European Review for Medical and Pharmacological Sciences 2020; 24: 9265-9273

MicroRNA-150-5p inhibits proliferation and invasion of osteosarcoma cells by down-regulating VEGFA

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether microRNA-150-5p was involved in osteosarcoma cell proliferation and invasiveness via modulating vascular endothelial growth factor A (VEGFA) expression.

PATIENTS AND METHODS: 10 pairs of osteosarcoma tissues and para-cancerous tissues were collected from patients with osteosarcoma in our center from February 2012 to July 2018. Relative expression levels of microRNA-150-5p and VEGFA in tissues and cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Luciferase reporter gene assay was conducted to illustrate the binding interplay between microRNA-150-5p and VEGFA. Furthermore, proliferative and invasive potentials in HOS and MG-63 cells regulated by both microRNA-150-5p and VEGFA were determined using Cell Counting Kit-8 (CCK-8), colony formation assay, and transwell assay, respectively.

RESULTS: MicroRNA-150-5p was remarkably downregulated, while VEGFA was upregulated in osteosarcoma tissues compared with para-cancerous tissues ($p<0.05$). Similar results were observed in osteosarcoma cells and normal osteoblasts. Overexpression of microRNA-150-5p significantly inhibited the proliferation and invasion of osteosarcoma cells ($p<0.05$). Luciferase reporter gene assay demonstrated that microRNA-150-5p could target to VEGFA to negatively modulate its expression. In addition, the knockdown of VEGFA remarkably weakened osteosarcoma cell proliferative and invasive capacities ($p<0.05$).

CONCLUSIONS: MicroRNA-150-5p weakens proliferative and invasive potentials in osteosarcoma cells by downregulating VEGFA level. All our findings suggest that microRNA-150-5p/VEGFA axis is a promising target for osteosarcoma treatment.

Key Words:
MicroRNA-150-5p, VEGFA, Cell proliferation, Cell invasion.

Introduction

Osteosarcoma is a kind of malignant tumor that is likely to occur in the skeletal system of children and adolescents. It is originated from mesenchymal tissue, accounting for 35% of malignant bone tumors. Osteosarcoma is most likely to occur in the metaphysis of long tubular bone, such as proximal tibia and distal femur, with high degree of malignancy and invasion ability[1]. Therefore, early diagnosis and treatment of osteosarcoma are difficult[2]. At present, surgery and chemotherapy are the main treatment methods for osteosarcoma. Statistics have shown that the 5-year survival of osteosarcoma patients is on the rise with the continuous improvement of medical technology[3]. However, due to the tendency of early lung metastasis and drug resistance to chemotherapy, the overall prognosis of patients with osteosarcoma is still far from satisfactory[4,5]. Currently, the diagnosis of osteosarcoma is mainly based on clinical symptoms and imaging examination, and there is a lack of clinical indicators for early diagnosis and prognosis evaluation. Therefore, there is an urgent need to find new and effective tumor markers for early diagnosis and treatment of osteosarcoma.

MicroRNA (miRNA) is a kind of non-coding, small-size RNA with about 22 nucleotides in length. By recognizing and complementary base pairing with the mRNA 3'untranslated region (3'UTR), miRNA leads to its degradation or translation inhibition. This may eventually mediate target mRNA expressions and their biological functions in cellular behaviors[6,7]. Some studies have confirmed that increasingly more miRNAs participate in the regulation of malignant tumors, including miR-144[8], miR-199[9], miR-193b[10] and miR-889[11].

MicroRNA-150-5p is abnormally expressed in a variety of tumors and can participate in the

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regulation of malignant phenotypes of tumor cells. Ma et al[12] have demonstrated that miR-150 is downregulated in colorectal cancer (CRC). The prognosis of CRC patients with lowly expressed microRNA-150-5p is poor. Meanwhile, chemotherapy response is significantly worse compared with those expressing high level of miR-150[12]. In addition, miR-150 inhibits invasiveness and metastasis of colon cancer cells by binding MUC4[13]. On the contrary, it has been found that upregulation of miR-150 promotes the proliferative and metastatic potentials of lung cancer by inactivating the SRC kinase signaling and inhibitor molecule 1[14]. So far, the exact role of microRNA-150-5p in the malignant development of osteosarcoma has not been fully elucidated. Therefore, the aim of this study was to investigate the role of microRNA-150-5p in the development of osteosarcoma, and to explore its underlying mechanism.

