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# MicroRNA-31 inhibits osteosarcoma cell proliferation, migration and invasion by targeting PIK3C2A

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**Abstract. – OBJECTIVE: To elucidate the role of microRNA-31 (miR-31) in osteosarcoma and the molecular mechanism of miR-31 in the proliferation, migration, and invasion of osteosarcoma.**

**PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to examine the expression of microRNA-31 in human osteosarcoma tissues. Pearson's chi-squared test was used to analyze the correlation between microRNA-31 and clinicopathological features. Proliferation, migration, invasion, and PI3K3C2A protein in treated osteosarcoma cells were detected by Cell Counting Kit-8 (CCK-8) assay, transwell assay without Matrigel, transwell assay with Matrigel, and Western blot analysis, respectively.**

**RESULTS: qRT-PCR showed that miR-31 was down-regulated in osteosarcoma tissues compared with paired para-tumor bone tissues. The lower level of miR-31 was closely associated with high-grade osteosarcoma, metastasis, and poor overall survival. CCK-8 and transwell assay showed that miR-31 inhibited osteosarcoma cells proliferation, migration, and invasion. According to luciferase assay, miR-31 inhibits osteosarcoma cell proliferation, migration, and invasion through inhibiting PIK3C2A. Reversely, overexpression of PIK3C2A inhibited partial effect of miR-31 on proliferation, migration, and invasion *in vitro*.**

**CONCLUSIONS: MiR-31 inhibits osteosarcoma cell proliferation, migration, and invasion by targeting PICK3C2A. MiR-31 can thus be used as a therapeutic target in osteosarcoma treatment.**

*Key Words:*
Osteosarcoma, Proliferation, Invasion, Migration, miRNA-31, PIK3C2A.

## Introduction

Osteosarcoma (OS) is the most common type of primary bone cancer in childhood and adolescence[1,2]. In the past, many therapies for OS have been described. However, the overall 5-year survival rate has not improved significantly owing to metastasis resulting in tumor recurrence[3]. Therefore, understanding the molecular mechanisms underlying metastasis is crucial for the development of effective therapeutic strategies for the treatment of osteosarcoma. In recent years, a great number of microRNAs (miRNAs), such as miR-34a[4], miR-187[5], miR-376c[6], miR-874[7], miR-200c[8], have been identified to specifically contribute to osteosarcoma progression and metastasis. MiR-31 is one of the frequently altered miRNAs in human cancers. Numerous studies have revealed that miR-31 expression was specifically altered in prostate cancer[9], gastric cancer[10], cervical cancer[11], and breast cancer, and miR-31 play an important role in cancer metastasis[12,13]. However, the roles of miR-31 in osteosarcoma are still unidentified, and the regulatory mechanisms of miR-31 were also unclear. To investigate the potential downstream mechanisms of miR-31 in osteosarcoma cell lines, we predicted its target genes according to sequence complementation using miRanda and TargetScan methods. PIK3C2A was one of candidate target. This leads to the hypothesis that miR-31 may play roles in osteosarcoma cell lines by inhibiting PIK3C2A. PIK3C2A belongs to the phosphoinositide 3-kinase (PI3K) family. The PI3K pathway regulates various cellular proces-

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ses, such as cell growth, proliferation, apoptosis, motility, differentiation, survival, and intracellular trafficking[14]. PIK3C2A was already proved to regulate cell proliferation and migration in some cancer cells[15]. In this study, our results indicated that miR-31 regulates osteosarcoma cell proliferation, migration, and invasion through PIK3C2A.

## Patients and Methods

### Tissue Sample

Osteosarcoma tissues and paired para-tumor bone tissues were collected from 43 patients, with their signed informed consent, during tumorectomy at people's Hospital of the Inner Mongolia Autonomous Region and Affiliated Zhongshan Hospital Dalian University between April 2007 and April 2017. All 43 cases had a definite pathological diagnosis, and the clinical stages of these patients were determined according to the TNM classification of the International Union Against Cancer (UICC). The Institute Research Medical Ethics Committee of the Inner Mongolia Autonomous Region of the Affiliated Zhongshan Hospital Dalian University granted approval for this study.

