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MicroRNA-191-5p promotes the development of osteosarcoma via targeting EGR1 and activating the PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: MicroRNA-191 (miR-191) has been reported to be abnormally expressed in human cancers and other diseases. The function of miR-191 was contradictory in different cancers. In the present study, we confirmed the specific function of miR-191-5p in osteosarcoma (OS).

PATIENTS AND METHODS: The effects of miR-191-5p on cellular behaviors of OS cells were investigated through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assay. The quantitative Real-time polymerase chain reaction (qRT-PCR) was applied to examine the expressions of miR-191-5p and early growth response gene 1 (EGR1). Western blot and immunocytochemical assay were used to detect the protein expression of EGR1. The binding relationship between miR-191-5p and EGR1 was confirmed by Dual-Luciferase reporter gene assay. Xenograft tumor formation assay was conducted to examine the *in vivo* effect of miR-191-5p on growth of OS.

RESULTS: MiR-191-5p was significantly upregulated in OS tissues, which was related to poor prognosis of OS patients. Moreover, miR-191-5p promoted cell proliferation, migration and invasion by regulating EGR1 in OS. Furthermore, EGR1 was downregulated in OS tissues, which was associated with poor prognosis of OS patients. MiR-191-5p was found to promote epithelial-mesenchymal transition (EMT) and PI3K/AKT pathway, thus promoting development of OS.

CONCLUSIONS: MiR-191-5p promoted the development of OS by targeting EGR1 and positively regulating PI3K/AKT signaling pathway.

Key words:
EGR1, Osteosarcoma, PI3K/AKT.

Introduction

Osteosarcoma (OS) is a malignant bone tumor characterized by tumor cells directly invading bone or bone marrow. OS mostly occurs in the young adolescent between and 20 years old, which is rarely seen in people above the age of 10 and after 30[2]. Moreover, OS incidence between males and females is 3:2. Currently, comprehensive treatments based on high-dose chemotherapy and surgery are widely applied[4]. The five-year survival rate of OS patients treated with standardized treatment is up to 70% and 90% of OS patients can retain their limbs[5]. Even all this, OS still has a high mortality rate among children and adolescents. Early diagnosis and timely treatment have greatly improved the survival rate of OS[6]. In particular, early diagnosis is also closely related to the prognosis of OS. Therefore, it is necessary to develop efficient biomarkers for the early diagnosis of OS. In recent years, MicroRNAs (miRNAs) have been identified as promising biomarkers involved in human cancers and other diseases[7]. Moreover, alternations of miRNA expressions have been reported to associate with pathogenesis and progression of OS[8]. For instance, miR-182 inhibits proliferation and promotes apoptosis in human OS cells by targeting HOXA9[9]. Besides, upregulated miR-148a in osteosarcoma promotes cell growth by targeting PTEN (gene of phosphate and tension homology deleted on chromosome ten)[10]. In various miRNAs, alternation of miR-191 expression has been widely reported, which is contradictory in different human cancers. MiR-191 is upregulated and functioned as an oncogenic regulator in human hepatocellular carcinoma[11], breast cancer[12] and colorectal cancer[13]. Inversely, the downregulation of miR-191 has been examined in renal cell carcinoma[14] and thyroid follicular tumors[15]. Those studies indicated that miR-191 might play an important role in the biology of human cancers. Wang et al[16] demonstrated that increased expression of miR-191 could act as a potential serum biomarker for diagnosis and prognosis in

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human OS. Therefore, we further investigated the specific functions of miR-191-5p related with the progression of OS. Early growth response gene 1 (EGR1) belongs to the EGR family of transcription factors, which has a high homologous with EGR2, EGR3, and EGR4[17]. Abnormal expression of EGR1 has been found in the progression of human diseases and cancers[18]. However, the effect of EGR1 varies in different cancers. Zheng et al[19] proposed that EGR1 is upregulated in gastric cancer which promotes tumor invasion and metastasis. EGR1 is capable of promoting growth and survival of prostate cancer cell[20]. On the contrary, the downregulation of EGR1 has been identified in breast cancer cells[21]. Li et al[22] indicated a crucial role of EGR1 in the transcriptional regulation of miR-20b in breast cancer. However, the interaction between EGR1 and miR-191-5p in OS is still unclear and need to be clarified. In the present work, miR-191-5p expression in OS tissues and cells was examined. The regulatory effects of miR-191-5p on cell proliferation, migration and invasion in OS, as well as epithelial-mesenchymal transition (EMT) and PI3K/AKT signaling pathway were evaluated. Then, we verified the relationship between miR-191-5p and EGR1 in OS. We hope these findings could contribute to better understanding the pathogenesis of OS and benefit the early diagnosis of OS.

