

# MicroRNA-431-5p Inhibits the Tumorigenesis of Osteosarcoma Through Targeting PANX3

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**Purpose:** This study aimed to evaluate the regulatory role of miR-431-5p on the tumorigenesis of osteosarcoma (OS) and the underlying mechanism involving *pannexin 3* (*PANX3*).

**Methods:** qRT-PCR was applied to measure the expression of miR-431-5p in OS tissues and cells. *PANX3* and miR-431-5p were overexpressed in U2OS and HOS cells. The cell viability and apoptosis were determined by MTT and FITC/PI double staining assay, respectively. Transwell assay was performed to detect cell migration and invasion. The protein expression of cleave-caspase-3 and MMP-2/-9 was detected by Western blot. The target relationship between miR-431-5p and *PANX3* was predicted by ENCORI and identified by DLR assay. The anti-tumor effect of miR-431-5p was further analyzed in a xenograft tumor model in mice.

**Results:** MiR-431-5p expression was down-regulated in OS tissues and negatively correlated with lymph node metastasis and TNM stage. Over-expression of miR-431-5p induced cell apoptosis, inhibited cell proliferation, migration and invasion, up-regulated cleave-caspase-3, and down-regulated MMP-2 and -9 in OS cells. Over-expression of miR-431-5p also inhibited the growth of tumor xenografts in mice. In addition, *PANX3* was a target of miR-431-5p. Over-expression of *PANX3* reversed the anti-tumor effect of miR-431-5p mimics on U2OS and HOS cells.

**Conclusion:** Up-regulation of miR-431-5p suppressed the tumorigenesis of OS via targeting *PANX3*.

**Keywords:** osteosarcoma, microRNA-431-5p, pannexin 3, proliferation, migration

## Introduction

Osteosarcoma (OS) is a severe tumor in bone that commonly occurs in adolescents with a high incidence at the age of 15–19 years.<sup>1,2</sup> Surgical resection, radiotherapy, and chemotherapy are common therapeutic strategies for OS.<sup>3</sup> Although substantial improvements have been made on the treatment of OS, the outcomes of OS cases, especially those with metastasis and recurrence remain poor.<sup>4</sup> Therefore, it is urgently needed to search for novel therapeutic targets for OS.

The emerging role of microRNAs (miRNAs) in controlling key pathways implicated in tumorigenesis makes their use as a powerful novel tool for the early detection, risk assessment and prognosis, as well as for the development of innovative anticancer therapies.<sup>5</sup> Massive miRNAs are involved in the tumorigenesis of OS.<sup>6</sup> A previous study based on gene expression profiling has shown that seven miRNAs are associated with OS, including miR-422a, -145, -194, -93, -125b, -193a-3p, and -140-5p.<sup>7</sup> Some miRNAs have been proved to be down-regulated in OS, such as miR-30a,<sup>8</sup> -33b,<sup>9</sup> -101,<sup>10</sup> -379,<sup>11</sup> -493-5p,<sup>12</sup> -567,<sup>13</sup> and

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-638.<sup>14</sup> These miRNAs exert valuable anti-tumor effect on OS through regulating diverse cellular processes.<sup>11–13</sup> For example, miR-379 and miR-567 can both suppress the proliferation, invasion, and migration of U2OS and MG-63 cells.<sup>11,13</sup> Over-expression of miR-493-5p induces apoptosis and suppresses the viability, migration, and invasion of U2OS cells.<sup>12</sup> MiR-431 is a specific miRNA that is involved in cancer development. MiR-431 is down-regulated in colorectal cancer tissues, and the up-regulation of miR-431 suppresses the migration of HCT116 and SW480 cells.<sup>15</sup> Sun et al have shown that miR-431 is down-regulated in HCC tissues, and miR-431 over-expression suppresses the invasion and migration of HCCLM3 cells.<sup>16</sup> Notably, a miRNA profile of OS patients has found that miR-431-5p is down-regulated in OS.<sup>17</sup> However, the regulatory role of miR-431-5p on OS remains unknown.

The regulatory effects of miRNAs on tumor cells are mainly achieved by modulating specific target genes.<sup>18,19</sup> Many regulatory interactions between miRNAs and target genes in OS have been found by previous studies, such as miR-101 and *ROCK1*,<sup>10</sup> miR-379 and *EIF4G2*,<sup>11</sup> miR-493-5p and *KLF5*,<sup>12</sup> and miR-567 and *FGF5*.<sup>13</sup> Pannexins (PANX1, 2 and 3) are a family of single membrane channel-forming glycoproteins that mainly function in cellular communication and autocrine/paracrine signaling.<sup>20</sup> *PANX3* plays a key role in osteoblast differentiation and osteogenesis.<sup>21</sup> Romano et al have shown that *PANX3* is up-regulated in an axillary sweat gland carcinoma with osteosarcomatous transformation, and high expression of *PANX3* strongly predicts OS.<sup>22</sup> A whole transcriptome analysis has revealed that *PANX3* is up-regulated in OS tissues.<sup>23</sup> However, the regulatory role of *PANX3* and its regulatory relationship with miR-431-5p on OS remain unclear.

Here, the expression of miR-431-5p in OS tissues and cells was determined. The regulatory role of miR-431-5p was evaluated on the proliferation, apoptosis, migration, and invasion of OS cells, and on the growth of tumor xenografts in mice. The regulatory relationship between *PANX3* and miR-431-5p was further investigated. Our study may reveal a promising target for the treatment of OS.

## Materials and Methods

### Tumor Samples

A total of 62 OS patients (33 male and 29 female; 21–78 years old) were screened from our hospital between

January 2016 and January 2019. OS tissues and adjacent normal tissues were collected by surgical resection and were confirmed histopathologically. Patients had not received chemotherapy and/or radiotherapy before resection. This study was approved by the Ethics Committee of The 89th Army Hospital of the Chinese People's Liberation Army in accordance with the Declaration of Helsinki, and informed consents were obtained from all subjects.