Patients and Methods

Sample Collection

The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). From February 2012 to July 2018, 10 pairs of osteosarcoma tissues and para-cancerous tissues were collected from osteosarcoma patients. All collected tissues were stored at -80°C for use. Samples were confirmed by pathological examination. Informed consent was obtained from patients and their families before the study. This study was approved by the Ethics Committee of Shuyang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Chinese Medicine.

Cell Culture

Osteosarcoma cell lines HOS and MG-63 (the Center for Cell Resources of the Chinese Academy of Medical Sciences, Beijing, China) and non-cancerous osteoblast cell line hFOB 1.19 [American Type Culture Collection (ATCC); Manassas, VA, United States] were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. Cell passage was conducted with 2-3 days interval. Cell culture reagents were provided by Gibco (Rockville, MD, USA).

Cell Transfection

MicroRNA-150-5p mimics, VEGFA siRNAs and corresponding negative controls were provided by GenePharma (Shanghai, China). Cells were first cultured to 50-60% of confluence in 6-well plates for transfection. After overnight culture, cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, DMEM containing 10% FBS was replaced. Transfection efficiency was verified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) at 24-48 h.

RNA Extraction and qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA in tissues and cells. Reverse transcription of miRNAs was conducted using TaqMan Micro-RNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), while the M-MLV reverse transcription system (Promega Corporation, Madison, WI, USA) was used for mRNAs. TaqMan Micro-RNA PCR Kit system was as follows: amplification at 92°C for 10 minutes, 40 cycles at 90°C for 10 s, and at 60°C for 1 minute. Relative expression level of microRNA-150-5p was calculated using the $2^{-\Delta\Delta Ct}$ method, with U6 as an internal control. Primer sequences used in this study were as follows: microRNA-150-5p (F: 5'-UCUCCCAACCCUUGUACCAGUG-3'; R: 5'-CUGGUACAAGGGUUGGGAGAUU-3'); VEGFA (F: 5'-TGGCTCACTGGCTTGCTCTA-3'; R: 5'-ATCCAACTGCACCGTCACAG-3'); GAPDH (F: 5'-GGTGGTCTCCTCTGACTTCAA-3'; R: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'); U6 (F: 5'-AGAGAAGATTAGCATGGCCCCTG-3'; R: 5'-ATCCAGTGCGGGTCCGAGG-3').

Cell Counting Kit-8 (CCK-8) Assay

Transfected HOS and MG-63 cells were first inoculated into 96-well plates at a density of 1.5 × 10^3 cells/well. At appointed time points, 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) and 100 μL of medium were mixed and added to each well, followed by incubation for 2 h at 37°C in dark. Absorbance at 450 nm was measured by a micro-plate reader to calculate cell viability.

Colony Formation Assay

Transfected cells were collected, counted, and inoculated into 6-well plates at a density of 1 × 10^3 cells/well. After culture for 14 days, visible colonies were washed with phosphate buffer solution

(PBS; Gibco, Rockville, MD, USA), fixed with 4% formaldehyde, and dyed with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China). Finally, the number of formed colonies were counted by naked eyes.

Transwell Assay

Transfected cells were suspended in serum-free medium and inoculated into the upper side of a 24-well transwell chamber at a density of 5×10^4 cells/well. Meanwhile, 500 μL of medium containing 20% FBS was added to the lower side. Subsequently, the cells were cultured for 48 hours. Next, a cotton swab was used to clear non-penetrating cells. Cells invading to the bottom were incubated with methanol and 0.5% crystal violet, followed by washing. Invading cells were observed under an inverted microscope (Olympus Corporation, Tokyo, Japan). Five fields of view were randomly selected for each sample (magnification ×200).

