assay. Cell cycles were explored using flow cytometer and cell protein expression levels after that the transfection was detected by Western blotting.

RESULTS: We found that ROC curve analysis showed that miR-299-5p was a sensitivity diagnostic criteria and GSEA indicated that miRNA-299-5p may regulate cell cycle. Gain of function assay showed that miR-299-5p promotes cell cycle transition and proliferation. Reversely, the opposite results were observed with loss of function assay. At last, Western blotting assay showed that miR-299-5p may promote cell cycle transition by regulating CDK family.

CONCLUSIONS: Our study demonstrated miR-299-5p can promote the progression of osteosarcoma by regulating the cell cycles.

Key Words:
Osteosarcoma, MicroRNAs, miR-299-5p, Proliferation, GEO.

Introduction

Osteosarcoma is the most common primary bone tumor in adolescents with high degree of malignancy, which is originated from mesenchymal tissue[1]. Current treatment of osteosarcoma mainly includes surgical resection combined with postoperative radiotherapy and chemotherapy, but the 5-year survival rate after amputation is 55% to 68%, indicating poor prognosis of osteosarcoma[2]. Although there is much progress in the etiology, development, diagnosis as well as treatment of osteosarcoma in recent years, the specific pathogenesis is still elusive. Therefore, it is of great importance to elucidate the underlying mechanism of osteosarcoma and find more potential therapeutic targets for this disease.

MicroRNA (miRNA) is a small noncoding RNA consisting of 20-25 nucleotides, which could bind to the 3'untranslated region (3'UTR) of messenger RNA (mRNA), resulting in the degradation or translational inhibition of mRNA thus to regulate target gene expressions[3]. It is reported[4] that miRNAs regulate about 60% of protein-coding genes in human. In the past few decades, miRNAs have been found to mediate many biological events including cell differentiation and cell proliferation together with apoptosis[5-7]. Previous studies[8] also suggest the important roles of miRNAs in various types of cancer. It has been found that miR-770 could inhibit chemotherapy resistance and tumor metastasis of triple-negative breast cancer by degrading STMN1. Moreover, researches have found that miR-494 may promote tumor progression by degrading APC in colorectal cancer[9]. However, few studies focused on miRNAs' functions in osteosarcoma. Thus, exploring the specific function of miRNAs in osteosarcoma might provide a new understanding of the pathogenesis.

MiR-299-5p is located on chromosome 14q32[10], while miRNAs transcribed from this region have been reported to affect ovarian cancer, melanoma, ependymomas and neuroblastoma[11-13]. Pre-

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vious studies[14] have established that miR-299-5p could promote angiogenesis of breast cancer cells by regulating osteopontin. In addition, miR-299-5p is reported to participate in CD34+ progenitor cells commitment[15]. However, the specific role of miR-299-5p in osteosarcoma remains unclear. Therefore, investigating the function of miR-299-5p in osteosarcoma and its relevant clinical significance might provide a new direction for target gene therapy of this disease.

Materials and Methods

Data Acquisition

MiR-299-5p expression in human osteosarcoma tissue was analyzed according to previously published datasets (gene expression omnibus accession GSE65071, GSE39040, GSE39055). GSE65071 includes miRNA profiling in human specimens from osteosarcoma patients' plasma. GSE39040 and GSE39055 include microRNA profiling and clinical outcomes in human osteosarcoma (Table I).

Cell Culture and Transfection

The osteosarcoma cell lines U2 and MG63 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). The miR-299-5p inhibitors, mimics, as well as their negative controls (miR-NC), were obtained from Guangzhou RiboBio (Guangzhou, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection with miR-299-5p mimics, inhibitors or miR-NC, following the instruction.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR

Total RNA was isolated from cells using TRIzol reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed to investigate the relative gene expressions. Primer sequences for the genes are as follows: miR-299-5p: Forward: ACACTCCAGCTGGGTGGTTTACCGTCCCAC, Reverse: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGTATGT, U6 Forward: CTCGCTTCGGCAGCACA, Reverse: AACGCTTCACGAATTTGCGT.