### Cell Culture

Four OS cell lines (U2OS, SaOS-2, SJSA-1 and HOS) and one normal human osteoblast cell line (hFOB 1.19) were purchased from BeiNa Chuanglian Biotechnology Research Institute (Beijing, China). Cells were cultured in DMEM containing 10% FBS at 37°C with 5% CO<sub>2</sub>.

### Cell Transfection

The miR-431-5p mimics, mimics negative control (mimics-NC), pcDNA3.1-PANX3 (pc-PANX3), and empty pcDNA3.1 (pcDNA3.1) were obtained from GenePharma (Shanghai, China). Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for cell transfection. Cells were randomly divided into Blank (cells without transfection), miR-431-5p mimics, mimics-NC, mimics-NC + pcDNA3.1, mimics-NC + pc-PANX3, miR-431-5p mimics + pcDNA3.1, and miR-431-5p mimics + pc-PANX3 groups. After 48 h of transfection, the transfected cells were used for the subsequent assays.

### MTT Assay

Cells ( $1 \times 10^4$  cells/well) were seeded into 96-well plates and cultured for 0, 24, 48 and 72 h, respectively. After 4 h of incubation with MTT, DMSO was added to dissolve the formazan crystals. The optical density (OD) at 490 nm was detected by a Microplate Reader.

### FITC/PI Double Staining Assay

Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Thermo Fisher Scientific). Briefly, cells ( $1 \times 10^5$  cells/well) were seeded into 6-well plates and incubated with Annexin V-FITC and PI in the dark for 15 min. The cell apoptosis was monitored by a flow cytometry (BD Biosciences, San Jose, CA, USA) using cytexpert software (V2.0). The apoptotic cells (%) were calculated as cells in the lower right quadrant (early apoptotic cells) + upper right quadrant (late apoptotic cells).

## Transwell Assay

Cells ( $1 \times 10^5$  cells) in FBS-free DMEM were seeded into the upper chamber and DMEM containing 10% FBS was added into the lower chamber. After 24 h of culturing, cells on the lower chamber were stained with 0.1% crystal violet for 20 min. The stained cells (migration cells) were counted under a microscope (Olympus, Tokyo, Japan) at five randomly selected fields. The upper chamber was pre-coated with Matrigel for detecting cell invasion.

## qRT-PCR

Total RNAs were extracted from tissues and cells using TRIZOL and were then transcribed into cDNA. qRT-PCR was performed on a real-time PCR system (ABI 7500) using the following program: 94°C for 5 min, 35 cycles of 94°C for 10 s, 56°C for 30 s and 72°C for 30 s. The relative expression level of specific genes was quantified using the  $2^{-\Delta\Delta Ct}$  method. The internal controls were GAPDH and U6. The primers were synthesized by Sangon Biotech Co, Ltd (Shanghai, China) and the sequences are shown in Table 1.

## Western Blot

The proteins were isolated by lysing cells in RIPA lysis buffer. The proteins were then separated by 10% SDS-PAGE and transferred onto PVDF membrane. After 2 h of blocking with 5% skim milk, the membrane was incubated with primary antibody (anti-PANX3, -cleave-caspase-3, -MMP-2, -MMP-9, -GAPDH; 1:1000, Thermo Fisher Scientific) at 4°C for 12 h. The membrane was then incubated with HRP-conjugated secondary antibody (1:1000, Thermo Fisher Scientific) for 2 h at 25°C. The bands were visualized using ECL kit and quantified by a Gel Imaging System. GAPDH was used as the internal control.

**Table 1** The Primers Used in qRT-PCR

Gene Name	Sequences
miR-23b-3p forward	5'-GAGCATCACATTGCCAGGG-3'
miR-23b-3p reverse	5'-GTGCAGGGTCCGAGGT-3'
U6 forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
U6 reverse	5'-CGCTTCACGAATTTGCGTGTCTAT-3'
PANX3 forward	5'-ATCATCAGCGAACTGGACAAT-3'
PANX3 reverse	5'-AAGTATCGTTCTTCCGAGCCT-3'
GAPDH forward	5'-CACCGTCAAGGCTGAGAAC-3'
GAPDH reverse	5'-GGTGAAGACGCCAGTGGA-3'

## Dual-Luciferase Reporter (DLR) Assay

A binding site of miR-431-5p at 3'-UTR of *PANX3* was predicted by ENCORI. The fragments of *PANX3* containing the predicted binding site (Wt) and mutant site (Mut) were synthesized and inserted into pGL3-promoter vector (PANX3-Wt/PANX3-Mut). U2OS and HOS cells were co-transfected with miR-431-5p mimics/mimics-NC and PANX3-Wt/PANX3-Mut using Lipofectamine 3000 for 48 h. After transfection, cells were lysed in Lysis Buffer. Luciferase Assay Reagent was added into the lysate and the intensity of Firefly luciferase was detected by a Microplate Reader. Subsequently, Stop & Glo Reagent was added and the intensity of Renilla luciferase was detected. The relative luciferase activity was calculated as the ratio of Firefly luciferase/Renilla luciferase.

## Subcutaneous Tumor Xenografts in Mice

Nude mice (BALB/c, male, four-week-old, N = 18) were obtained from SLAC (Shanghai, China) and maintained in a controlled environment at 23–25°C, 50–60% humidity, and 12-h light/dark cycle with free access to food and water. Mice were randomly divided into Blank, mimics-NC, and miR-431-5p mimics groups (N = 6 each group). U2OS cells ( $1 \times 10^7$  cells) transfected with mimics-NC or miR-431-5p mimics were subcutaneously injected into the right flank of mice. Mice injected with non-transfected U2OS cells were considered as the Blank group. The tumor volume was measured every 5 days after the injection using a caliper and calculated using the following formula:  $(A \times B^2)/2$  (A, the longest diameter; B, the shortest diameter). At the 30th day post-injection, mice were anesthetized with pentobarbital sodium (60 mg/kg) and killed by cervical dislocation. The tumor xenografts were removed and weighed. All animal experiments were approved by the Ethics Committee of The 89th Army Hospital of the Chinese people's Liberation Army in accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011, National Institutes of Health, USA).