Patients and Methods

Clinical Tissues

63 human OS tissues and adjacent samples were acquired from the First Affiliated Hospital of the Zhejiang University after receiving signed informed consent. None of the patients received any treatment before the operation. Tissues were frozen in liquid nitrogen and then stored in -80°C refrigerator for subsequent experiments. This study was approved by the Institutional Ethics Committee of The First Affiliated Hospital of Zhejiang University.

Cell Culture

OS, MG63, Saos2 cell lines and human fetal osteoblastic cell line hFOB1.19 were used for the experiment. These cell lines were provided by the Institutes for Biological Sciences (Shanghai, China). Cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and cultured at 37°C with 5% CO_2.

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied for extracting total RNA according to the standard method. Synthesis of complementary deoxyribose nucleic acid (cDNA) was performed by the PrimeScript Reverse Transcriptase (TaKaRa, Otsu, Shiga, Japan) based on the manufacturer's instructions. Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) was carried out through the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on ABI 7500. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-191-5p and EGR1. The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: EGR1: 5'-GAGCGAACCGGGTCCTTAC-3', R: 5'-GTCTAGATGGGTTCCGTGGA-3'; miR-191-5p: 5'-CTGGTGCAGGGTCCGAGGTATTCGCACCAGCAGCACATCCTCCTG-3', R: 5'-ACCATCGTGTCGTTTCAAGG-3'; U6: F: 5'-CTCGCTTCGGCAGCACATATACTAAAAT-3', R: 5'-ACGCTTCACGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATACGACCAAATCCGTTGACTCCGACCTTCAC-3'.

Cell Transfection

miR-191-5p mimic or inhibitor, miR-191-5p plasmid and negative control (NC) were obtained from Ribobio (Guangzhou, China). MG63 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] Assay

Transfected cells were cultured in 96-well plates with 2×10^3/well and incubated for 24, 48, 72 h and 96 h. 20 µL MTT solution (Thermo Fisher Scientific, Waltham, MA, USA) was applied per well and incubated for 4 h at 37°C. Finally, the absorbance at 490 nm (OD=490 nm) was detected with a spectrophotometer.

Transwell Assays

Transwell chambers (8-µm pore size membranes) were employed to measure the abilities of cell migration and invasion. The bottom chamber was added with 10% FBS and incubated at 37°C with 5% CO_2. The upper surface pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell invasion. Cell migration assay was conducted without Matrigel pre-coating. 2×10^4 cells were cultured in the bottom cham-

ber with serum-free medium. 24 h later, the migrated or invasive cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of migrated or invasive cells using Image J software (NIH, Bethesda, MD, USA).

Western Blot Analysis

The protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated through a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skim milk, the membranes were incubated with primary antibodies of E-cadherin, N-cadherin, vimentin and AKT, p-AKT, GAPDH primary antibodies (1:1000; Abcam, Cambridge, MA, USA) overnight at 4°C. After washing, they were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:3000; Santa Cruz Biotechnology, Dallas, TX, USA). Then, the protein expression levels were measured by electrochemiluminescence (ECL, Pierce, Waltham, MA, USA).

Dual Luciferase Assay

The wild or mutant type of EGR1 3'-untranslated region (3'-UTR) were inserted into the pmirGLO Luciferase vector (Promega, Madison, WI, USA) to perform Luciferase reporter experiments. Then, wild or mutant type 3'-UTR and miR-191-5p mimics were transfected into MG63 cells. Subsequently, the Dual-Luciferase Assay System (Promega, Madison, WI, USA) was applied to detect luciferase activity.

Immunohistochemical Assay

The section of OS tissues was dewaxed, hydrated and washed twice with Phosphate-Buffered Saline (PBS, Gibco, Grand Island, NY, USA) for 5 min. After blocking with 5% goat serum diluted with PBS), sections were incubated with anti-EGR1 (1:100) antibody at 37°C for 1-2 h. After PBS washing three times, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 1 h. After washing 3 times with PBS, diaminobenzidine (DAB) mixture (Abcam, Cambridge, MA, USA) was used for color development of the section. The section was washed, counterstained, dehydrated, transparentized and mounted. Images were captured using a microscope.