Cell Counting Kit-8 (CCK8)

Cells transfected with miRNA mimics or inhibitors as well as their negative controls were digested with trypsin and seeded (2×10^3/well) into 96-well plates. Cell proliferative activity at different time points (24 h, 48 h, 72 h, 96 h) was detected by measuring the absorbance at 450 nm with the CCK8 assay kit (Beyotime, Shanghai, China). Cells were added with 10 μL/well CCK8 reagent and incubated for 2 h. The absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells were seeded (10^3/plate) into 60 mm plates and incubated for 14 days; after that, they were fixed with paraformaldehyde and stained with crystal violet solution for colony formation observation.

Flow Cytometry Analysis

Cells were harvested after transfection, while the cell supernatants were fixed in 200 μL 75% ethanol containing ribonuclease (RNase) A (1 mg/mL) for 30 min at 37°C. Cell suspensions were stained with propidium iodide (PI) solution in the dark for 30 min. Stained cells were analyzed using a FACS flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blotting Analysis

Cells were digested with trypsin and total protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocked in nonfat milk, the immunoblots were incubated with the following primary antibodies: anti-p16 (1:1000, Abcam, Cambridge, MA, USA), p21 (1:500, Abcam, Cambridge, MA, USA), Cyclin D (1:1000, Abcam, Cambridge, MA, USA), Cyclin E (1:1000, Abcam, Cambridge, MA, USA) and anti-GDK (1:1000, Abcam, Cambridge, MA, USA). Protein bands were detected using enhanced chemiluminescence (ECL) machine (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

In this study, differential miRNAs were analyzed by limma R package and prognostic analysis by survival function. All experiments were repeated 3 times. Data were represented as mean ± SD. *t*-test or x^2-test was performed to calculate differences between two groups. Prognostic analysis was obtained by log-rank. In addition,

gene and clinical data were tested by single- and multi-factor COX regression. The ROC curve and charts were drawn with Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) or GraphPad Software (La Jolla, CA, USA). $p<0.05$ was considered statistically significant.

Results

MiR-299-5p is Aberrantly Expressed in Osteosarcoma and Negatively Correlated with Prognosis

To investigate the expression of miR-299-5p in osteosarcoma patients, we analyzed the expression matrix GSE65071 by limma R package and found that serum miR-299-5p was highly expressed in osteosarcoma patients comparing to normal people (Figure 1A). Next, we performed analysis on the correlation between miR-299-5p expression and prognosis in osteosarcoma patients by Kaplan-Meier along with log-rank method. Expression values larger than the median value (median expression value = 8.437213) were considered as the high expression group, while values lower than the median were considered the low expression group. Analysis showed that the miR-299-5p overexpression group had a shorter survival time relative to the miR-299-5p low expression group (Figure 1B). These results suggested that miR-299-5p might play a crucial role in osteosarcoma.

miR-299-5p Promotes Cell Cycle Conversion in Osteosarcoma

According to the receiver operating characteristic curve (ROC) test, we found that miR-299-5p could predict the prognosis of osteosarcoma patients (AUC = 0.704) (Figure 2A). GSEA analysis indicated that miR-299-5p mainly affected the cell cycle (Figure 2B-D). To explore the role of miR-299-5p in osteosarcoma progression, we examined the expression of miR-299-5p in osteosarcoma cells. Results showed miR-299-5p expression in osteosarcoma cell lines U2 and MG-63 were significantly higher than that in human osteoblast hFOB1.19 (Figure 2E). In addition, miR-299-5p inhibitor and mimic sequences were constructed and transfected into U2 and MG-63 cells with high transfection efficiency (Figure 2F-G). Using CCK8 assay we found that miR-299-5p interference decreased the proliferation in U2 and MG-63 cells. In contrast, overexpression of miR-299-5p could increase cell proliferation (Figure 2H-I). Colony formation experiments also confirmed that miR-299-5p could promote proliferation in U2 and MG-63 cells (Figure 3A-B). Besides, we detected the cell cycle by flow cytometry, which revealed that cells blocked in G0/G1 phase after interfering with miR-299-5p, whereas overexpressing miR-299-5p rescued this blocking effect. (Figure 4A-B). These data demonstrated that miR-299-5p could promote cell proliferation via accelerating cell cycle conversion.