## Statistical Analysis

The data were expressed as the mean  $\pm$  standard deviation and analyzed using SPSS 22.0 software. The differences among multi-groups were determined by one-way ANOVA followed by Tukey's post hoc test. The differences between two groups were determined by Student's *t* test. The correlation between miR-431-5p

and *PANX3* expression was analyzed by Pearson correlation test. A  $p < 0.05$  was considered to be significantly different.

## Results

### The miR-431-5p Was Down-Regulated in OS

The expression of miR-431-5p was determined in OS tissues and cells. MiR-431-5p expression in OS tissues was significantly lower than that in adjacent normal tissues ( $P < 0.001$ , Figure 1A). MiR-431-5p expression in OS cell lines (U2OS, SaOS-2, SJS-A-1 and HOS) was also significantly lower than that in hFOB 1.19 cells ( $P < 0.001$ , Figure 1B). We then analyzed the correlation between miR-431-5p expression and clinicopathological parameters of OS patients. The results showed that miR-431-5p expression was negatively correlated with the TNM stage and lymph node metastasis ( $P < 0.05$ ) (Table 2).

### Over-Expression of miR-431-5p Inhibited the Malignant Behavior of OS Cells

MiR-431-5p was over-expressed in U2OS and HOS cells by the transfection of miR-431-5p mimics ( $P < 0.001$ , Figure 2A). The cell viability in the miR-431-5p mimics group was significantly decreased compared with that in the mimics-NC group ( $P < 0.05$ , Figure 2B). On the contrary, the apoptosis rate in the miR-431-5p mimics group was significantly increased compared with that in the mimics-NC group ( $P < 0.001$ , Figure 2C). Transwell assay showed that the transfection of miR-431-5p mimics

**Table 2** The Correlation Between the Expression of miR-431-5p and the Clinicopathological Parameters of Patients with OS

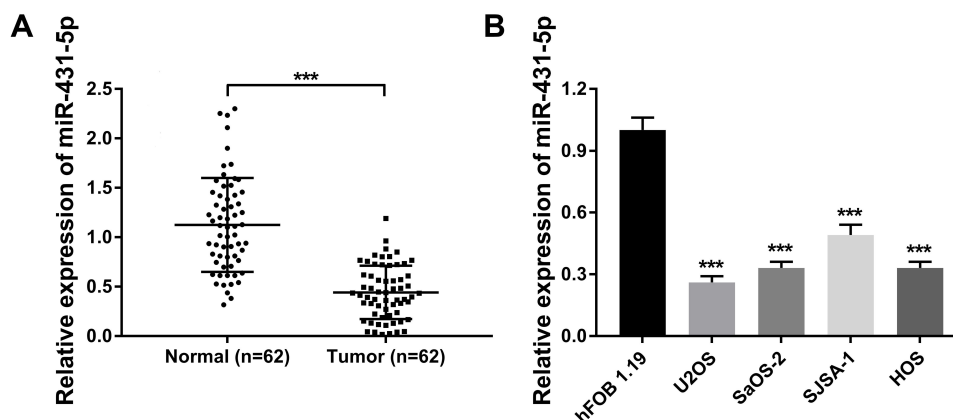
Parameters	Cases	miR-431-5p		$\chi^2$	P
		Low (31)	High (31)		
Age (years)				0.26	0.607
< 60	36	17	19		
≥ 60	26	14	12		
Gender				0.060	0.799
Male	33	17	16		
Female	29	14	15		
Histological grade				0.370	0.544
Well and Moderate	14	6	8		
Poor	48	25	23		
TNM stage				4.35	0.037*
I-II	24	8	16		
III-IV	38	23	15		
Lymph node metastasis				6.22	0.013*
Yes	41	25	16		
No	21	6	15		

**Notes:** Patients with OS were divided into high and low expression group according to the median expression level. \*Represented significantly different at  $P < 0.05$ .

promoted the migration and invasion of U2OS and HOS cells ( $P < 0.001$ , Figure 2D and E).