### Cell Culture

Human osteosarcoma cell line MG-63 was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, CA, USA). All media were supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco, Grand Island, CA, USA). All cells were cultured at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Prediction of MiRNA Targets

Prediction of miRNA target genes uses miRanda and TargetScan methods. The target genes predicted by both methods were included in further analysis.

### Plasmid Construction

The PIK3C2A fragment containing miR-31 binding site was amplified and cloned into the KpnI and XhoI restriction sites (Promega, Madison, WI, USA) of pcDNA3.1 vector to synthetize pcDNA3.1-PIK3C2A-wild-type (pcDNA3.1-PIK3C2A-wt); pcDNA3.1-PIK3C2A-mutant-type (pcDNA3.1-PIK3C2A-mut) was also gained by using a QuikChange Site-Directed Mutagenesis Kit (Agilent, Beijing, China). These two plasmids were used to construct PIK3C2A overexpression cell models.

### Transfections

The MG63 osteosarcoma cells were seeded in 24-well plates at a density of $5\times10^5$ cells/wells. Then, they were incubated overnight. MiR-31 mimics, miR-31 inhibitors, and plasmids were transfected into osteosarcoma cells by using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. After transfection, total RNA and proteins from MG63 osteosarcoma cells were isolated at 48 h. The sequence of PIK3C2A siRNA used before[16] as follows: siRNA: PIK3C2A (5'-AAGGTTGGCACTTACAAGAAT-3')

### Cell Counting Kit-8 (CCK-8) Assay

Proliferations of MG63 osteosarcoma cells were determined using a CCK-8 (Dojindo, Kumamoto, Japan). The MG63 osteosarcoma cells were seeded in 96-well plates at a density of 2000 cells/well. Then, MG63 osteosarcoma cells were incubated in 10% CCK-8 and 2 h at 37°C in a humidified incubator. Optical density value was measured at 450 nm. After transfection, proliferation rates of MG63 osteosarcoma cells were determined at 24, 48, and 72 h.

### Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The procedure was carried out as previously described[17]. In brief, total RNA of osteosarcoma tissues or cells were extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The expressions of miRNAs and genes were determined by the qRT-PCR kit (TaKaRa, Otsu, Shiga, Japan) using a StepOne Plus Real-Time PCR System (Life Technologies, Shanghai, China) and a SYBR Green kit (TaKaRa, Otsu, Shiga, Japan). U6 and β-actin were used as an internal control. All reactions were performed in triplicate. The relative expressions of miRNAs and genes were quantified. Primers were synthesized by Sangon Biotechnology (Shanghai, China) and the sequences were as follows: PIK3C2A 5'-CTTACTCATTGCTTCACCAGTGG-3' and 5'-GCCTCAATCCAGGTCACAGCTA-3'. U6 5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. miR-31 5'-TAATACTGCCTGGTAATGATGA-3' and 5'-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGATACGACAGCTAT-3'), and β-actin (5'-ATGATATCGCC GCGCTCG-3', 5'-CGCTCGGTGA GGATCTTCA-3').

### Western Blot

Osteosarcoma tissues and cells were split by using a radioimmunoprecipitation assay lysis buffer. Proteins were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. A 5% non-fat milk was used to block the membranes; then, they were incubated with PIK3C2A antibodies (Invitrogen, Carlsbad, CA, USA; dilution rates of 1:1000) and β-actin antibodies (Abcam, Cambridge, MA, USA; dilution rates of 0.5 mg/mL). After an extensive wash of membranes, the secondary antibodies (Abcam, Cambridge, MA, USA) were added into the system. The enhanced chemiluminescence (ECL) system was used to detect the immunoreactive protein bands. After the film is shot, Image-Pro Plus 6.0 software is adopted for semi-quantitative analysis.

### Migration and Invasion Assay

Invasion of MG63 osteosarcoma cells was determined with a transwell chamber (Corning, Corning, NY, USA) with Matrigel (Invitrogen, Carlsbad, CA, USA), and migration was determined in the same way but without Matrigel (Invitrogen, Carlsbad, CA, USA). The cells in the logarithmic growth phase in each group were digested and counted after 48 h of miR-31 cell transfection. Osteosarcoma cells were seeded onto the membrane of the upper chamber of the transwell at a concentration of $3 \times 10^5$ mL in 2 mL of DMEM. The medium in the upper chamber was serum-free. The medium in the lower chamber contained 5% fetal calf serum as a source of chemoattractants. After 24 h, migrated cells were fixed in 100% methanol for 30 minutes. Those non-migrated cells were then removed by cotton swabs. Finally, the cells on the bottom surface of the membrane were stained for 20 minutes with the 0.1% crystal violet. Eventually, pictures were taken in eight random views under a microscope for records.