miR-299-5p Regulates Osteosarcoma Cell Cycle Via Regulating Cyclin Proteins

To investigate the molecular mechanism of miR-299-5p in regulating osteosarcoma, we detected the key proteins in cell cycle by Western blot experiments. Results showed that the protein levels of Cyclin E, Cyclin D, and CDK, which could promote G1 phase, were significantly downregulated after interference with miR-299-5p. Meanwhile, the protein levels of p16 and p21, which could block the G1 phase, significantly increased after miR-299-5p interference (Figure 5A-B). These results suggested that miR-299-5p

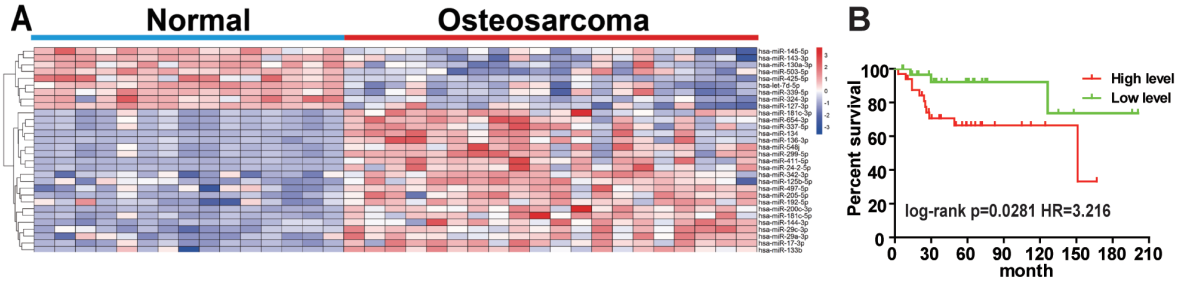

Figure 1. miR-299-5p is upregulated in osteosarcoma and conversely correlated with OS patients' overall survival. ***A,*** miR-299-5p is up-regulated in serum of OS patients. ***B,*** miR-299-5p is conversely correlated with OS patients' overall survival in GSE39055.

