ORIGINAL ARTICLE

WILEY **Cancer Science**

CXCR4-mediated osteosarcoma growth and pulmonary metastasis is suppressed by MicroRNA-613

Yong Zhu[1] | Lanhua Tang[2] | Shushan Zhao [1] | Buhua Sun[1] | Liang Cheng[1] |
Yifu Tang[1] | Zhongwei Luo[1] | Zhangyuan Lin[1] | Jianxi Zhu[1] | Weihong Zhu[3] |
Ruibo Zhao[1] | Bangbao Lu[1] | Haitao Long[1] (iD)

[1]Department of Orthopedic Surgery, Xiangya Hospital, Central South University, Changsha, China

[2]Department of Oncology, Xiangya Hospital, Central South University, Changsha, China

[3]Department of Orthopedic Surgery, The First People's hospital of Chenzhou, Chenzhou, China

Correspondence
Haitao Long, Department of Orthopedic Surgery, Xiangya Hospital, Central South University, Changsha, China.
Email: doclong2008@aliyun.com

Osteosarcoma is the most common primary bone malignancy. Recently, studies showed chemokine receptor 4 (CXCR4) played a critical role in osteosarcoma. However, the regulation of CXCR4 is not fully understood. microRNAs are short, non-coding RNAs that play an important roles in post-transcriptional regulation of gene expression in a variety of diseases including osteosarcoma. miR-613 is a newly discovered miRNA and has been reported to function as a tumor suppressor in many cancers. In this study, we confirmed that both Stromal Cell-Derived Factor (SDF-1) and CXCR4 could be prognostic markers for osteosarcoma. Meanwhile this study found that SDF-1/CXCR4 pathway regulated osteosarcoma cells proliferation, migration and reduced apoptosis. Besides, we demonstrated that miR-613 was significantly downregulated in osteosarcoma patients. Elevated expression of miR-613 directly suppressed CXCR4 expression and then decreased the proliferation, migration and induced apoptosis of osteosarcoma cells. Moreover, our study found that CXCR4 promoted the development of lung metastases and inhibition of CXCR4 by miR-613 reduced lung metastases. These data indicated that CXCR4 mediated osteosarcoma cell growth and lung metastases and this effect can be suppressed by miR-613 through directly downregulating CXCR4.

KEYWORDS
CXCR4, lung metastasis, miR-613, SDF-1, steosarcoma

1 | INTRODUCTION

Osteosarcoma, deriving form primitive bone-forming mesenchymal cells, is the most common primary bone malignancy.[1] The incidence rate of osteosarcoma has a bimodal age distribution. It typically occurs during adolescence and has the second peak in older adulthood.[2] Usually the osteosarcoma occurs in long bones.

The etiology of osteosarcoma is currently being studied but still not well understood.[3] Several studies showed that age, gender and height are risk factors for osteosarcoma.[4] The overall survival (OS) of osteosarcoma has been improved significantly because of effective chemotherapy combination.[5] Studies showed that complete surgical resection with an addition of chemotherapy has increased the 5-year OS to approximately 70%,[5] but improvements in osteosarcoma survival during the last decade have been limited.[6]

Zhu and Tang equally contributed to this study.

Chemokine receptor 4 (CXCR4) is a seven-transmembrane G protein-coupled chemokine receptor and selectively binds the CXC chemokine Stromal Cell-Derived Factor (SDF-1).[7] SDF-1, also knows as CXCL12, is a homeostatic chemokine, whose major function is in regulating hematopoietic cell trafficking and secondary lymphoid tissue architecture. CXCR4 is normally expressed on many cell types including lymphocytes, hematopoietic stem cells, endothelial and epithelial cells.[8] Besides, CXCR4 is also the chemokine receptor most commonly expressed in tumor cells such as osteosarcoma cells. Several studies had demonstrated CXCR4 is involved in tumor cell proliferation, migration and invasion.[9]