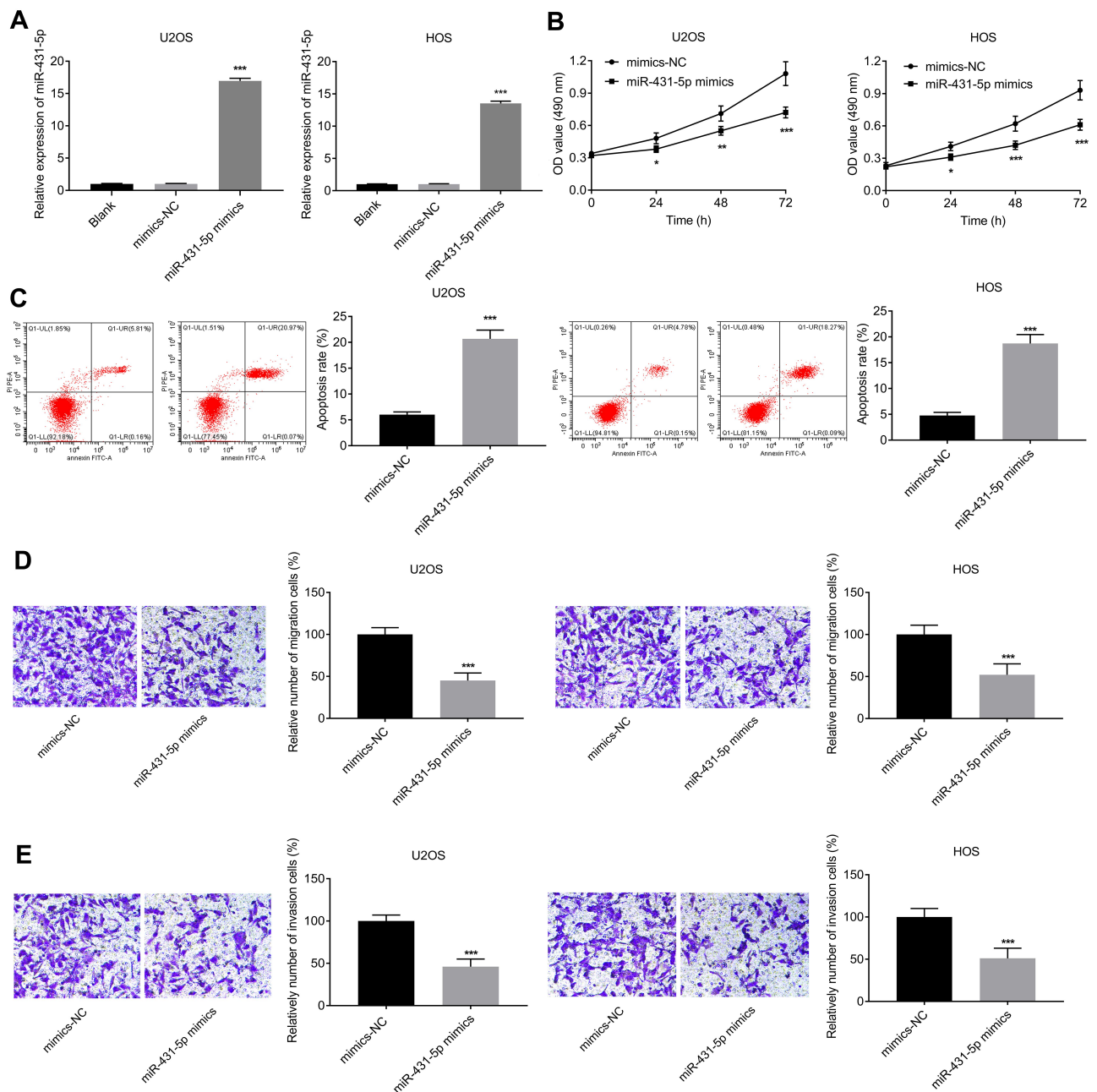
### PANX3 Was a Target of miR-431-5p

A binding site of miR-431-5p was predicted at 3'-UTR of *PANX3* by ENCORI (Figure 3A). The target



**Figure 1** The expression of miR-431-5p in OS tissues and cells. (A) Relative expression of miR-431-5p was detected in OS tissues and adjacent normal tissues by qRT-PCR (N = 62). (B) Relative expression of miR-431-5p was detected in OS cell lines (U2OS, SaOS-2, SJS-A-1 and HOS) and normal human osteoblast cells (hFOB 1.19) by qRT-PCR. \*\*\* $P < 0.001$  vs Normal (A) and hFOB 1.19 (B).