### Luciferase Assay

The MG63 osteosarcoma cells were seeded in 24-well plates at a density of $5\times10^5$ cells/well. Then, they were incubated for 24 hours. The procedure was carried out as previously described[18]. In the luciferase reporter gene assay, the MG63 osteosarcoma cells were co-transfected with 0.8 μg of pcDNA3.1-PIK3C2A-wt/pcDNA3.1-PIK3C2A-mut plasmid (Promega, Madison, WI, USA), and 200 nM miR-31 mimics, miR-31 inhibitor with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 h after transfections, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to evaluate fluorescence intensity changes according to the manufacturer's protocol.

### Statistical Analysis

Pearson's $x^2$-test was used to analyze the correlation between microRNA-31 and clinicopathological features. All data are listed as mean ± standard deviation (SD) from individual experiments. The *t*-test for two groups and ANOVA (followed by Least Significant Difference as its Post-Hoc Test) for more than two groups were used for calculation. The Kaplan-Meier method was applied to calculate data from the survival curves. All data presented in this study have been repeated at least three times from three independent and are presented as mean ± SD. $p$-values $< 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS19 (IBM, Armonk, NY, USA).

## Results

### Expression of MiR-31 and PIK3C2A mRNA in Osteosarcoma Tissue and Paired Para-Tumor Bone Tissues

We examined the expression of miR-31 in 43 pairs of osteosarcoma tissue and paired para-tumor bone tissues by qRT-PCR. The qRT-PCR results demonstrated that the miR-31 expression in osteosarcoma tissue was markedly lower than paired para-tumor bone tissues (Figure 1A). The results were validated by the non-parametric sum of ranks of paired samples, and the difference was significant ($p < 0.05$). We tried to assess whether the lower miR-31 was correlated with the final survival time of osteosarcoma. Our Kaplan-Meier analysis and log-rank test revealed that high expression of miR-31 was correlated with osteosarcoma patients' overall survival (Figure 1B) ($p < 0.01$). Further correlation analysis confirmed that the low level of miR-31 was closely correlated with clinicopathological features, especially with clinical stage ($p = 0.011$) and distant metastasis ($p = 0.047$) (Table I).

### MiR-31 Inhibits Proliferation, Migration, and Invasion of Osteosarcoma Cell Lines

We transfected miR-31 mimics and miR-31 inhibitor into MG63 cells (Figure 2A). We inve-

**Table I.** Association of miR-31 expression with clinic pathological features of osteosarcoma.

| Features | Number of cases | miR-31 | | *p*-value |
|---|---|---|---|---|
| | | High | Low | |
| Ages | | | | 0.88 |
| <18 | 29 | 11 | 18 | |
| >18 | 14 | 5 | 9 | |
| Gender | | | | 0.771 |
| Female | 24 | 9 | 15 | |
| Male | 19 | 7 | 14 | |
| Clinical stage | | | | 0.011 |
| I+IIA | 14 | 9 | 5 | |
| IIB/III | 29 | 7 | 22 | |
| Distant metastasis | | | | 0.047 |
| Absent | 16 | 9 | 7 | |
| Present | 27 | 7 | 20 | |
| Tumor size (cm) | | | | 0.782 |
| < 5 | 15 | 6 | 9 | |
| ≥ 5 | 28 | 10 | 18 | |

*p*-value obtained from Pearson v2-test or Fisher's exact test.

stigated the potential impact of miR-31 on proliferation, migration, and invasion of osteosarcoma cell in MG63 cell lines. The MG63 osteosarcoma cells were transfected with miR-31 mimics or inhibitors or negative control. The CCK-8 assay showed that the proliferation of MG63 osteosarcoma cells was inhibited by miR-31 mimics, which is evidenced by slower increases of OD450 values in miR-31-mimics-transfected osteosarcoma cell lines compared with non-transfected cell lines. Meanwhile, miR-31 inhibitors significantly promoted the proliferation of the MG63 osteosarcoma cells (Figure 2B). Moreover, transwell assay showed that miR-31 inhibitors significantly promoted the migration and invasion of MG63 osteosarcoma cells. And miR-31 mimics inhibited cell