Western Blot

Radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) was used to extract total proteins. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) kit (Pierce™; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). After sealing with 5% skimmed milk for 2 hours at room temperature, the membranes were incubated with primary antibodies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or anti-VEGFA (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. On the next day, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 for three times, followed by incubation with goat anti-mouse monoclonal antibody (1:2000) for 2 hours at room temperature. Immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL; Bio-Rad Laboratories Inc., Hercules, CA, USA) method. AlphaEase FC software (version 4.0.1; Protein Simple, San Jose, CA, USA) was finally utilized for grey value analyses.

Luciferase Reporter Gene Assay

Binding sequences in the 3'UTR of microRNA-150-5p and VEGFA were predicted by TargetScan (http://www.targetscan.org/). HOS and MG-63 cells in 24-well plates were cultured to 40-50% of confluence. Subsequently, they were co-transfected with microRNA-150-5p mimic/NC and pmirGLO-VEGFA-3'UTR-mutant (Mut)/pmirGLO-VEGFA-3'UTR-wild type (Wt). Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was applied to assess Luciferase activity at 48 h.

Statistical Analysis

GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation (SD). The Student's *t*-test was applied to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

MicroRNA-150-5p was Downregulated in Osteosarcoma Tissues and Cells

MicroRNA-150-5p was significantly downregulated in osteosarcoma tissues compared with adjacent normal tissues ($p<0.05$, Figure 1A). Similarly, compared with human normal osteoblast hFOB 1.19, microRNA-150-5p was identically downregulated in osteosarcoma HOS and MG-63 cells ($p<0.05$, Figure 1B). To further investigate its role in osteosarcoma, microRNA-150-5p overexpression model was conducted by transfection of microRNA-150-5p mimics *in vitro*. Transfection efficacy was determined by qRT-PCR (Figure 1C-1D).

MicroRNA-150-5p Inhibited Proliferative and Invasive Abilities of Osteosarcoma

To uncover the specific role of microRNA-150-5p in the progression of osteosarcoma, several functional experiments were performed. CCK-8 and colony formation assay indicated that the viability of HOS and MG-63 cells was remarkably inhibited after upregulation of microRNA-150-5p ($p<0.05$, Figure 2A-2C). Transwell assay was conducted to examine the invasive ability of osteosarcoma cells. The results showed that overexpression of microRNA-150-5p remarkably inhibited the invasiveness of HOS and MG-63 cells ($p<0.05$, Figure 2D). Therefore, we believe that microRNA-150-5p can remarkably inhibit proliferative and invasive abilities in osteosarcoma.