Xenograft Tumor Formation Assay

Nude mice (4 weeks old) were provided by the Shanghai Lab Animal Research Center (Shanghai, China). All animal researches were approved by the Animal Care and Use Ethics Committee of Zhejiang University Animal Center. 1st, 3×10^6 cells transfected with pre-miR-191-5p plasmid or negative control were injected into the right hind flank of nude mice. The tumor volumes were observed every 3 days after 7-day incubation. 4 weeks later, mice were sacrificed by CO_2 asphyxiation and tumors were harvested for further studies.

Statistical Analysis

The data were analyzed using Statistical Product and Service Solutions (SPSS 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, CA, USA). Data were shown as mean ± SD analyzed by Student's *t*-test. The relationship between miR-191-5p expression and clinic-pathological features of OS patients was analyzed by χ^2 test. Kaplan-Meier analysis was applied to draw the survival curve, and the log-rank test was used to compare the survival differences. A significance was defined at $p<0.05$.

Results

MiR-191-5p Was Upregulated in OS Tissues

Primarily, miR-191-5p expression in OS tissues was detected by qRT-PCR. We found that miR-191-5p was markedly upregulated in OS tissues in comparison with normal tissues (Figure 1A). Moreover, we also analyzed the relationship between miR-191-5p expression and their clinic-pathological characteristics of OS patients. As shown in Table I, the expression of miR-191-5p was positively correlated to TNM stage ($p=0.034$). In addition, we also identified that miR-191-5p expression was negatively related to prognosis of OS patients ($p=0.022$, Figure 1B). Based on those results, we considered that miR-191-5p might be involved in the pathogenesis of OS and predict its prognosis.

MiR-191-5p Promoted Cell Proliferation, Migration and Invasion in OS

MiR-191-5p expression in U2OS, MG63, Saos2 and hFOB1.19 cell lines was detected. Similarly, miR-191-5p was also upregulated in U2OS, MG63, Saos2 cells contrast to that of hFOB1.19 cells (Fig-

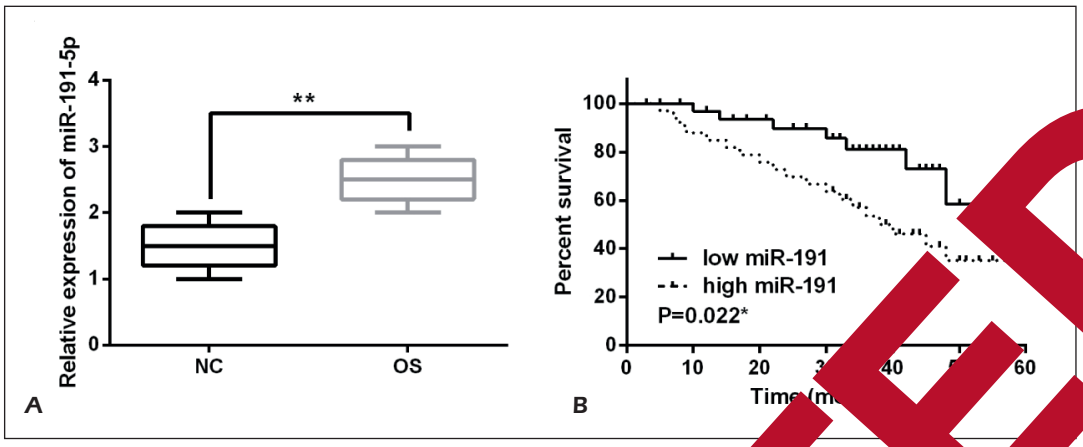


Figure 1. MiR-191-5p was upregulated in OS tissues. **A**, The expressions of miR-191-5p in OS tissues detected by qRT-PCR. **B**, Lower miR-191-5p expression was related to longer overall survival in OS patients.

ure 2A). Next, transfection efficacy of miR-191-5p mimics and inhibitor in MG63 cells was verified by qRT-PCR. As shown in Figure 2B, miR-191-5p expression was significantly enhanced by transfection of miR-191-5p mimics, and was reduced in MG63 cells transfected with miR-191-5p inhibitor. MiR-191-5p overexpression promoted the proliferation of MG63 cells, while miR-191-5p knockdown inhibited proliferative potential (Figure 2C, D). Similarly, cell migration was enhanced by miR-191-5p overexpression (Figure 2E, F), consistently, miR-191-5p promoted cell invasion as well (Figure 2G, H). MiR-191-5p was found to promote cell proliferation, migration and invasion in OS.