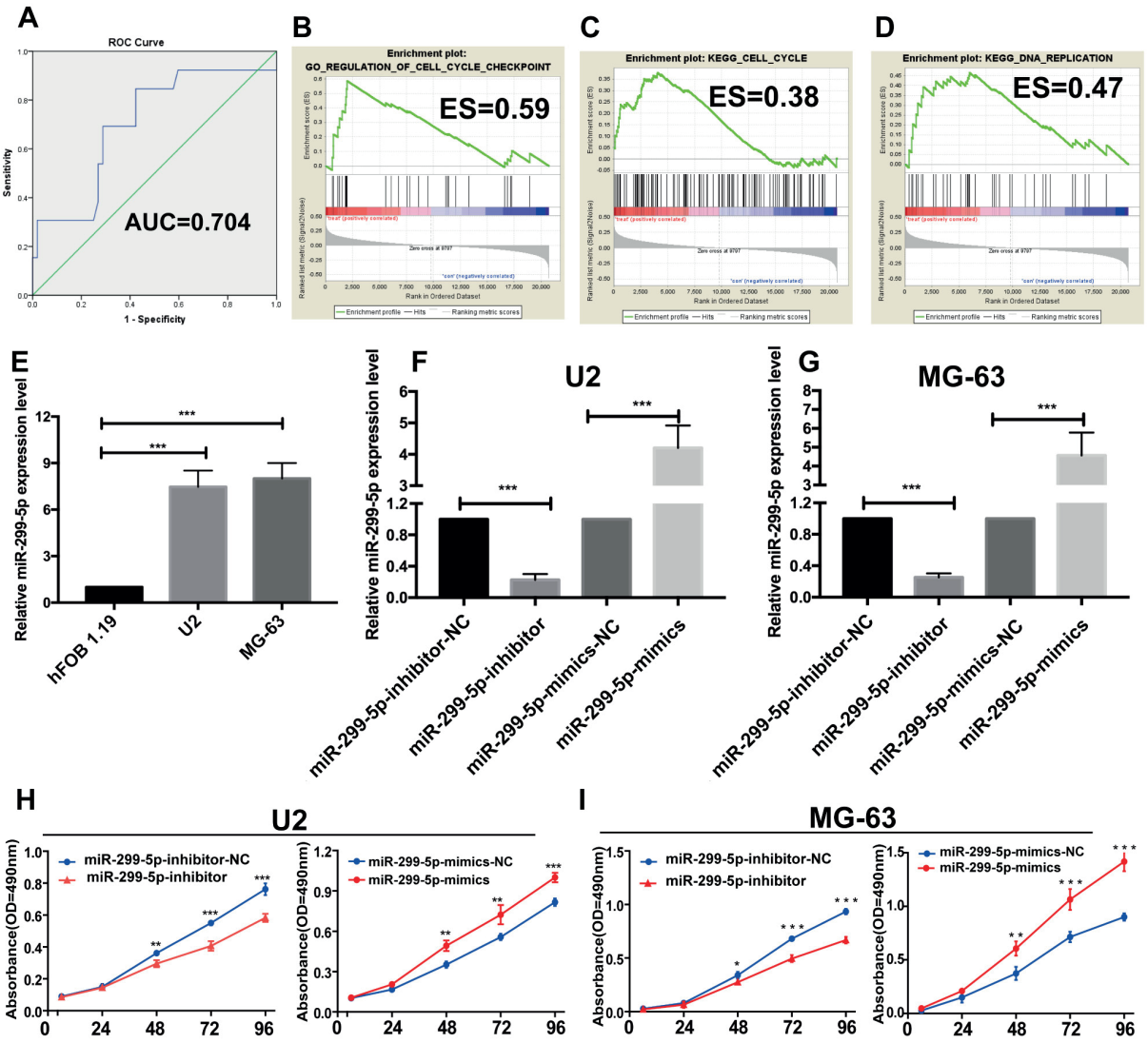

Figure 2. miR-299-5p promotes OS cell proliferation by CCK-8 assay. ***A,*** ROC analysis indicates that miR-299-5p is a diagnostic indicator in osteosarcoma. ***B,*** GO analysis indicates that miR-299-5p regulates cell cycle. ***C-D,*** KEGG analysis predict that miR-299-5p regulates cell cycle. ***E-F,*** CCK-8 assay showed that miR-299-5p could promote U2 and MG-63 cell proliferation. $^{*}p<0.05$, $^{**}p<0.01$,$^{***}p<0.001$.

regulated osteosarcoma cell cycle via downregulating cyclin proteins.

Discussion

Osteosarcoma is the most common bone tumor with high malignancy. Current therapeutic strategies mainly include surgical treatment combined with chemotherapy; however, current treatment could not work effectively with poor prognosis[16]. Genetic therapy is a hot topic in the treatment of osteosarcoma; then, seeking an effective therapeutic gene targets for osteosarcoma is urgently needed. Previous reports[17] show that miRNAs have a wide range of gene regulatory functions in a series of biological process such as embryonic development, cell proliferation, apoptosis and hematopoietic function. In addition, many miRNAs have been found to be aberrantly expressed in osteosarcoma during its occurrence and development. Therefore, it is suggested that some