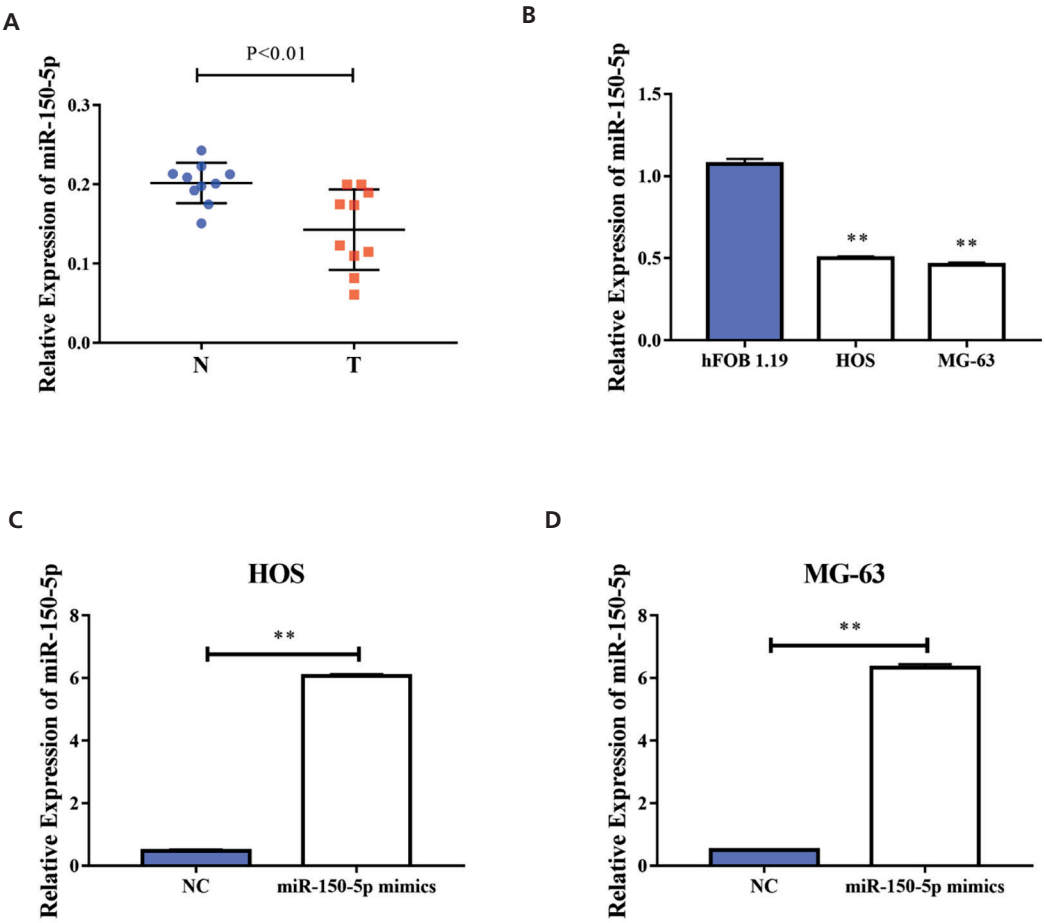

Figure 1. MicroRNA-150-5p was down-regulated in osteosarcoma tissues and cells. **A,** QRT-PCR analysis showed that the expression level of microRNA-150-5p was significantly reduced in osteosarcoma tissues compared with adjacent tissues. **B,** QRT-PCR assay showed that the expression level of microRNA-150-5p was significantly down-regulated in osteosarcoma cells HOS and MG-63 compared with human normal osteoblast hFOB 1.19. **C,** Transfection efficiency of microRNA-150-5p mimics in HOS. **D,** Transfection efficiency of microRNA-150-5p mimics in THP-1. (**p<0.01).

MicroRNA-150-5p Could Target VEGFA

Potential binding targets for microRNA-150-5p and VEGFA were predicted through bioinformatics tools (Figure 3A). Subsequently, wild-type and mutated sequences of VEGFA were constructed. Luciferase activity was significantly reduced in the VEGFA-WT 3'UTR after transfection of microRNA-150-5p in HOS and MG-63 cells (p<0.05). However, no significant difference was observed in VEGFA-MUT 3'UTR (p>0.05, Figure 3B-3C). This indicates that VEGFA is the downstream gene of microRNA-150-5p. Meanwhile, transfection of microRNA-150-5p mimic markedly downregulated VEGFA expression (p<0.05, Figure 3D). To further analyze the interaction between microRNA-150-5p and VEGFA, si-VEGFA was transfected in HOS and MG-63 cells. The results demonstrated that VEGFA was markedly downregulated in transfected cells, while microRNA-150-5p was upregulated at the mRNA level (p<0.05, Figure 3E-3F). Western blot experiments showed the same results at the protein level (Figure 3G). The above results indicate that microRNA-150-5p can target bind to VEGFA and inhibit its expression.

VEGFA Was Highly Expressed in Osteosarcoma and Promoted Cell Proliferation and Invasion

QRT-PCR results showed that VEGFA was remarkably upregulated in osteosarcoma and was negatively correlated with microRNA-150-5p expression (p<0.05, Figure 4A-4B). Notably, the proliferation ability of HOS and MG-63 cells was

remarkably inhibited after knockdown of VEGFA (p<0.05, Figure 4C-4D). The same results were observed in colony formation assay (Figure 4E). To further evaluate its effect on invasiveness, transwell assay was conducted. The results showed significantly weakened invasive ability in osteosarcoma cells with VEGFA knockdown (p<0.05). These results suggest that knockdown of VEGFA inhibits proliferative and invasive abilities of osteosarcoma cells.