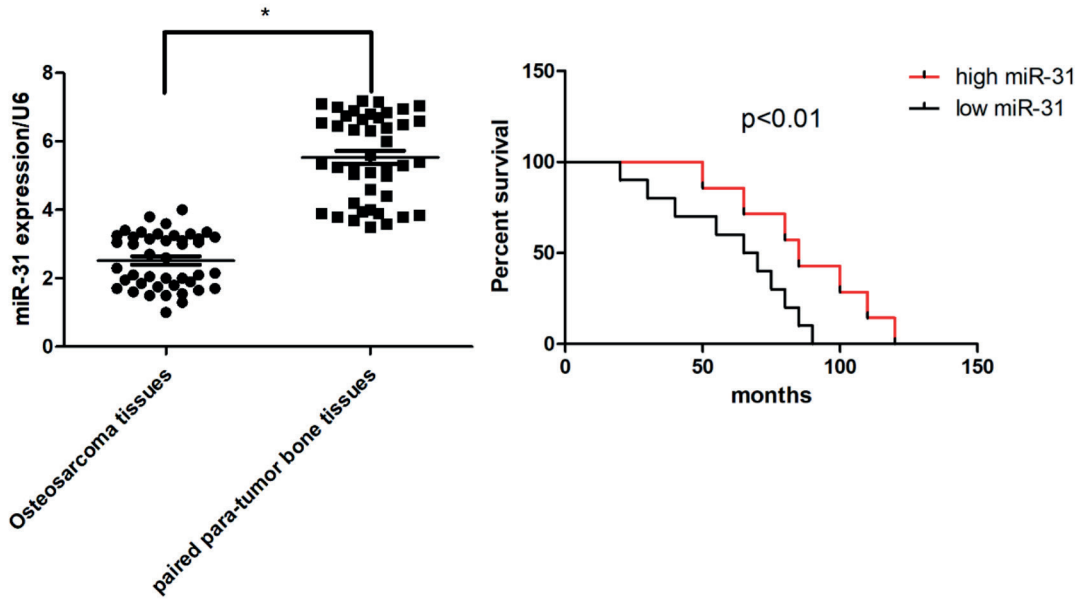


**Figure 1.** MiR-31 is up-regulated in osteosarcoma tissues and is associated with disease progression. (A) Quantitative RT-PCR (qRT-PCR) analysis of miR-31 expression in osteosarcoma tissues and paired noncancerous bone tissues (n = 43). (B) Kaplan-Meier analyses of the associations between miR-31 expression level and overall survival of patients with osteosarcoma (the log-rank test was used to calculate *p*-values). Patients were divided into two groups according to the median miR-31 expression in osteosarcoma. *$p < 0.05$.

migration and invasion in MG63 osteosarcoma cells (Figure 2C, 2D).

### MiR-31 Inhibits Proliferation, Migration, and Invasion of Osteosarcoma Cell Lines Through PIK3C2A

To explore downstream targets of miR-31, bioinformatics analysis was performed using two online algorithms, TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do). PIK3C2A was identified as a putative target gene. We first explore the function of PIK3C2A in osteosarcoma cell line MG63 with PIK3C2A siRNA. We showed that knockdown PIK3C2A with PIK3C2A siRNA inhibits proliferation, migration, and invasion of MG63 (Figure 2B-D). According to the prediction of miRanda and TargetScan, there was complementarity between has-miR-31 and the 3'untranslated region (UTR) of PIK3C2A (Figure 3A).

Then, we transfected miR-31 miRNA mimics, miR-31 inhibitors, and inactive controls into MG63 osteosarcoma cell lines, respectively. Western blots and qRT-PCR revealed that overexpression of miR-31 reduced the protein and mRNA levels of PIK3C2A in osteosarcoma cells. Conversely, miR-31 inhibitor increased the protein and mRNA levels of PIK3C2A in osteosarcoma cells (Figure 3B, 3C). The effect of miR-31 on the translation of PIK3C2A mRNA into protein was then assessed by using a luciferase reporter assay. The enforced expression of miR-31 significantly reduced the luciferase activity of the reporter gene with the wild-type construct but not with the mutant PIK3C2A 3'UTR construct (Figure 3D). These results reveal that miR-31 targets the 3'UTR region of PIK3C2A to inhibit its expression.