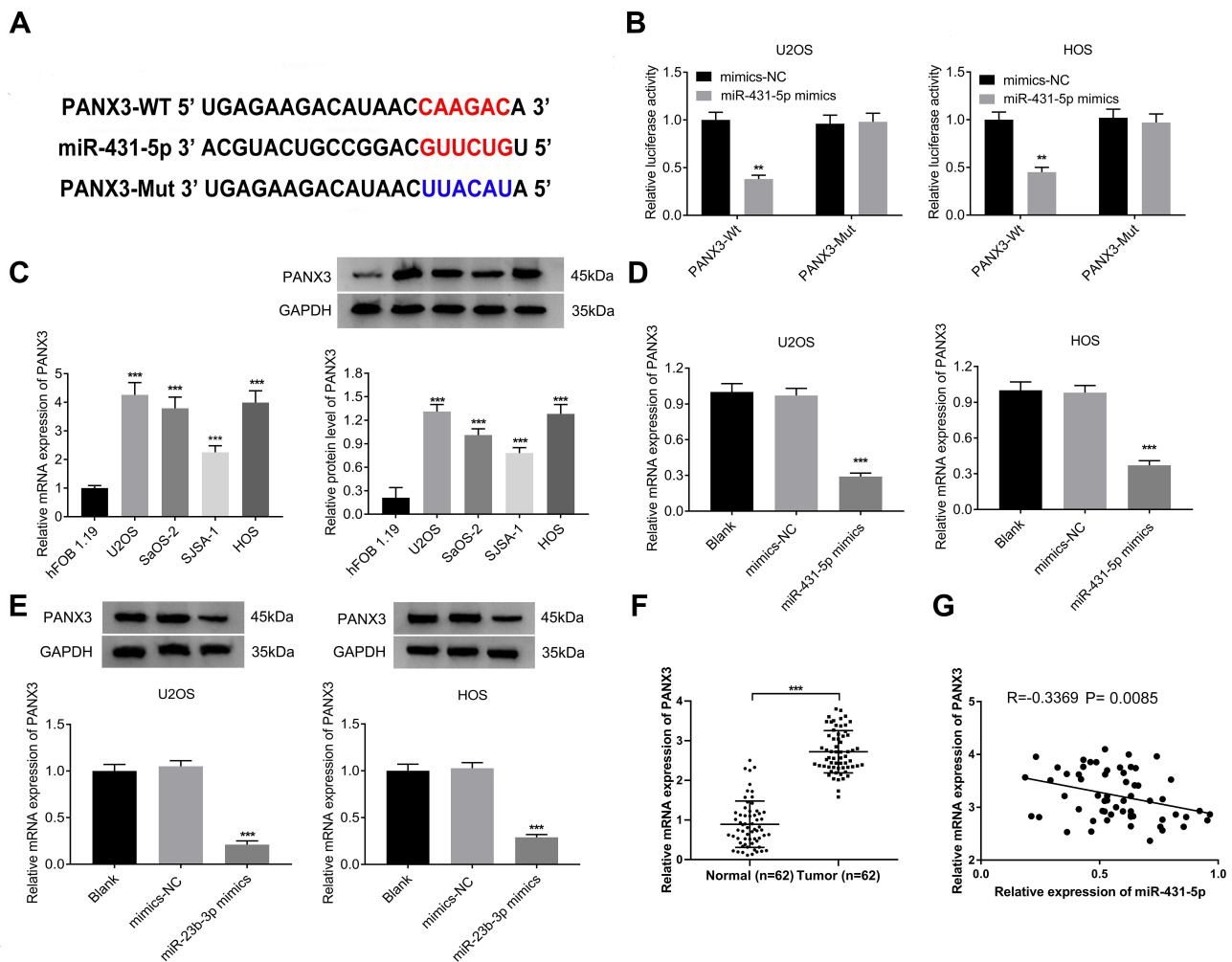




**Figure 2** Over-expression of miR-431-5p inhibited the proliferation, migration, and invasion, and inhibited the apoptosis of OS cells. **(A)** Relative expression of miR-431-5p was detected in U2OS and HOS cells by qRT-PCR. **(B)** The viability of U2OS and HOS cells was detected by MTT assay. **(C)** The apoptosis of U2OS and HOS cells was detected by FITC/PI double staining assay. **(D)** The migration of U2OS and HOS cells was detected by transwell assay. **(E)** The invasion of U2OS and HOS cells was detected by transwell assay. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs Blank **(A)** and mimics-NC **(B-E)**.

relationship between miR-431-5p and *PANX3* was further confirmed by DLR assay ( $P < 0.01$ , Figure 3B). The mRNA and protein expression of *PANX3* in OS cell lines (U2OS, SaOS-2, SJS-1 and HOS) were higher than those in normal human osteoblast cells (hFOB 1.19) ( $P < 0.001$ , Figure 3C). MiR-431-5p mimics significantly decreased the mRNA and protein

expression of *PANX3* in U2OS and HOS cells ( $P < 0.001$ , Figure 3D and E). In addition, the mRNA expression of *PANX3* in OS tissues was higher than that in adjacent normal tissues ( $P < 0.001$ , Figure 3F). Pearson correlation test showed that *PANX3* expression was negatively correlated with miR-431-5p expression in OS tissues ( $P = 0.0085$ , Figure 3G).



**Figure 3** PANX3 was a target of miR-431-5p. (A) A binding site of miR-431-5p at 3'-UTR of PANX3 was predicted by ENCORI. (B) The luciferase activity of U2OS and HOS cells was detected by DLR assay. (C) The mRNA and protein expression of PANX3 were detected in OS cell lines (U2OS, SaOS-2, SJS-A-1 and HOS) and normal human osteoblast cells (hFOB 1.19). (D) The mRNA expression of PANX3 in U2OS and HOS cells was detected by qRT-PCR. (E) The protein expression of PANX3 in U2OS and HOS cells was detected by Western blot. (F) The mRNA expression of PANX3 in OS tissues and adjacent normal tissues was detected by qRT-PCR (N = 62). (G) The correlation between the mRNA expression of PANX3 and miR-431-5p in OS tissues was analyzed by Pearson correlation test. \*\*P < 0.01, \*\*\*P < 0.001 vs mimics-NC (B), hFOB 1.19 (C), Blank (D and E).

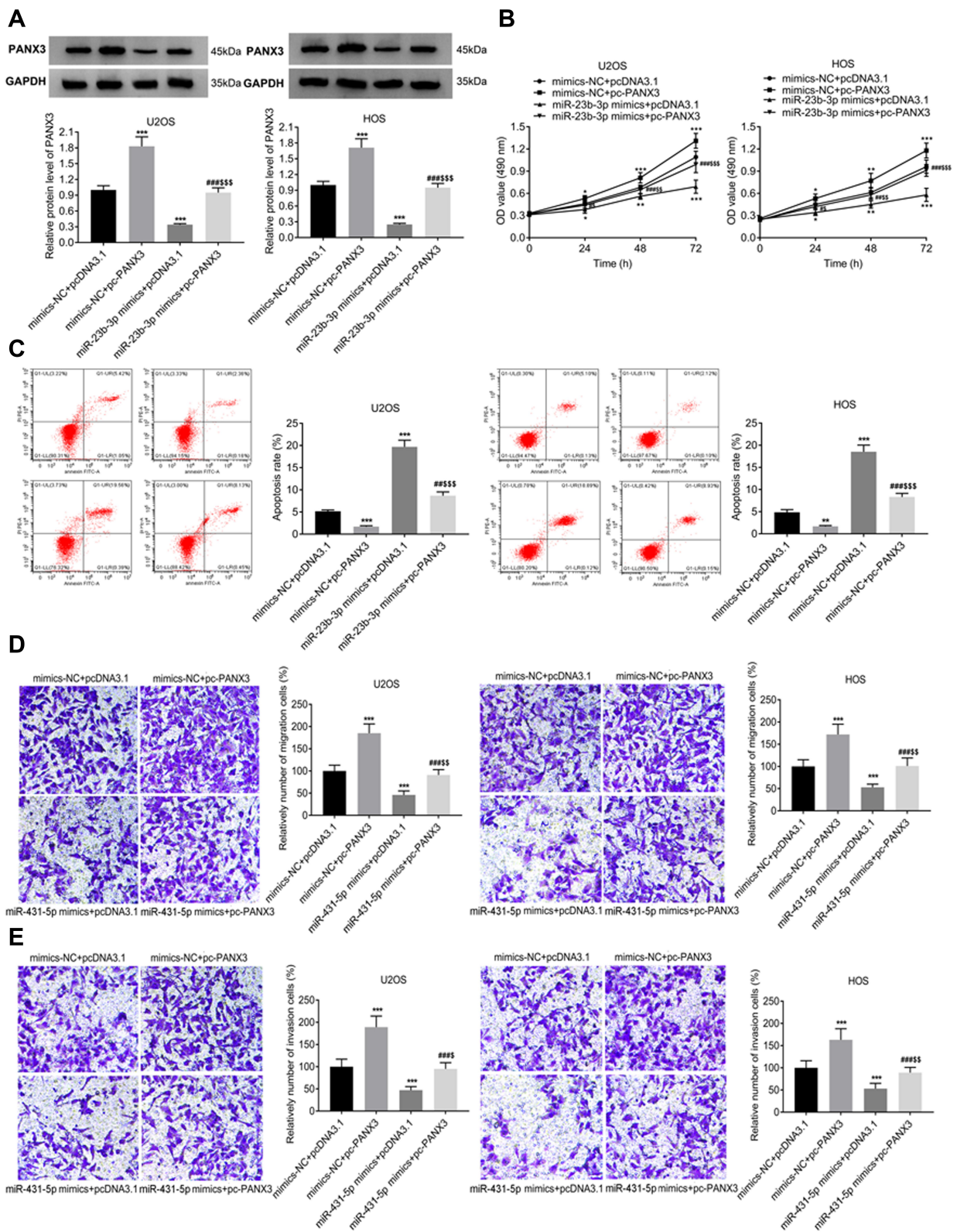
## Over-Expression of PANX3 Reversed the Anti-Tumor Effect of miR-431-5p Mimics on OS Cells