Discussion

Osteosarcoma is characterized by high malignancy and poor prognosis. Even with the combination of surgery and chemotherapy, the current therapeutic effect of osteosarcoma is still far from satisfactory. Gene level therapy has always been a hot topic in the study of osteosarcoma, in particular, to search for effective therapeutic targets. Current studies[7,15] have suggested that miRNA has a wide range of gene regulation functions. It can regulate various levels of gene activity and participate in a series of biological activities, including embryonic development, cell phenotypes, and energy metabolism. Many scholars have investigated the role of miRNAs in the development of osteosarcoma. All these findings suggest that the biological behavior of osteosarcoma is closely related to the members of the miRNA family[16,17]. For example, overexpression of miR-488 can inhibit proliferative and migratory potentials in osteosarcoma cells[18]. Meanwhile, miR-544 is remarkably upregulated in human osteosarcoma tissues and promotes osteosarcoma metastasis by regulating AXIN2[19].

Tumor growth depends on intravascular blood nutrition, therefore, angiogenesis is crucial for the development of all types of tumors[20]. Vascular endothelial growth factor (VEGF), especial-

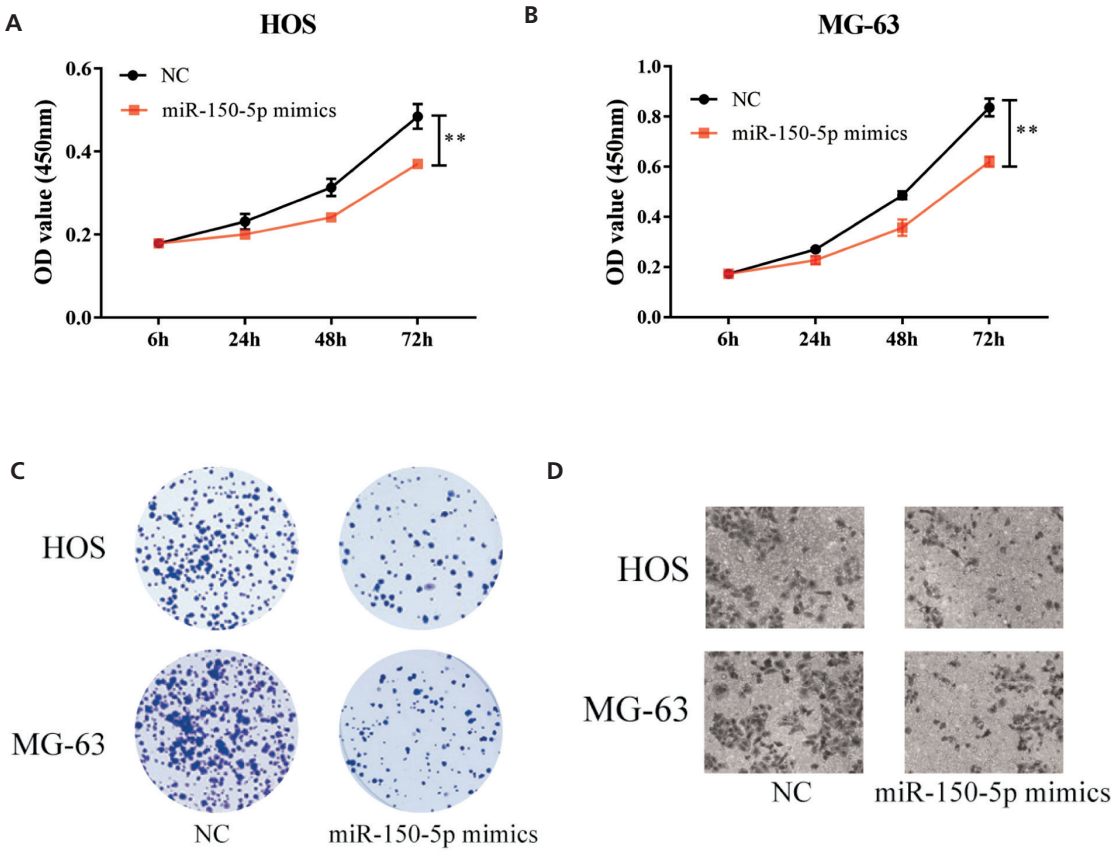

Figure 2. MicroRNA-150-5p inhibited osteosarcoma cell proliferation and invasion. **A,** CCK-8 assay showed that up-regulation of microRNA-150-5p cells significantly weakened the proliferation of HOS cells. **B,** CCK-8 assay showed that up-regulation of microRNA-150-5p obviously weakened the proliferation of MG-63 cells. **C,** Colony formation assay showed that up-regulation of microRNA-150-5p cells remarkably inhibited the proliferation of HOS and MG-63 cells (× 20). **D,** Transwell assay showed that upregulation of microRNA-150-5p attenuated the invasion of HOS and MG-63 cells (× 20). **p<0.01.