microRNAs(miRNAs) are a class of 20-24 nucleotide non-coding RNAs that regulate genes by binding to the 3′-untranslated region (3′-UTR) of target mRNAs in multicellular organisms. They are involved in post-transcriptional regulation of gene expression in a variety of diseases including osteosarcoma and other cancers. Recently studies have shown that miRNAs play an important role in osteosarcoma and several miRNAs have been identified, such as miR-132, miR-21, miR-215 and miR-143. miR-613 is a newly discovered miRNA and has been reported to function as a tumor suppressor in many cancers.[10] However, its function and molecular basis in the osteosarcoma is incompletely understood.

We have performed miRNA expression profiling in both osteosarcoma tissue and cell line. The results demonstrated that miR-613 expression was significantly downregulated. Over-expression of miR-613 decreased osteosarcoma cells proliferation and induced apoptosis. We also identified CXCR4, which is upregulated in osteosarcoma and plays an important role in the development of lung metastases, as a novel target of miR-613, and upregulation of miR-613 could decrease lung metastases in osteosarcoma. Our data here suggested that miR-613 mediated CXCR4 translational repression may be an additional therapeutic strategy for osteosarcoma.

2 | MATERIALS AND METHODS

2.1 | Patients

All the patients, participants and their guardians were provided written informed consent for this study. In addition, ethical approval was obtained from the Ethics Committee of the Xiangya Hospital, Central South University. Fresh specimens were obtained from all the participants who underwent tumor resection or biopsy at the Xiangya Hospital between March 2005 and December 2014. Following resection, the tissues were divided into two parts. One was frozen in liquid nitrogen and then stored at −80° for RT-PCR or western blot. The other part was formalin-fixed, paraffin-embedded and then stored in the Department of Pathology of the Xiangya Hospital. All slides were evaluated for diagnosis by two similarly experienced pathologists. To determine plasma concentration of SDF-1 and miRNA in each participant, 5 mL of venous whole blood with EDTA-anticoagulated was drawn in totally. Whole blood samples were centrifuged for 10 minutes at 3000 RPM immediately to separate plasma and then stored at −80° till further analysis. Estimation of plasma SDF-1 was carried out by using commercially available ELISA kit (R&D Systems) with a detection range of 156 to 10 000 pg/mL SDF-1. The optical density of the samples was measured by using a microplate reader. No patients received preoperative chemotherapy or radiotherapy before the samples were collected.

2.2 | Cell culture and reagents

The cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were incubated at 37° in a standard mixture of 95% air and 5% CO_2. The media and antibiotics were purchased from Biyuntian(Chian) and FBS was obtained from Gibco (USA). For passaging, cells were detached with trypsin/EDTA and subsequently replanted. The reagents were purchased from Sigma-Aldrich or R&D Systems.

2.3 | Real time PCR analysis

Total RNA was extracted from tissues or cells by using TRIzol reagent (Invitrogen, USA). Samples were homogenized in TRIzol reagent and RNA was precipitated with isopropanol, applied to RNeasy spin columns, eluted, and treated with RNase-free DNase (Invitrogen) for 30 minutes at 37°, followed by heat inactivation at 75° and storage at −80°. Transcript levels were quantified using real-time quantitative reverse transcription-polymerase chain reaction. Syber green was used for analysis of SDF-1, CXCR4 and GADPH mRNAs by using the following primers: SDF-1, forward: 5′-tgatcgtctgactggtctta-3′, reverse: 5′-cttaggggatttggaagttt-3′, CXCR4, forward: 5′-tcatcagtctggaccgctacc-3′, reverse: 5′-cgttggcaaagatgaagtcg-3′, GAPDH, forward: 5′-agggctgcttttaactctggt-3′, reverse: 5′-ccccacttgattttggaggga-3′. Polymerase chain reaction was performed using a LightCycler®480 system (Switzerland). Each cDNA sample was run in triplicate.