### Overexpression of PIK3C2A Abates miR-31 Induced Inhibition of Proliferation, Migration, and Invasion in Osteosarcoma Cell Lines

To confirm the effect of PIK3C2A on proliferation, migration, and invasion induced by miR-31 in osteosarcoma, plasmids expressing PIK3C2A or control vector were transfected into

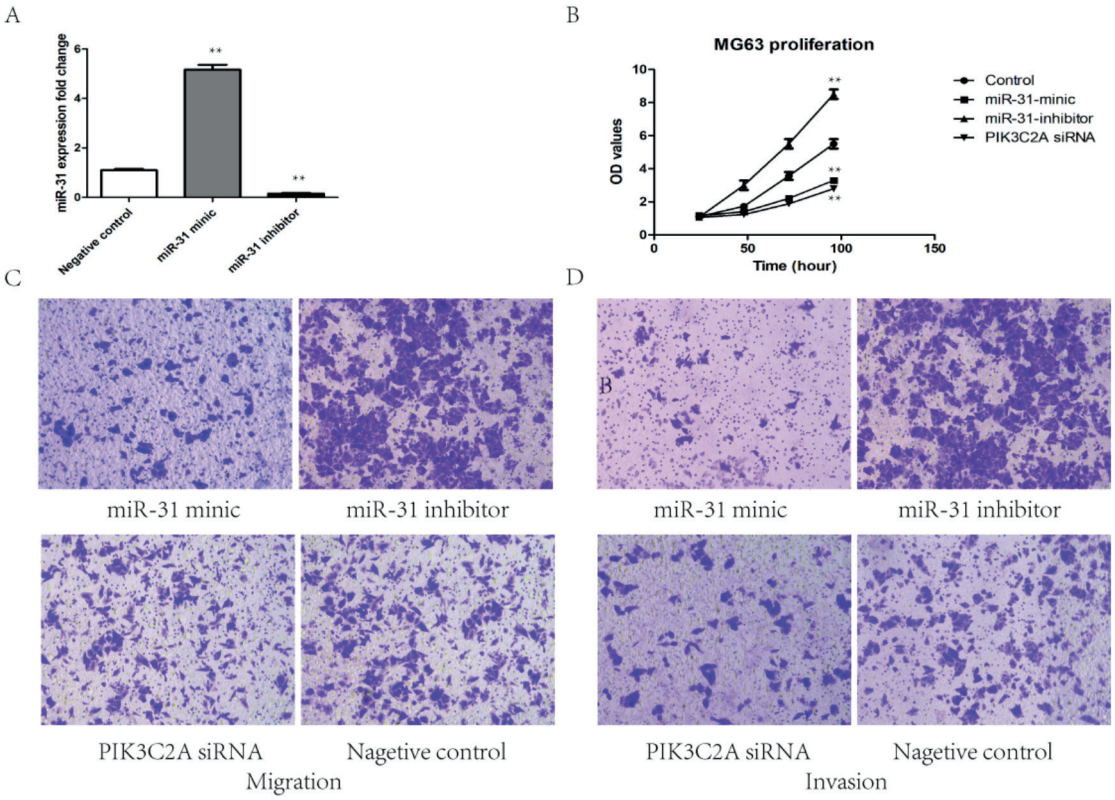


**Figure 2.** MiR-31 mimics promotes osteosarcoma cell MG63 proliferation, migration and invasion. ***(A)*** The expression of miR-31 was determined by qRT-PCR in MG63 cell line. ***(B)*** CCK-8 assay showed miR-31 mimics and PIK3C2A siRNA inhibited MG63 cells proliferation. ***(C-D)***. Transwell assay showed cells migration and invasion abilities were enhanced by miR-31 inhibitor, and inhibited by PIC3C2A siRNA.