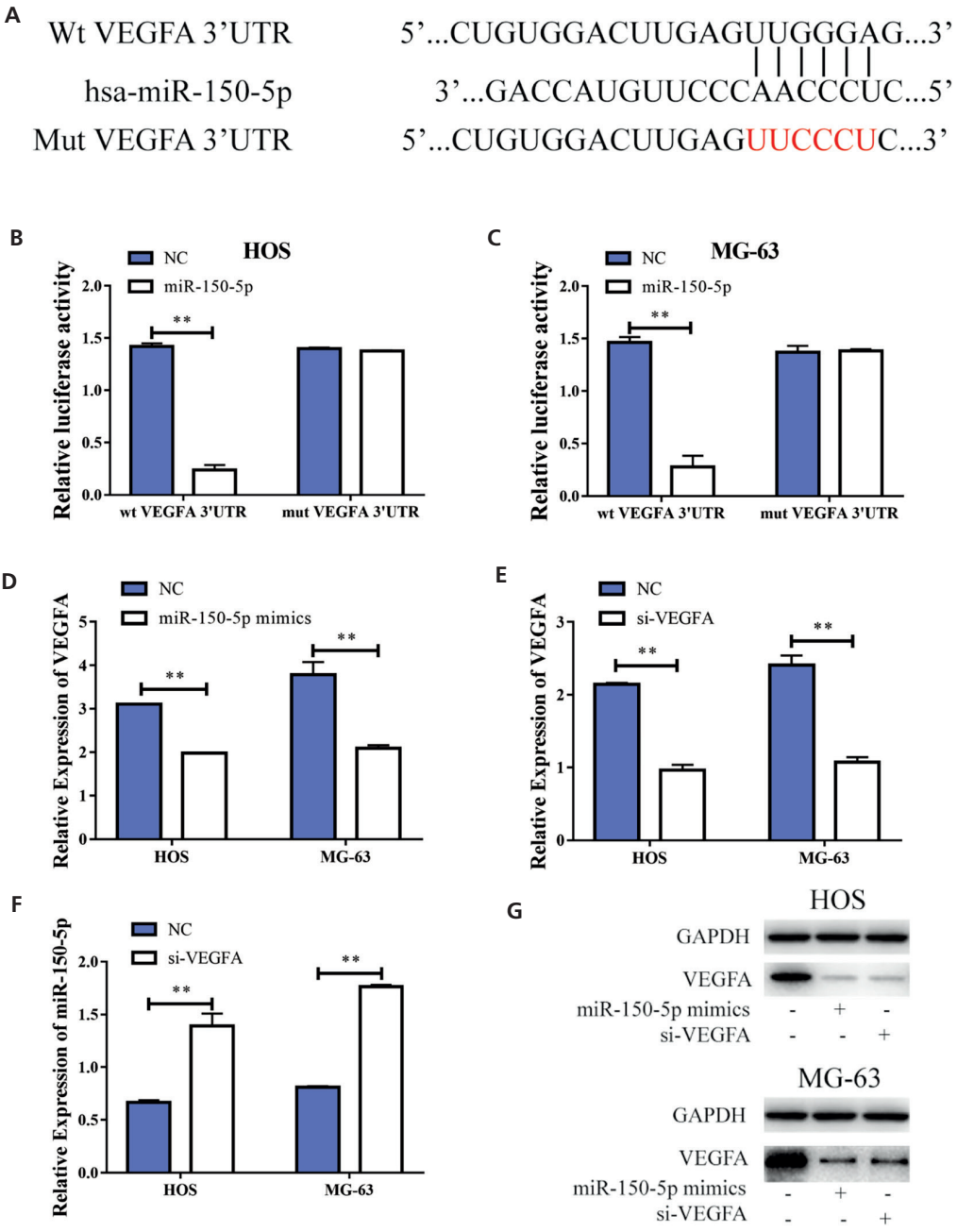

Figure 3. MicroRNA-150-5p could target VEGFA. **A,** Bioinformatics tools predicted potential binding sites for microRNA-150-5p and VEGFA. **B,** Dual luciferase reporter gene assay showed that microRNA-150-5p could target bind to VEGFA in HOS cells. **C,** Dual-Luciferase reporter gene assay indicated that microRNA-150-5p could target bind to VEGFA in MG-63 cells. **D,** Upregulation of microRNA-150-5p decreased VEGFA expression levels. **E,** Transfection efficiency of si-VEGFA in HOS and MG-63 cells. **F,** The expression level of microRNA-150-5p was inhibited after down-regulation of VEGFA expression. **G,** Western blot showed that the protein expression of VEGFA decreased after up-regulation of microRNA-150-5p in HOS and MG-63 cells. The protein expression level of VEGFA decreased significantly after transfection of si-VEGFA. (**$p<0.01$).

ly VEGFA, is an important angiogenic factor for vascular endothelial formation. Qiu et al[21] have confirmed that it can affect tumor progression. VEGFA binds to its receptor, such as VEGFR1, and activates PI3K/AKT signaling, thereby promoting tumor proliferation and angiogenesis[22,23]. Therefore, VEGFA/VEGFR1 signaling is considered as a therapeutic target for several malignant