PANX3 was over-expressed in U2OS and HOS cells. The protein expression of PANX3 in the mimics-NC + pc-PANX3 group was increased compared with that in the mimics-NC + pcDNA3.1 group ( $P < 0.01$ ). pc-PANX3 reversed the inhibiting effect of miR-431-5p mimics on the protein expression of PANX3 in U2OS and HOS cells ( $P < 0.01$ , Figure 4A). In addition, the cell viability, migration, and invasion were enhanced in the mimics-NC + pc-PANX3 group ( $P < 0.05$ ). pc-PANX3 reversed the inhibiting effects of miR-431-5p mimics on the viability,

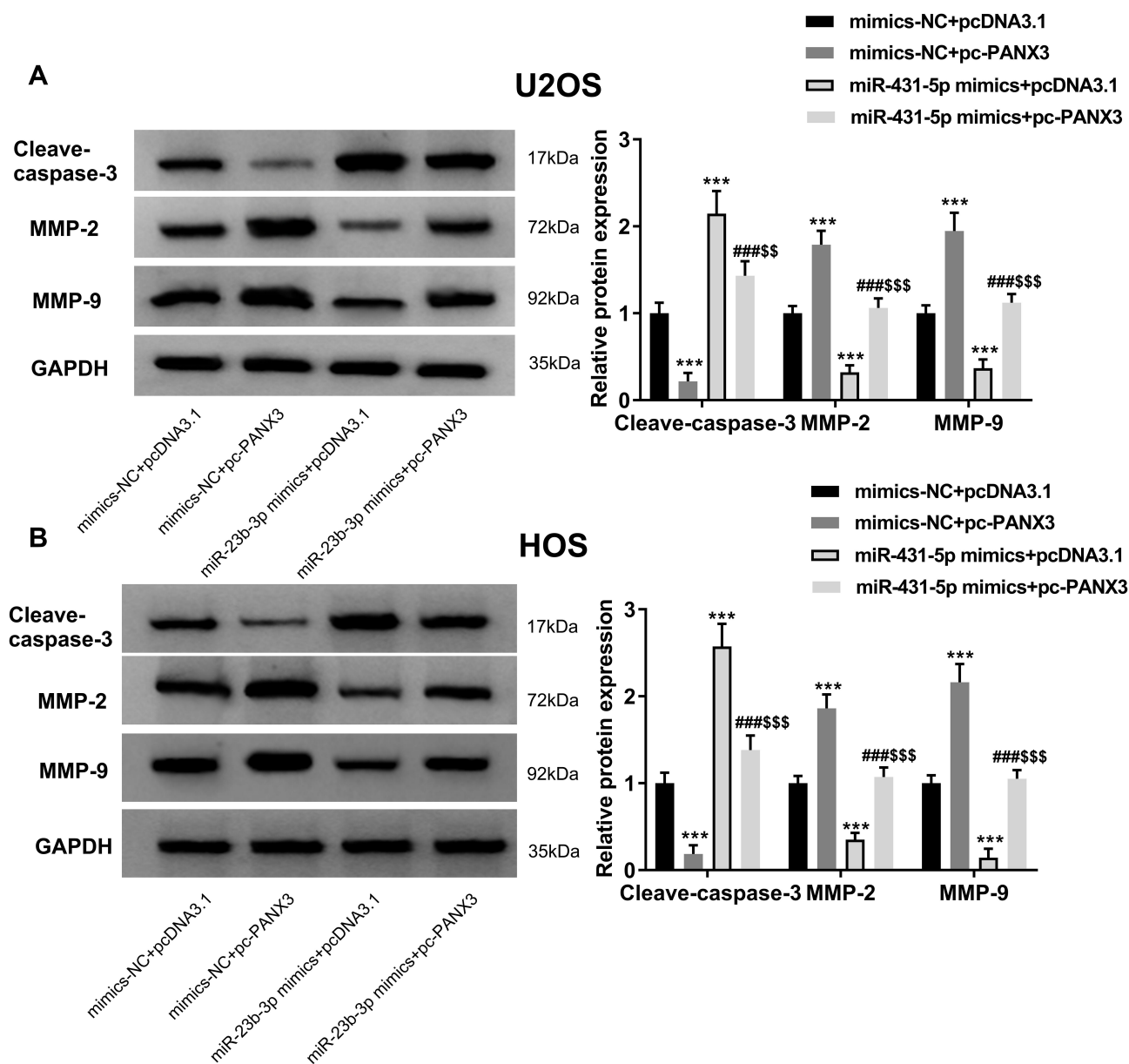
migration, and invasion of U2OS and HOS cells ( $P < 0.01$ , Figure 4B, D and E). On the contrary, pc-PANX3 reduced the apoptosis rate of U2OS and HOS cells, and reversed the promoting effect of miR-431-5p mimics on cell apoptosis ( $P < 0.01$ , Figure 4C).