To detect miR-613, Total RNA was isolated from tissue samples or plasma by using the miRNeasy mini kit and miRNeasy Serum/plasma kit from QIAGEN (Dusseldorf, Germay) in accordance with the manufacturer's protocols. cDNA was synthesized with gene-specific primers. Reverse transcription reactions were conducted using 50 ng RNA, 50 nM/L stem-loop RT primers, 3.33 U/μL multiscribe reverse transcriptase, 0.25 mM/L of each dNTP, RT buffer and 0.25 U/μL Rnase inhibitor. cDNA product was subsequently used for RT-PCR analysis. RT-PCR was performed in 96-well plates using the PCR reaction mixtures included 1.33 μL RT product, TaqMan universal PCR master mix and 1 μL primers and probe mix fram the TaqMan micro RNA assay kit on a StepOne Plus real time PCR machine (Applied Biosystems). U6 small nuclear RNA was used as the internal control for miRNA. The following primers were used for detecting the miRNA: miR-613, forward: 5′-tggcaggaatgttccttct-3′, reverse: 5′-tgcagggtccgaggtat-3′, u6, forward: 5′-ctcgcttcggcagcaca-3′, reverse: 5′-aacgcttcacgaatttgcgt-3′. For data analysis, the ΔΔCt method was used.[11] For each gene, the fold change was calculated as a difference.

2.4 | Immunohistochemistry

Hematoxylin and eosin-stained samples were reviewed by two experienced pathologists to determine the diagnosis and characterize the tumor. The formalin-fixed, paraffin-embedded tissue samples were sectioned at a thickness of 4 μm prior to heating at 60° in an oven for ≥60 minutes. The slides were deparaffinized with xylene, hydrated and pretreated with phosphate-buffered saline. Subsequently, 3% hydrogen peroxide was used to block endogenous peroxidase activity for 15 minutes. Slides were incubated overnight with primary antibodies (CXCR4; Abcam, USA)and then complexed with a biotinylated secondary antibodies (Beijing Zhongshan Jinqiao Biotec, China) for 30 minutes at room temperature. Streptavidin-peroxidase was applied and EnVision™ and universal 3,3′-diaminobenzidine detection kits (Gene Tech Biotechnology Co., Ltd., Shanghai, China) was used with an extra washing step. The slides were counterstained with hematoxylin and mounted. Negative controls were obtained by substituting the primary antibody with PBS In each samples, 15 random selected fields were evaluated in three random locations (five slides per location) and only representative images were presented here.

2.5 | Migration assay

The migration assay was performed using Transwell assay (Corning Inc. USA) in 24-well dishes. Prior to the assay, OS cells (MG-63) were pretreated for 24 hours in FBS-free medium. Then, 1×10^5 cells in 200 μL of FBS-free DMEM medium were placed in the upper chamber, and 500 μL of the same medium containing 100 ng/mL SDF-1 in the lower chamber. Additionally, 500 μL FBS-free and 10% FBS DMEM medium was placed in the lower chamber as negative and positive controls, respectively. Plates were incubated for 48 hours at 37° in 5% CO_2; culture medium was then removed and filters were washed with phosphate-buffered saline (PBS) twice. The cells on the upper side of filters were removed with cotton-tipped swabs. The cells on the underside of the filters were fixed in 95% alcohol for 10 minutes and stained with 0.1% crystal violet for 15 minutes. Cells that migrated from the upper to the lower side of the filter were counted under a light microscope by counting 10 random fields at a magnification of ×100. This assay was performed according to the manufacturer's instructions.