EGR1 Was a Direct Target of miR-191-5p in OS Cells

Further, EGR1 was predicted as a target gene of miR-191-5p in the database of TargetScan (http://www.targetscan.org) (Figure 3A). Then, we conducted Luciferase reporter gene assay to confirm that. Consistent with the prediction, we observed that Luciferase activity was reduced in MG63 cells co-transfected with miR-191-5p

Table I. Relationship between miR-191-5p expression and their clinic-pathological characteristics of OS patients.

Characteristics	C	miR-191-5p		*p*-value
		High	Low	
Age (yea				0.332
≥20	35	20	15	
<2	28	18	10	
G				0.651
M	33	23	10	
Fem	30	15	15	
or size				0.218
m	38	25	13	
m	25	13	12	
				0.034*
	23	11	12	
III-IV	40	27	13	
mph node metastasis				0.339
	30	16	14	
es	33	22	11	

Statistical analyses were performed by the χ^2 test.
*$p<0.05$ was considered significant.

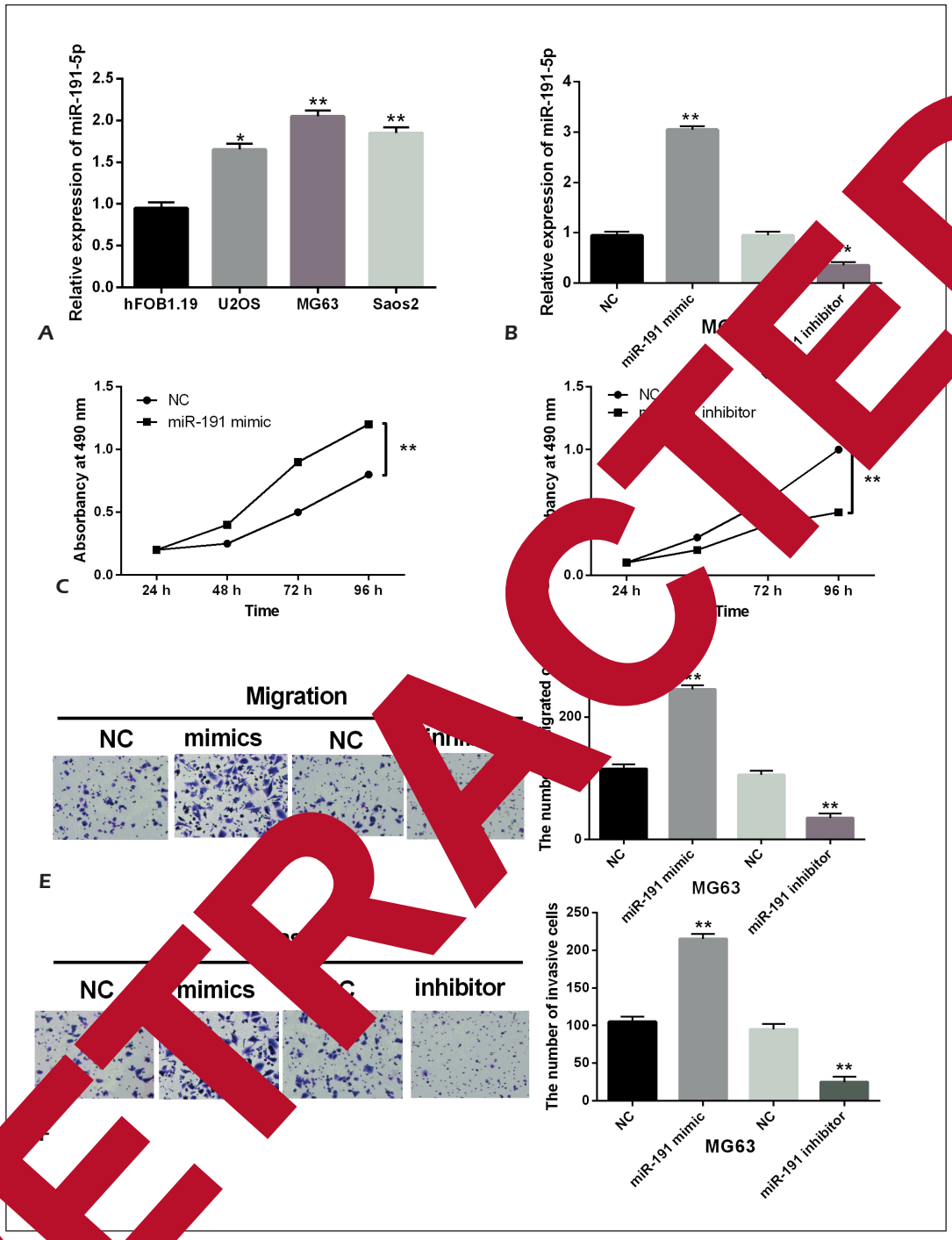


Fig. 2. MiR- omoted cell proliferation, migration and invasion in OS. **A**, MiR-191-5p expression in U2OS, MG63, Saos2 and B1.19 cell l . **B**, MiR-191-5p expression was examined in MG63 cells with miR-191-5p mimics or inhibitor via qRT-PCR. **C** roliferation was measured in cells containing miR-191-5p mimics or inhibitor via MTT assay. **E-F**, Cell migration and n cells containing miR-191-5p mimics or inhibitor was detected by transwell assay. *p<0.05, **p<0.01.

and EGR1-Wt vector. However, the Luciferase activity of EGR1-Mut was not changed by transfection of miR-191-5p mimics (Figure 3B). Furthermore, the expression level of EGR1 was examined to be negatively correlated with miR-191-5p expression in OS tissues (p<0.05, R^2=0.331; Figure 3C). Moreover, mRNA expression of EGR1 was declined by transfection of