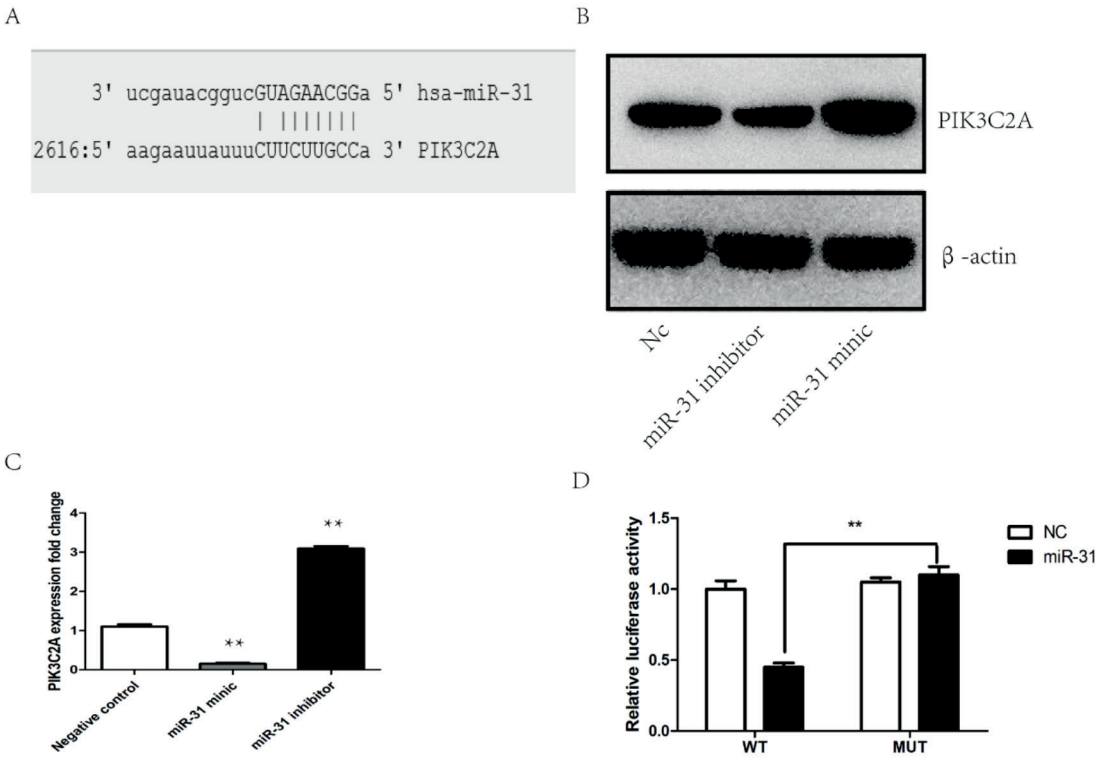


**Figure 3.** MiR-31 directly binds the 3'-UTR of PIK3C2A. (A) The putative binding site in the 3'-UTR of PIK3C2A. (B, C) miR-31 mimics significantly decreased the PIK3C2A expression at both mRNA and protein levels. (D) Weakened fluorescence was presented when co-transfection of miR-31 mimics and pcDNA-3.1-PIK3C2A-wt was detected by luciferase assays. $^{**}p < 0.01$ *vs.* co-transfection of miR-31 mimics and pcDNA-3.1- PIK3C2A –mut.