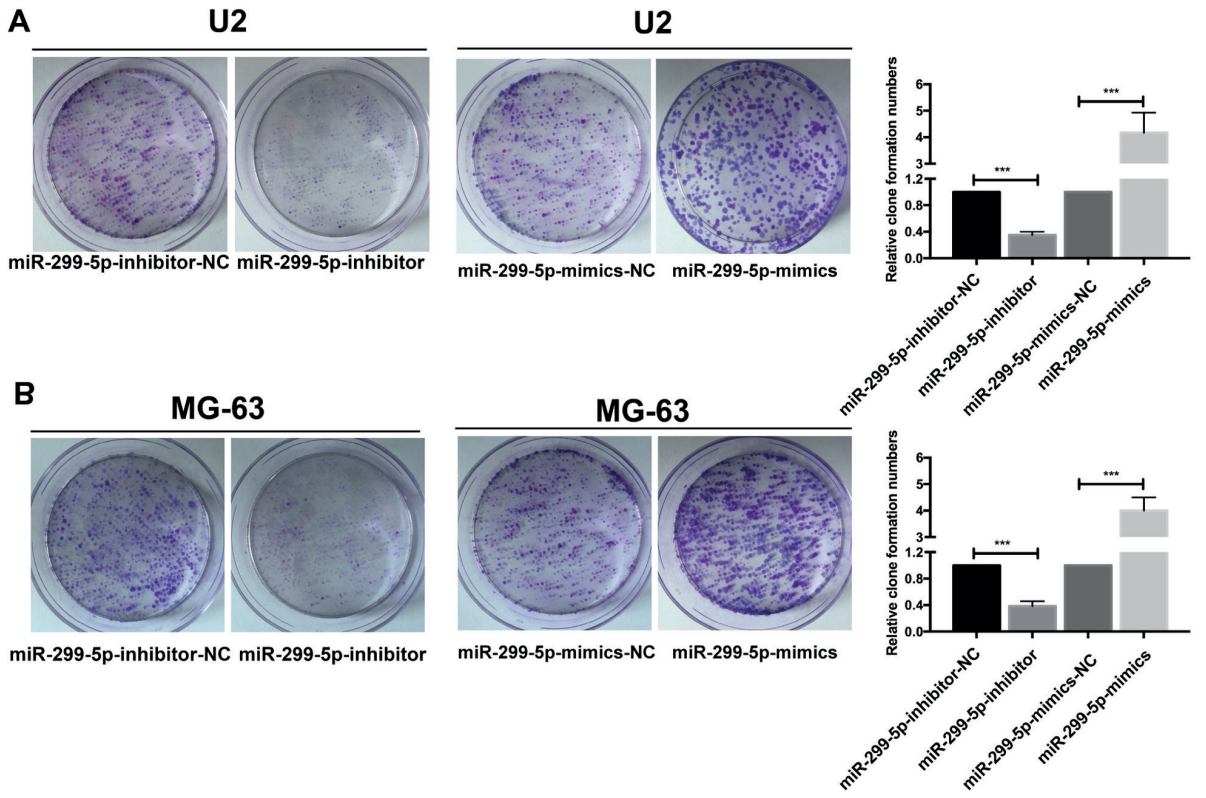

Figure 3. miR-299-5p enhances OS cell proliferative ability by colon formation assay. ***A-B,*** Colony formation assay showed that miR-299-5p promoted U2 and MG-63 cell growth. $^{***}p<0.001$.

miRNAs contribute to the malignant biological phenotype of osteosarcoma[18-20].

MiR-299-5p is located at chromosome 14q32.31, which contains transcripts of up to 54 miRNAs. MiRNAs in this region have been shown to be associated with a variety of tumors, while most of them target the PRC2 gene by reducing the formation of the PRC2 complex[10]. Additionally, miR-299-5p has been reported with aberrant expression in many diseases like oral squamous cell carcinoma and prostate cancer[21]. In metastatic breast cancer and high-risk neural tumor, for instance, miR-299-5p expression was significantly decreased[18,22]. Besides, miR-299-5p could negatively regulate OPN, a target gene that promotes tumor proliferation and formation in hepatoma cells[19]. Meanwhile, miR-299-5p has been found to be significantly up-regulated in non-small cell lung cancer cell line[20]. Although miR-299-5p plays a crucial role in many tumors, its function in osteosarcoma remains unknown.

In our investigation, the serum level of miR-299-5p was significantly elevated in osteosarcoma patients based on our bioinformatics analysis, and the expression of miR-299-5p was inversely proportional to the patient's prognosis. Meanwhile, the receiver operating characteristic curve test (ROC) showed that miR-299-5p was a sensitive indicator of osteosarcoma diagnosis. Furthermore, CCK-8 assay together with colony formation experiment showed that miR-299-5p could promote osteosarcoma cell proliferation.

To explore the possible target of miR-299-5p, we conducted the GSEA analysis and found that miR-299-5p might regulate cell cycle progression. Flow cytometry confirmed that miR-299-5p could promote the cell cycle transition. To investigate the mechanism how miR-299-5p regulating cell cycle, we further identified the potential target genes. Many molecules have been found to regulate the cell cycle. There are three major classes of proteins involved in this process: cyclin, cyclin-dependent kinase (CDK) as well as cyclin-dependent kinase inhibitor (CKI). These genes are known as cell division cycle genes (CDC). Among them, CDKs are the core of regulatory networks in cell cycle. Cyclins positively regulate CDKs while CKIs exert a negative reg-