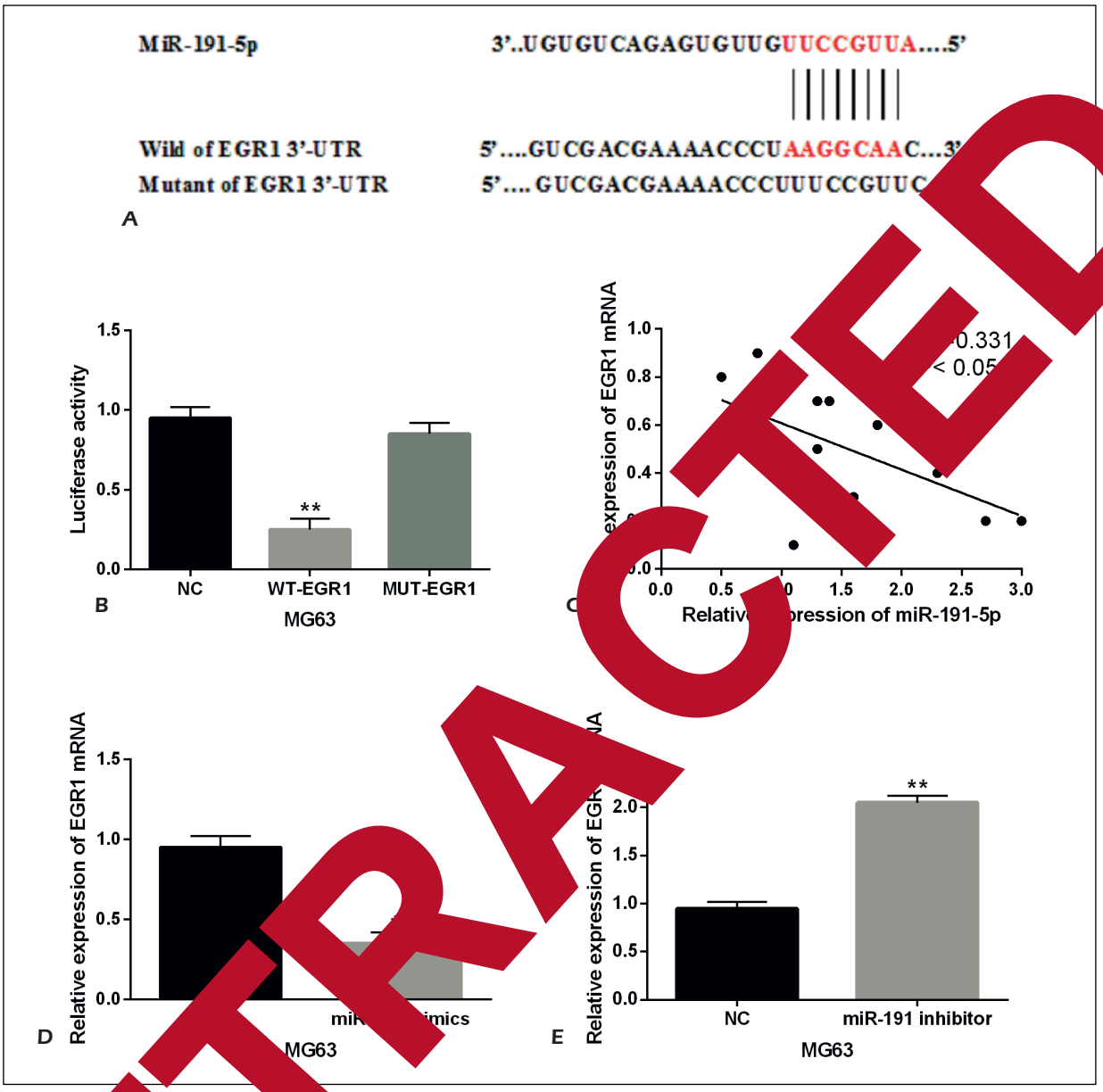


Figure 3. EGR1 was a direct target of miR-191-5p in OS cells. **A**, The binding sites of miR-191-5p on the 3'-UTR of EGR1. **B**, Luciferase reporter assay. **C**, The correlation between miR-191-5p and EGR1. **D-E**, The expression of EGR1 were observed in MG63 cells containing miR-191-5p mimics or inhibitor **$p<0.01$.

miR-191-5p into MG63 cells (Figure 3D) and decreased after transfection of miR-191-5p inhibitor (Figure 3E). Therefore, miR-191-5p was identified to directly target EGR1 and had a negative association with EGR1.

EGR1 was Downregulated in OS Tissues