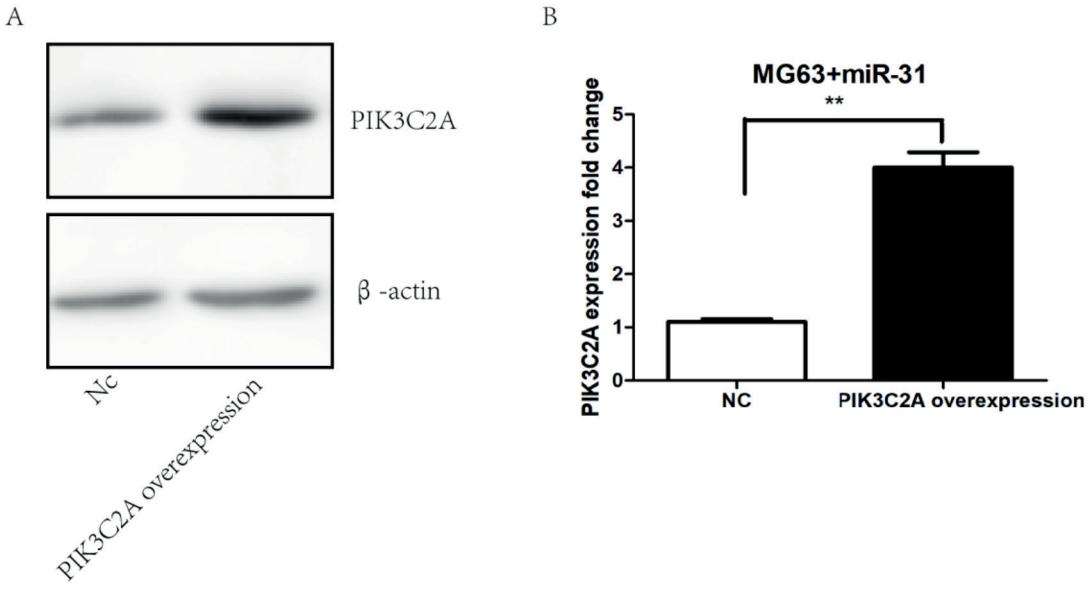


**Figure 4.** Validate the overexpression of PIK3C2A at mRNA (A) and protein (B) level. $^{**}p<0.05$.

MG63 cells, which containing miR-31 mimic. The expression of PIK3C2A was detected by qRT PCR and Western blot (Figure 4). Compared with cells co-transfected with control vector and miR-31 mimics, the expression of PIK3C2A was markedly increased in cells co-transfected