## Over-Expression of miR-431-5p Up-Regulated Cleave-Caspase-3 and Down-Regulated MMP-2 and -9 in OS Cells via Targeting PANX3

In order to further identify the regulatory role of miR-431-5p on the apoptosis and metastasis of OS cells, the expression of apoptotic marker cleave-caspase-3 and metastasis markers



**Figure 4** Over-expression of PAX3 reversed the anti-tumor effect of miR-431-5p mimics on OS cells. **(A)** The protein expression of PAX3 in U2OS and HOS cells was detected by Western blot. **(B)** The viability of U2OS and HOS cells was detected by MTT assay. **(C)** The apoptosis of U2OS and HOS cells was detected by FITC/PI double staining assay. **(D)** The migration of U2OS and HOS cells was detected by transwell assay. **(E)** The invasion of U2OS and HOS cells was detected by transwell assay. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs mimics-NC+pcDNA3.1; #P < 0.05, ##P < 0.01, ###P < 0.001 vs mimics-NC+pc-PANX3; \$P < 0.05, \$\$P < 0.01, \$\$\$P < 0.001 vs miR-431-5p mimics+pcDNA3.1.



**Figure 5** Over-expression of *PANX3* reversed the regulatory effects of miR-431-5p mimics on the expression of cleave-caspase-3, MMP-2 and -9 in OS cells. (A) The protein expression of cleave-caspase-3, MMP-2 and -9 in U2OS cells was detected by Western blot. (B) The protein expression of cleave-caspase-3, MMP-2 and -9 in HOS cells was detected by Western blot. \*\*\* $P < 0.001$  vs mimics-NC+pcDNA3.1; #### $P < 0.001$  vs mimics-NC+pc-PANX3; \$\$ $P < 0.01$ , \$\$\$ $P < 0.001$  vs miR-431-5p mimics+pcDNA3.1.

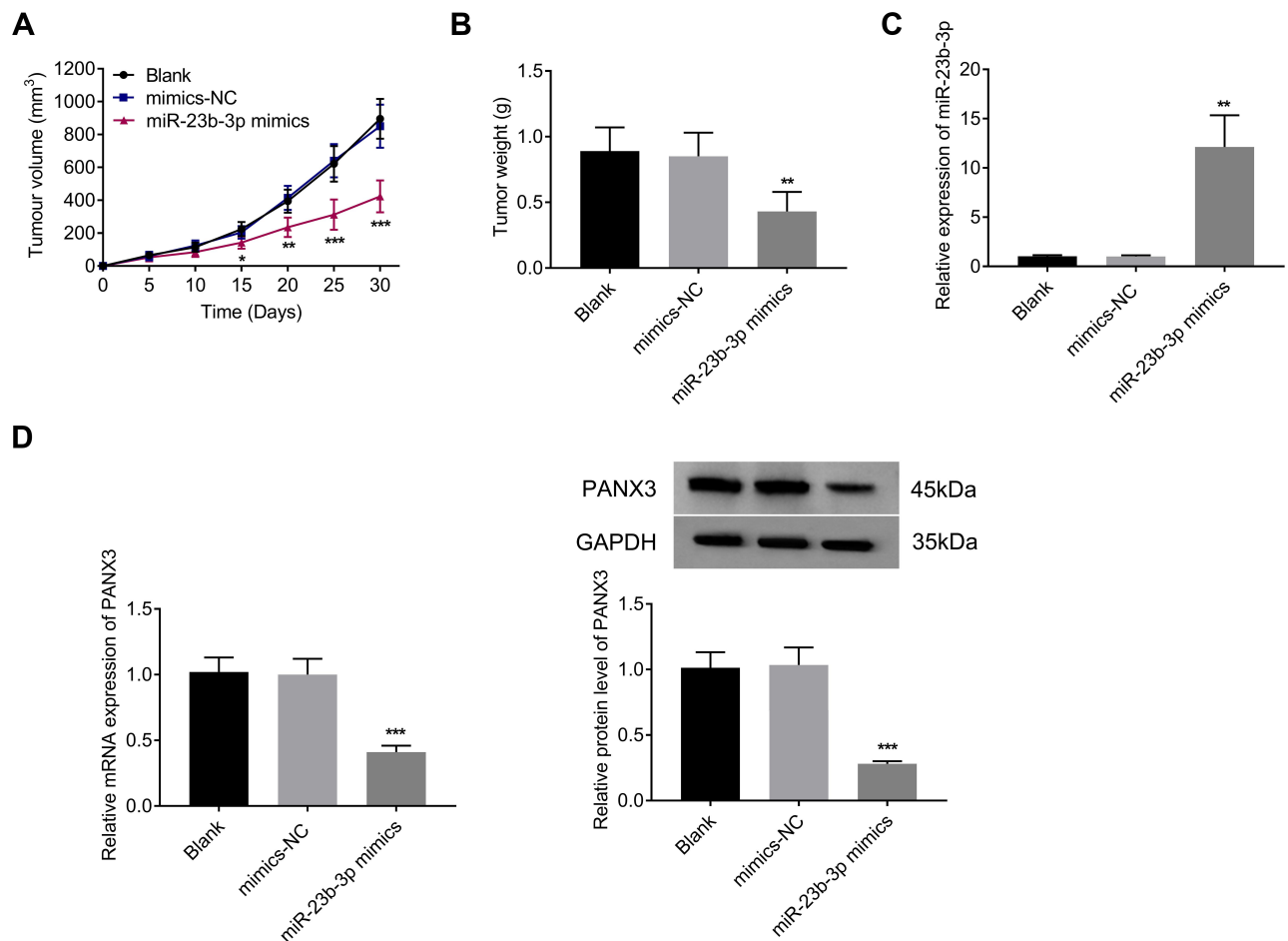
MMP-2 and -9 were detected. As shown in Figure 5A and B, the protein expression of cleave-caspase-3 was significantly increased by the transfection of miR-431-5p mimics in U2OS and HOS cells ( $P < 0.001$ ). Over-expression of *PANX3* significantly reversed the promoting effect of miR-431-5p mimics on the protein expression of cleave-caspase-3 ( $P < 0.01$ ). In addition, the protein expression of MMP-2 and -9 was significantly decreased by the transfection of miR-431-5p mimics in U2OS and HOS cells ( $P < 0.001$ ). Over-expression of *PANX3* significantly reversed the inhibiting

effect of miR-431-5p mimics on the protein expression of MMP-2 and -9 ( $P < 0.001$ ) (Figure 5A and B).