2.6 | Western blot analysis

Tissues or cellular lysates were obtained using RiPA lysis buffer (Beyotime institute of Biotechnology, Shanghai, China), protease and phosphatase inhibitors (Thermo Scientific). Protein concentrations were calculated using the Pierce BCA protein assay kit (Thermo Scientific). Equivalent amounts of total protein (30 μg) from each sample were separated by electrophoresis on 10% SDS-polyacrylamide gels at 80 volts and transferred to nitrocellulose membranes. After blocking with Tris-buffered saline with 0.2% of Tween 20 containing 5% skim milk for 1 hour, the membranes were incubated overnight at 4° with 1:1000 dilution of primary antibodies targeting CXCR4, cleaved Caspase 3, cleaved PARP (Abcam, USA) and GAPDH (Kangchen bio, China) followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Sigma) at room temperature for one hour. Expression of proteins was analyzed using the Odyssey infrared laser imaging system and the intensity of the bands was analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).[12]

2.7 | Luciferase reporter assay

A full-length human CXCR4 3′-untranslated region and wild-type (WT) and mutant (Mut) target sequences for miR-613 were cloned on either side of the 3′-region of the luciferase coding sequence in the pmiR-GLO-REPORT™ vector to construct the pmiR-GLO-REPORT™-CXCR4 vector. The construct was confirmed by DNA sequencing. Cells were seeded into plates at approximately 1×10^4 cells per well and transfected with a mixture of 50 ng of pmiR-GLO-REPORT™ using a riboFECT CP transfection kit. Luciferase activity was measured 16 hours after transfection using a Dual-Luciferase Reporter Assay System (Promega) in tandem with a Promega GloMax 20/20 luminometer according to the manufacturer's instructions. The relative firefly luciferase activities of the UTR construct and pathway reporter constructs were analyzed as previously reported.[13]

2.8 | Apoptosis analysis by flow cytometry

Cells were harvested and rinsed twice with PBS. The samples were diluted with 100 μL of 1× annexin-binding buffer, and then 5 μL of FITC-labeled enhanced-annexin V and 5 μL (20 μg/mL) of propidium iodide (Biolegend) were added into the cell suspension. The cells were incubated in the dark for 20 minutes at room temperature. Flow cytometry was conducted on a Beckman Coulter Gallios instrument. The results were analyzed according to the manufacturer's instructions. The experiments were performed independently three times, and the data from a representative experiment are shown.

2.9 | Animal experiments

6-week-old female nude mice were purchased from the Chinese Academy of Sciences (Shanghai, China). The mice were housed under standard conditions with a 12-hours light-dark cycle and fed with sufficient water and food. All the animal procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of Central South University. A single-cell suspension of ₁$10^6$ MG-63 cells in 10 μL of PBS was injected into the right proximal tibia medullary cavity to establish an orthotopic xenograft animal model of OS. Two weeks after transplantation, the mice were randomly allocated to the miR-613 agomir or antagomir group and negative control (scrambled) group. The tumors were intratumorally injected with 2 nmol/L miR-613 agomir, antagomir or control every 2 days respectively. After 3 weeks injection, all the mice were

euthanized to observe the primary tumor size and their tumors were weighed and imaged. Tumor and lung weight was described as the mean ± standard deviation (SD). Lung metastasis was detected by metastatic nodules and hematoxylin and eosin staining.

2.10 | Statistical analysis

All of the results are represented as the mean ± (SD). Two-tailed Student's t test, one-way analysis of variance or Mann-Whitney U test was used to calculate statistical significance. The Kaplan-Meier method and log-rank test were used for the survival analysis. All of the statistical analyses were performed with SPSS software (version 17.0) or GraphPad Prism V5.0. $P < .05$ was considered to indicate a statistically significant result.

3 | RESULTS

3.1 | Participant characteristics

All the participant characteristics are displayed in Tables 1 and S1. The basic characteristics between the osteosarcoma patients and normal healthy controls had no significant difference. The mean patient age was 27.1 ± 14.8 years (range, 8-66 years), and the median follow-up was 22.0 months (range: 5-60 months). After original diagnosis, distant metastases occurred in 49 patients at a mean of 16.3 months (ranges 4-51 months). Of these patients, 10 had bone metastases and 43 had lung metastases (4 patients had both bone and lung metastases). The median overall survival of patients with osteosarcomas was 25.8 months.