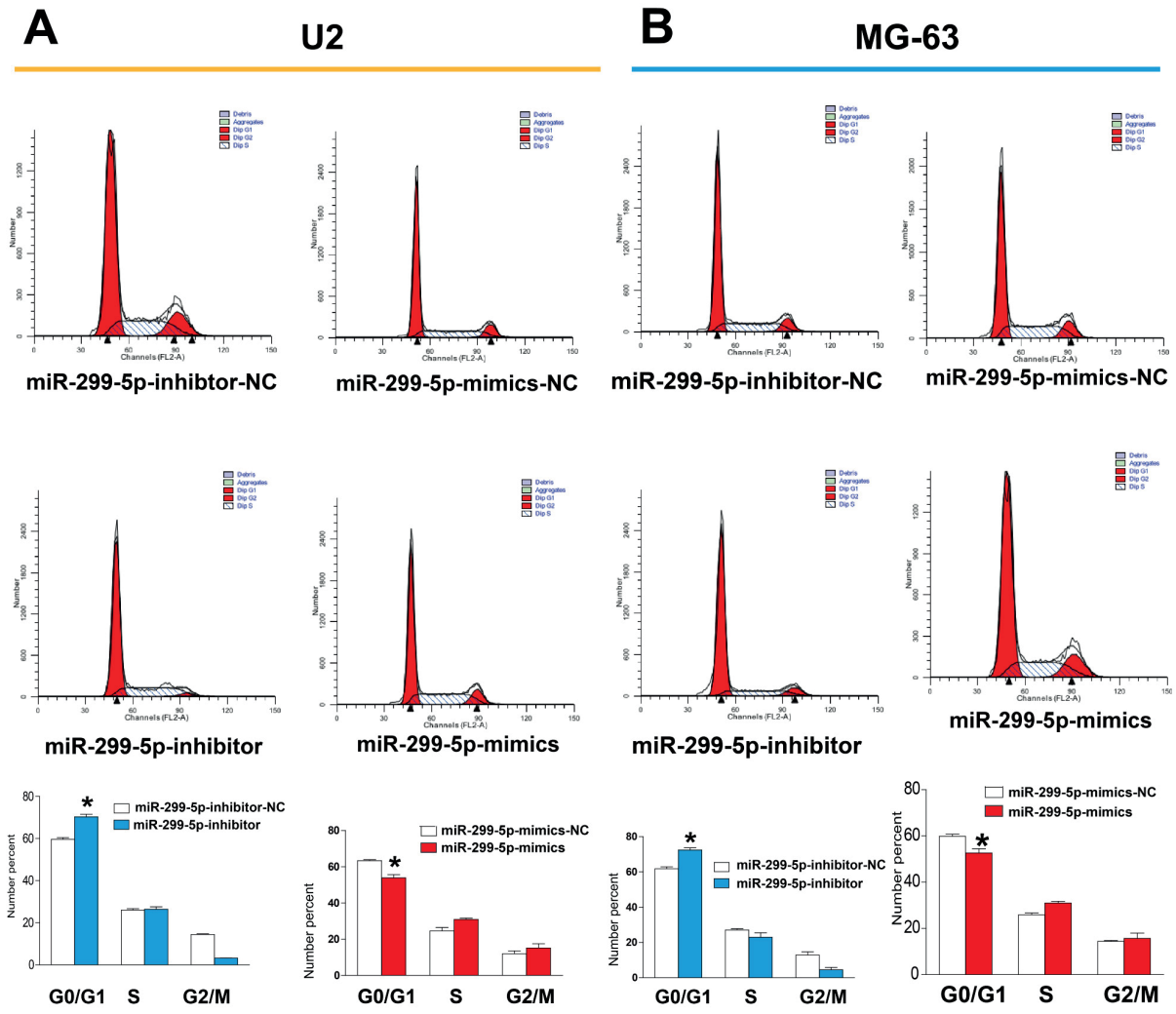

Figure 4. miR-299-5p promotes OS cell cycle transition. ***A-B,*** miR-299-5p accelerated osteosarcoma cell cycle transition. n.* $p<0.05$