Subsequently, EGR1 expression was analyzed in OS tissues. IHC showed a positive detection of EGR1 protein expression in the cell nucleus of OS tissues (Figure 4A). Moreover, the protein expression intensity of EGR1 significantly decreased in OS tissues in comparison with the adjacent normal tissues (Figure 4B). Besides, high EGR1 expression was found to be correlated with good prognosis of OS patients (p=0.043, Figure 4C). EGR1 was suspected to participate in the tumorigenesis of OS and miR-191-5p might promote the development of OS *via* targeting EGR1.

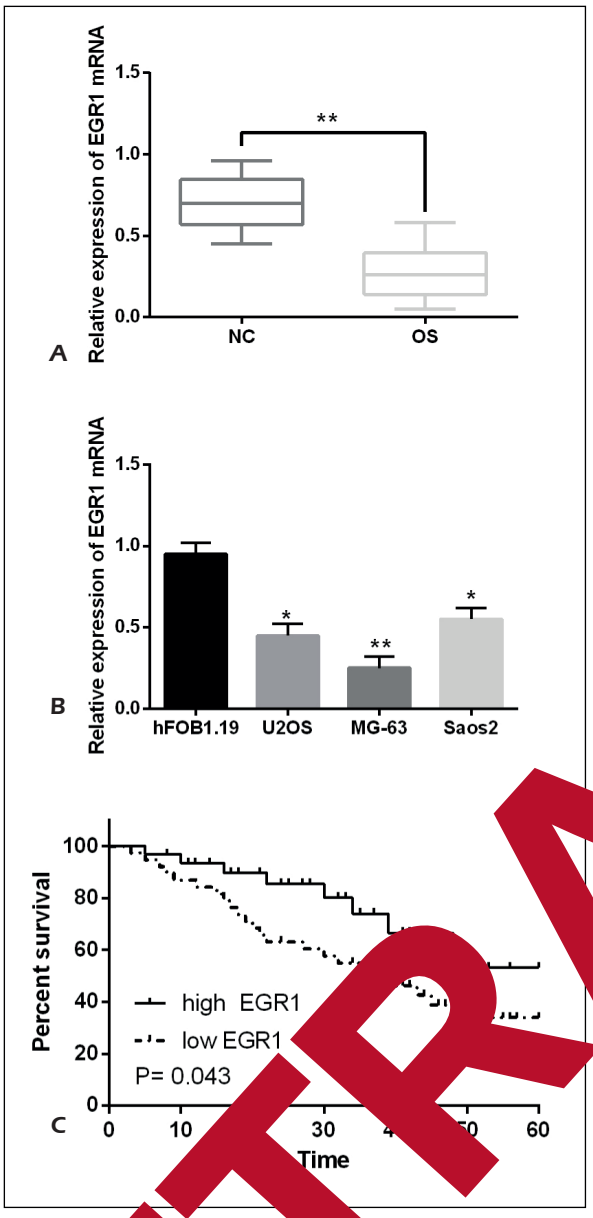
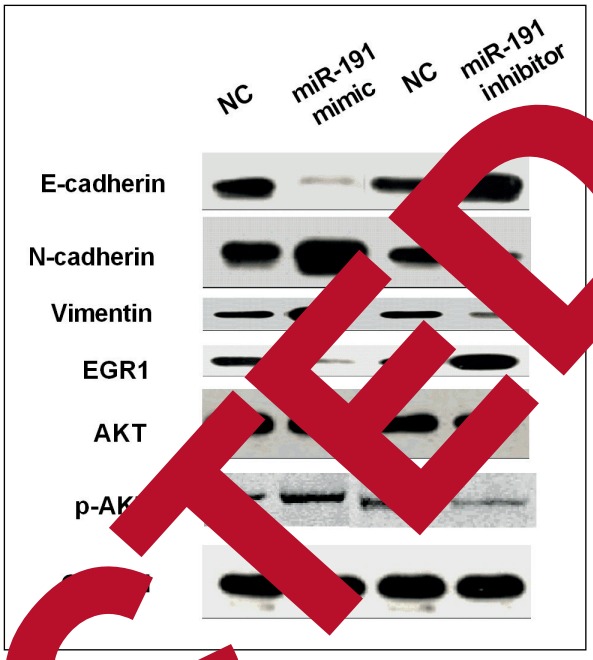