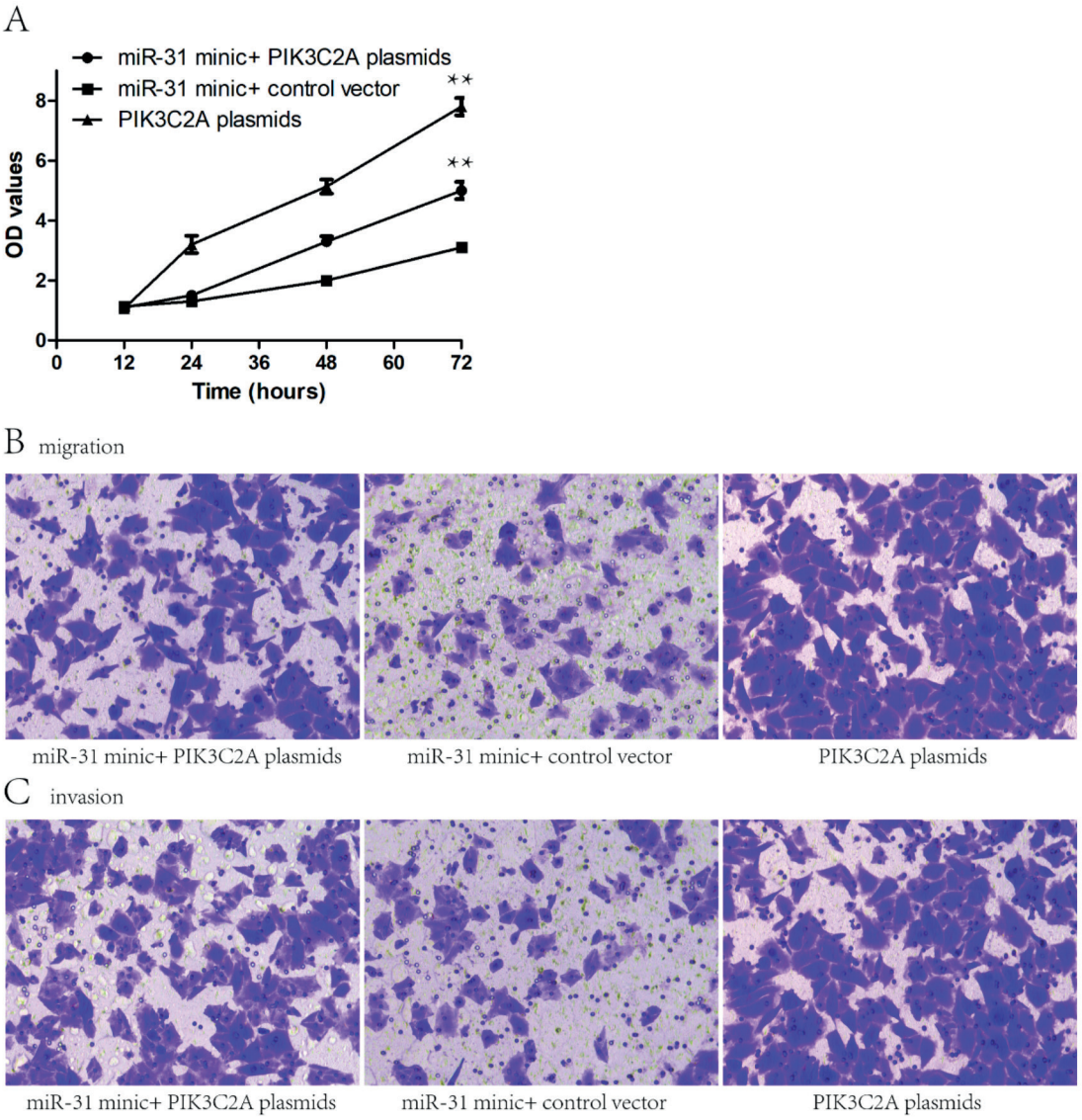


**Figure 5.** Overexpression of PIK3C2A attenuates the miR31-induced inhibition of proliferation, migration and invasion in MG63 cells. ***(A)*** CCK-8 assay showed co-transfecting PIK3C2A plasmids attenuates the miR-31 mimics induced inhibition of MG63 proliferation. ***(B-C)*** Transwell assay showed cells migration and invasion abilities were enhanced by PIK3C2A overexpression. $^{**}p<0.05$

with PIK3C2A and miR-31 mimics at mRNA and protein levels. We further examined the effect of PIK3C2A on proliferation, migration, and invasion in cells co-transfected with miR-31 mimics and PIK3C2A expression plasmids or control vectors. As shown in (Figure 5A-D), over-expression of PIK3C2A significantly abated the inhibition of proliferation, migration, and invasion compared with co-transfected with control vector and miR-31 mimics.

## Discussion

Cancer development is a complex process which requires transcription and posttranscriptional regulation process. MiRNAs were found playing important roles in the pathogenesis of various cancers. The miR-31 is a novel tumor-related miRNA recently discovered in various cancer[10-14]. But the relationship between miR-31 and osteosarcoma has never been

reported before. In this study, we analyzed the expression pattern of miR-31 in osteosarcoma tissues compared with paired para-tumor bone tissues. We have shown that miR-31 was lower in osteosarcoma tissues compared with paired para-tumor bone tissues. And the results revealed that miR-31 is also lower in osteosarcoma cell lines. Here, our data revealed that the decreased expression of miR-31 was significantly associated with poor 5-year overall survival. Furthermore, miR-31 mimics and miR-31 inhibitor were transfected into osteosarcoma MG-63 cells. As results showed, miR-31 inhibited MG63 cell proliferation, migration, and invasion on osteosarcoma cells. According to prediction, we assume that miR-31 may affect the biological behavior of osteosarcoma partly by affecting the expression of PIK3C2A protein. PIK3C2A is also known as CPK, PI3-K-C2A, and PI3-K-C2 (ALPHA). The protein encoded by this gene belongs to the phosphoinositide 3-kinase (PI3K) family[16]. The PI3K pathway regulates various cellular processes, such as cell growth, proliferation[19], autophagy[20], survival[21], and angiogenesis[22]. In this study, we found miR-31 directly target PIK3C2A, which confirmed our hypothesis. And down-regulation of miR-31 markedly enhanced cell proliferation, migration, and invasion in osteosarcoma cells. Western blotting showed that miR-31 mimic reduced PIK3C2A expression and inhibition of miR-31 increased PIK3C2A expression. Overexpression of PIK3C2A attenuated the miR-31 induced inhibition of proliferation, migration, and invasion in *vitro*, indicating that PIK3C2A promoted osteosarcoma cells proliferation, migration, and invasion.

## Conclusions

We showed that miR-31 was significantly decreased in human osteosarcoma and that down-regulation of miR-31 correlated with poor prognosis. MiR-31 suppressed cell proliferation, migration, and invasion of osteosarcoma by targeting PIK3C2A. Taken together, miR-31 and PIK3C2A can be used as a therapeutic target for osteosarcoma treatment.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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