### Over-Expression of miR-431-5p Inhibited the Growth of Tumor Xenografts in Mice

Subcutaneous tumor xenografts in mice were used to identify the anti-tumor role of miR-431-5p in vivo. The tumor volumes in the miR-431-5p mimics group were decreased compared with that in the Blank group beginning from the 15th day ( $P < 0.05$ , Figure 6A). At the





**Figure 6** Over-expression of miR-431-5p inhibited the growth of tumor xenografts in mice. **(A)** Tumor volume (N = 6). **(B)** Tumor weight (N = 6). **(C)** The expression of miR-431-5p was detected in tumor xenografts by qRT-PCR. **(D)** The mRNA and protein expression of PANX3 were detected in tumor xenografts. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs Blank.

30th day post-injection, the tumor weight in the miR-431-5p mimics group was lower than that in the Blank group ( $P < 0.01$ , Figure 6B). Mimics-NC did not significantly influence the growth of tumor xenografts in mice. In addition, miR-431-5p mimics significantly increased the expression of miR-431-5p and decreased the expression of PANX3 in tumor xenografts ( $P < 0.01$ , Figure 6C and D).

## Discussion

MiRNAs are key regulators in the tumorigenesis of OS. Some miRNAs are down-regulated in OS, such as miR-133a,<sup>24</sup> -137,<sup>25</sup> -150,<sup>26</sup> -223,<sup>27</sup> -505,<sup>28</sup> and -564.<sup>29</sup> In consistent with these miRNAs, miR-431-5p was also down-regulated in OS tissues and cells in this study. Our findings indicate that miR-431-5p may be a tumor suppressor in OS. The down-regulation of miRNAs is usually associated with the clinical characteristics of OS patients. Li et al and Qu et al have shown that miR-505 and miR-

150 are both negatively associated with TNM stage and metastasis status in patients with OS.<sup>26,28</sup> Dong et al have shown that miR-223 is negatively related to distant metastasis, advanced stage, and poor survival time in patients with OS.<sup>27</sup> Here, TNM stage and lymph node metastasis were negatively correlated with miR-431-5p expression in OS tumors. The clinical predictive value of miR-223 on OS is consistent with the above miRNAs. MiR-223 may be a potential diagnostic and prognostic marker for OS.

MiRNAs play key roles in the occurrence and progression of OS by regulating cellular processes. For example, miR-567, -192, and -152 can inhibit the proliferation, migration, and invasion of MG-63 and/or U2OS cells.<sup>13,30,31</sup> Here, miR-431-5p was over-expressed in OS cells to identify the regulatory role of miR-431-5p on OS cells. The up-regulation of miR-431-5p promoted the apoptosis and inhibited the proliferation, migration, and invasion of U2OS and HOS cells. Our findings are similar



to the above previous researches, and illustrate that miR-431-5p acts as a tumor suppressor in OS cells. In addition, we also found that the up-regulation of miR-431-5p up-regulated apoptotic marker cleave-caspase-3 and down-regulated metastasis markers MMP-2 and -9 in U2OS and HOS cells. These results further illustrate that miR-431-5p can promote the apoptosis and inhibit the metastasis of OS cells. The anti-tumor effect of miR-431-5p on OS is not only limited at the cellular level but also reflected in animal models. In this study, subcutaneous tumor xenografts were established in mice to identify the anti-tumor role of miR-431-5p *in vivo*. The results showed that the tumor volume and weight were decreased by the miR-431-5p mimics in mice. These findings indicate that the over-expression of miR-431-5p can inhibit the tumor growth *in vivo*.

The anti-tumor effects of miRNAs on OS are always achieved by modulating specific target genes. In this study, *PANX3* was identified as a target of miR-431-5p, and miR-431-5p mimics down-regulated *PANX3* in U2OS and HOS cells. These findings indicate that miR-431-5p negatively regulated its target gene *PANX3* in OS cells. *PANX3* is a member of the gap junction *PANX* family that plays an important role in coordinating tissue homeostasis.<sup>32</sup> Previous studies have revealed that some *PANX* members are involved in the processes of multi-types of cancers. For example, silencing of *PANX1* suppresses the proliferation of malignant glioma cells.<sup>33</sup> Over-expression of *PANX1* inhibits the spheroid formation and growth of rhabdomyosarcoma cells.<sup>34</sup> Loss of *PANX1* attenuates the melanoma progression by reversion to a melanocytic phenotype.<sup>35</sup> In this study, over-expression of *PANX3* inhibited apoptosis and promoted the proliferation, migration, and invasion of U2OS and HOS cells. The function of *PANX3* in OS is similar to that of *PANX1* in glioma and melanoma. We speculate that miR-431-5p may inhibit the tumorigenesis of OS through down-regulating *PANX3*. This speculation was subsequently confirmed by the fact that over-expression of *PANX3* significantly reversed the anti-tumor effect of miR-431-5p mimics on OS cells.

## Conclusions

In conclusion, miR-431-5p was down-regulated in OS tissues and negatively correlated with lymph node metastasis and TNM stage in OS patients. Over-expression of miR-431-5p induced the apoptosis and inhibited the proliferation, migration, and invasion of OS cells through targeting *PANX3*. Over-expression of miR-431-5p also

inhibited the growth of tumor xenografts in mice. MiR-431-5p and its target *PANX3* may be therapeutic targets for OS. However, further studies on the upstream regulatory factors and other target genes of miR-431-5p are still needed.

## Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of The 89th Army Hospital of the Chinese People's Liberation Army in accordance with the Declaration of Helsinki, and informed consents were obtained from all subjects. All animal experiments were approved by the Ethics Committee of The 89th Army Hospital of the Chinese people's Liberation Army in accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011, National Institutes of Health, USA).

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no conflicts of interest to disclose for this work.

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