Figure 4. ... was downregu... OS tissues. **A-B**, The protein e... sion of EGR1 in OS t... detected by immunohist... istry. **C**, ...igh EGR1 expression was related to lon... erall su... in OS patients. **$p<0.01$.



...re 5. MiR-191... romoted EMT and AKT pathway in C... estern blot ana... of E-cadherin, N-cadherin, Vimentin... and p-A... MG63 cells contained miR-191-5p mimi...

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...re, Western blot showed that the up-...gulation of miR-191-5p suppressed E-cadherin ...ession and promoted expressions of N-cadh... nd Vimentin (Figure 5). On the contrary, the downregulation of miR-191-5p inhibited expressions of N-cadherin and Vimentin, but enhanced E-cadherin expression level (Figure 5).

...re, we considered that miR-191-5p over-...p... on promoted cell metastasis by regulating EMT. Moreover, we observed the protein expressions of AKT and p-AKT in MG63 cells transfected with miR-191-5p mimics or inhibitor to verify whether miR-191-5p regulated the PI3K/AKT signaling pathway in OS. Western blot showed that increased miR-191-5p expression apparently promoted expressions of AKT and p-AKT in MG63 cells (Figure 5). Inversely, decreased miR-191-5p expression inhibited expressions of AKT and p-AKT expression (Figure 5). MiR-191-5p was considered to promoted EMT and PI3K/AKT pathway in OS.

MiR-191-5p Promoted the Tumor Growth in vivo

Finally, we subcutaneously injected MG63 cells containing miR-191-5p stable transfection plasmid or miR-NC into nude mice. As shown in Figure 6A, the overexpression of miR-191-5p markedly enlarged the tumor volume compared with the control group. In Figure 6B, we also found the tumors with miR-191-5p stable transfection plasmid grew more quickly than that with miR-NC. These findings showed that miR-191-5p promoted the tumor growth of OS *in vivo*.