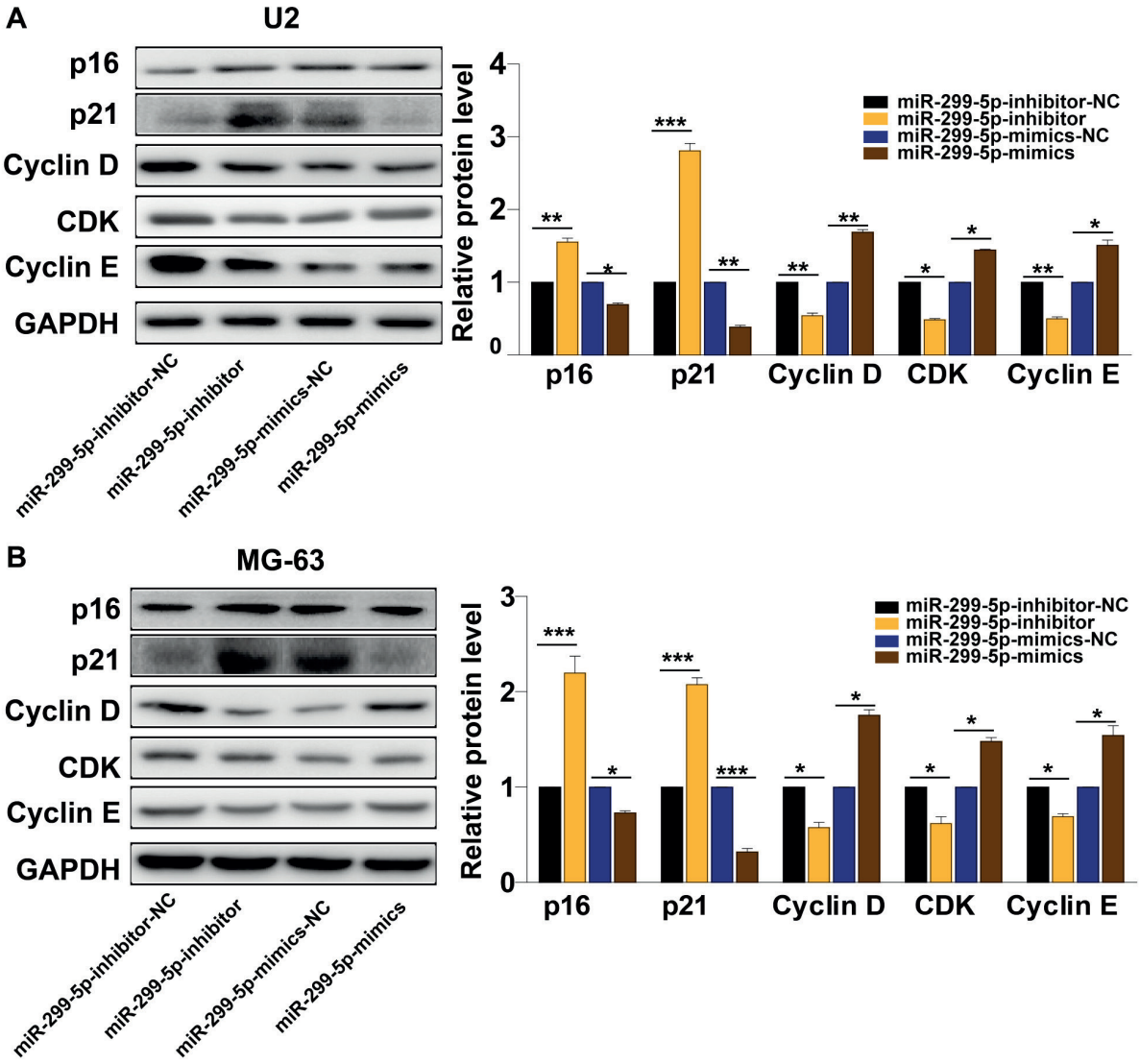

Figure 5. The mechanisms of miR-299-5p regulate cell cycle. ***A-B,*** P16 and P21 were down-regulated in miR-299-5p mimics group and up-regulated in miR-299-5p inhibitor group. Inversely, Cyclin D, Cyclin E and CDK were up-regulated in miR-299-5p mimics group and down-regulated in miR-299-5p inhibitor group. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

ulatory effect on CDKs[23,24]. Western blotting results indicated that miR-299-5p could mediate G1 phase via targeting cyclin E, cyclin D, CDK.

To summarize, our study demonstrated the clinical significance of miR-299-5p in osteosarcoma, providing a potential target for gene therapy of this disease.

Conclusions

We observed that the expression of miR-299-5p was significantly higher in osteosarcoma patients than that in normal individuals, thereby predicting poor prognosis. In addition, miR-299-5p could accelerate tumor progression by promoting the cell cycle.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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