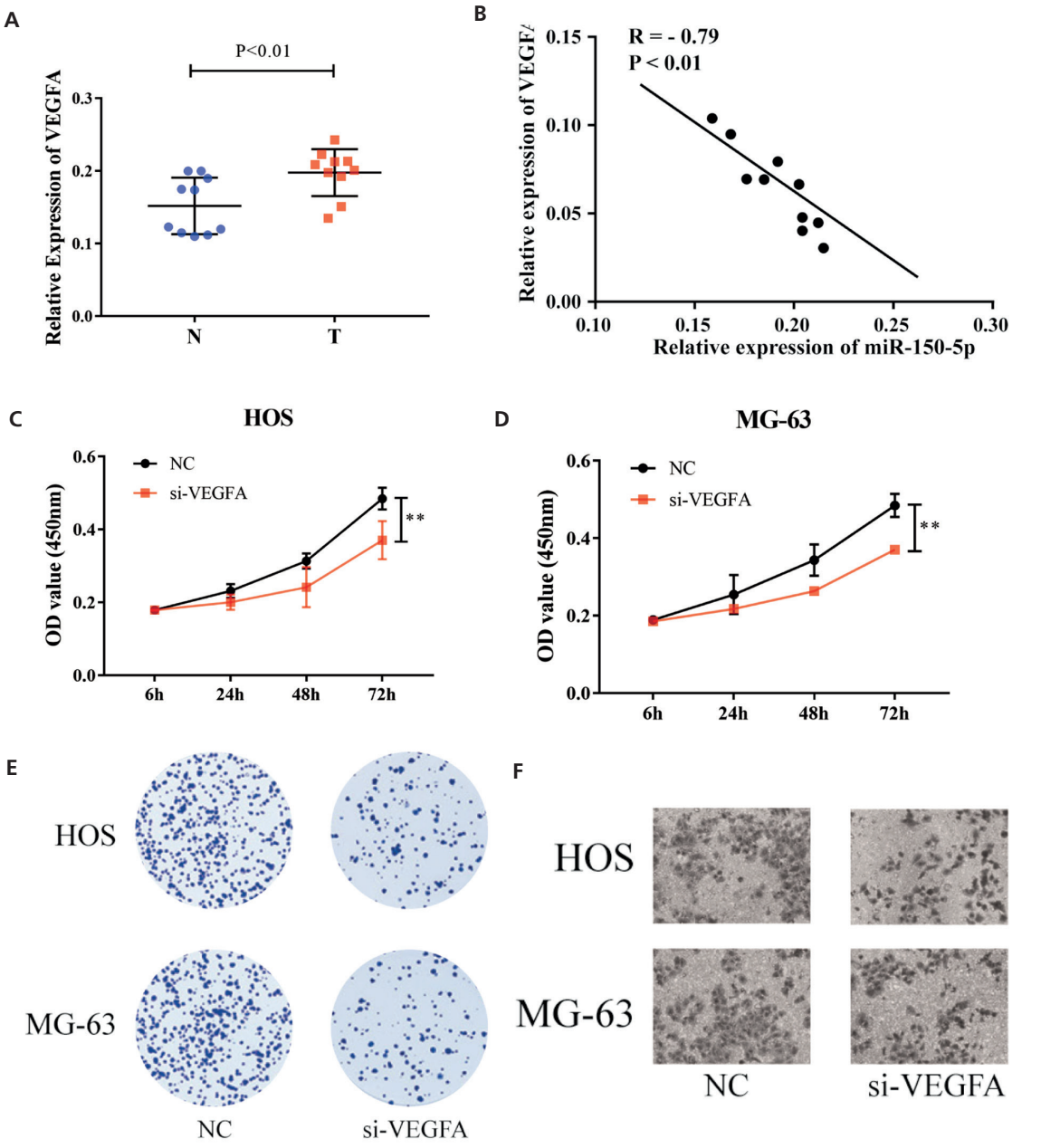

Figure 4. VEGFA was highly expressed in osteosarcoma and promoted cell proliferation and invasion. **A,** VEGFA was highly expressed in osteosarcoma tissues and promoted cell proliferation and invasion; VEGFA was highly expressed in osteosarcoma tissues. **B,** A negative correlation was observed between the expressions of VEGFA and microRNA-150-5p in osteosarcoma tissues. **C,** CCK-8 assay showed that the proliferation ability of HOS cells was reduced after down-regulation of VEGFA. **D,** CCK-8 assay showed that the proliferation of MG-63 cells was weakened after down-regulation of VEGFA. **E,** Colony formation assay showed that the proliferation of HOS and MG-63 cells was downregulated after down-regulation of VEGFA (× 20). **F,** Transwell experiments showed that the invasive ability of HOS and MG-63 cells was inhibited after down-regulation of VEGFA (× 20). (**p<0.01).

tumors. In fact, anti-angiogenesis therapy against VEGF has been shown to inhibit the progression of advanced cancer[24]. More importantly, high expression of VEGF indicates poor prognosis of osteosarcoma patients. This suggests that VEGFA/VEGFR1 signaling may exert a crucial role in the development of osteosarcoma[25,26]. However, the regulatory mechanism of VEGFA and VEGFR1 in the development of osteosarcoma remains unclear.

In this study, the results showed that microRNA-150-5p was significantly downregulated in osteosarcoma tissues compared with para-cancerous tissues. Meanwhile, VEGFA decreased remarkably and was negatively correlated with the expression level of microRNA-150-5p. Compared with normal human osteoblasts, microRNA-150-5p was lowly expressed in osteosarcoma cells as well. After overexpression of microRNA-150-5p, proliferative and invasive abilities of osteosarcoma cells were remarkably inhibited. MicroRNA-150-5p VEGFA was found to be the downstream gene of microRNA-150-5p and displayed a negative correlation between each other. Subsequent functional assays showed that knockdown of VEGFA remarkably inhibited proliferative and invasive abilities of osteosarcoma cells. Therefore, we concluded that microRNA-150-5p weakened the proliferative and invasive potentials in osteosarcoma by downregulating VEGFA.

Conclusions

MicroRNA-150-5p inhibits the proliferative and invasive potentials of osteosarcoma by downregulating VEGFA. The novelty of this study was that microRNA-150-5p/VEGFA is a promising target for osteosarcoma treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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