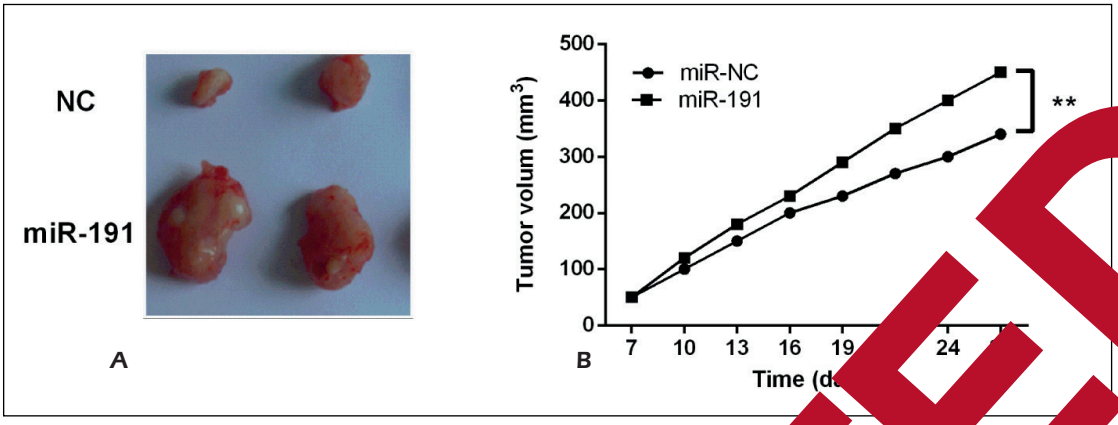

Figure 6. MiR-191-5p promoted the tumor growth *in vivo*. **A**, Tumor si... Cell grow... <0.0...

Discussion

In recent years, increasing miRNAs have been reported to associate with the pathogenesis of human cancers. MiRNAs could serve as therapeutic targets in human cancers[23]. To date, more and more miRNAs have been proposed to regulate different biological processes of OS. For example, mi...26 was found to inhibit proliferation, migratio... sion and EMT in OS by targeting ZEB1[24]. Ji... [25] demonstrated that miR-140 suppressed OS t... growth by enhancing anti-tumor immune respo... and blocking mTOR signaling. M...r, miR-2... was reported to predict poor ...gnos... OS pa... tients[26]. However, further i...tigation ...ill need to be done to verify the ...f miR... OS. Here, we found that mi...191... ...egulat...n OS tissues and cell l... The up... ...tion of miR-191-5p was identi... promote c... ...liferation, migration and i... well as EM... ...d PI3K/AKT pathway ...OS.

MiR-1... ...as been fo... ...o act as a tumor promot... regulating the ...-p53 pathway in intra... ...tic ch... ...giocarcinoma[27]. Same as the ab... ...udy, ...also id...ntified the carcinogenesis o... ...5p in ... In addition, Shi et al[28] ...icate... miR-... ...was upregulated in human g... carc... ...hich was similar to our result in this w... Moreover, the overexpression of ...191 has also been demonstrated to predict ... p... ...s and promote proliferation and invasion in esophageal squamous cell carcinoma[29]. ...e current research, we found that the upregula... of miR-191-5p was related to poor prognosis of OS patients. Besides, Huang et al[30] proposed that miR-191 promoted OS cells proliferation by suppressing Chk2. Our work showed that miR-191-5p promoted th... ...liferation of OS cells *via* ...g EGR1. Li... [31] demonstrated that m... ...91 targeted EGR1 a... ...d suppressed intimal ...kening after c... ...otid injury. We also confirmed ... EGR1 was a... ...ect target of miR-191-5p and p... ...pated in t... ...rogression of OS.

... et ... ...dicated that the specific function o... ...as sophisticated in the pathogenesis of human cancers and diseases, which could ... as tumor suppressor-gene or oncogene in ... cancers. EGR1 has been reported to promote cell growth and survival in prostate cancer. On the contrary, the suppressive function of EGR1 has been identified in several cancers and diseases. For instance, miR-183 was found to function as an oncogene *via* targeting EGR1 to promote tumor cell migration[32]. In our research, we found that miR-191-5p promoted the migration of OS cells *via* targeting EGR1. In addition, miR-301b has been identified to promote the proliferation and EMT of bladder cancer cells by targeting EGR1[33], which was consistent with our findings. EGR1 served as a direct target that was downregulated in acute lymphoblastic leukemia[34], Alzheimer's disease[35] and B cell lymphomas[36]. In OS, the downregulation of ERG1 was also examined and a negative correlation between EGR1 and miR-191-5p was detected in OS tissues. Therefore, we considered that miR-191-5p promoted the development of OS *via* targeting EGR1.

Conclusions

Increased expression of miR-191-5p was identified to be related to poor prognosis of OS patient. Moreover, miR-191-5p promoted cell proliferation, migration and invasion in OS by inhibit-

ing EGR1 expression. MiR-191-5p also promoted EMT and PI3K/AKT signaling pathway in OS. Besides, miR-191-5p promoted OS tumor growth *in vivo*. These findings would help us develop novel diagnostic and therapeutic approaches of OS.

Conflict of Interests

The authors declare that they have no conflict of interest.

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