TABLE 1 Clinical characteristics of osteosarcoma patients and the expression status of SDF-1 and CXCR4

Parameters	No. of cases	CXCR Positive	CXCR Negative	P value
Gender				
Male	112	73	39	>.05
Female	99	68	31	
Age				
<18	108	71	37	>.05
≥18	103	70	33	
Tumor site				
Femur/tibia	132	86	46	>.05
Others	79	55	24	
SDF-1				
High	106	88	18	<.01
Low	105	53	52	
Metastasis				
Absent	162	101	61	.01
Lung/Bone	49	40	9	

3.2 | Elevated CXCR4 and SDF-1 expression in osteosarcoma patients and there are prognostic markers for osteosarcoma

ELISA was used to examine plasma level of SDF-1 in samples obtained from osteosarcoma and normal control participants. Plasma SDF-1 levels were significantly higher among the osteosarcoma group than the normal control group (Figure 1A, $P < .01$). To explore the associations of SDF-1 expression with overall survival, all patients were divided into two groups by using the median value of SDF-1 expression (330.4 pg/mL) in plasma as a cutoff point: SDF-1 high (n = 106, mean ± SD: 491.6 ± 121.4) and SDF-1 low (n = 105, mean ± SD: 214.0 ± 81.7) groups. Kaplan-Meier curve analysis showed that the patients with high SDF-1 expression had significantly shorter overall survival than those with low SDF-1 expression ($P < .01$, Figure 1B). CXCR4 protein expression was examined by IHC and a larger proportion of osteosarcoma samples exhibited CXCR4 in expression (141/211) than of normal control participants (8/42)($P < .05$, Figure 1C and Table S1). Kaplan-Meier curve analysis demonstrated that CXCR4 positive expression was significantly associated with shorter overall survival (Figure 1D, $P < .05$). Based on SDF-1 and CXCR4 expression levels, the osteosarcoma patients were divided into four groups which were SDF-1^{high}/CXCR4 + , SDF-1^{high} /CXCR4−, SDF-1^{low}/CXCR4 + and SDF-1^{low}/CXCR4−. Compared with other groups, SDF-1high/CXCR4 + group had significantly shorter overall survival time, while SDF-1low/CXCR4− group had statistic longer overall survival time (Figures 1E and S1A, $P < .01$).

3.3 | SDF-1 and CXCR4 expression in osteosarcoma cell lines and this pathway play a key role in proliferation, migration and apoptosis

SDF-1 and CXCR4 mRNA and protein expression were measured by RT-PCR and western blot or ELISA respectively on three different osteosarcoma cell lines MG-63, U2OS, and HOS osteosarcoma cells (Figure 2A-D). Both mRNA and protein level were elevated in these cell lines. MG-63 was used for further study because of high expression of mRNA and protein. The ability of SDF-1 inducing MG-63 cell proliferation was evaluated by adding SDF-1(0.1 μg/mL) into cell culture medium. CCK8 test results showed that the addition of SDF-1 significantly increased MG-63 proliferation (Figure 2E). To explore the role of CXCR4 in MG-63 proliferation, CXCR4 inhibitor AMD3100 and CXCR4 siRNA were used. AMD3100 and CXCR4 siRNA, which dramatically reduced CXCR mRNA and protein expression (Figure S1B,C), significantly decreased MG-63 proliferation than normal control (Figure 2F). To evaluate the role of SDF-1 in cell migration, AMD3100 and CXCR4 siRNA was used to block the association between SDF-1 and CXCR4. As shown in Figures 2G and S1D, 0.1 μg/mL of SDF-1 significantly increased MG-63 migration, and this effect was significantly decreased by AMD3100 and CXCR4 siRNA. Flow cytometry and western blot were used to study the apoptosis of MG-63 cells (Figure 2 H-J). The results indicated that
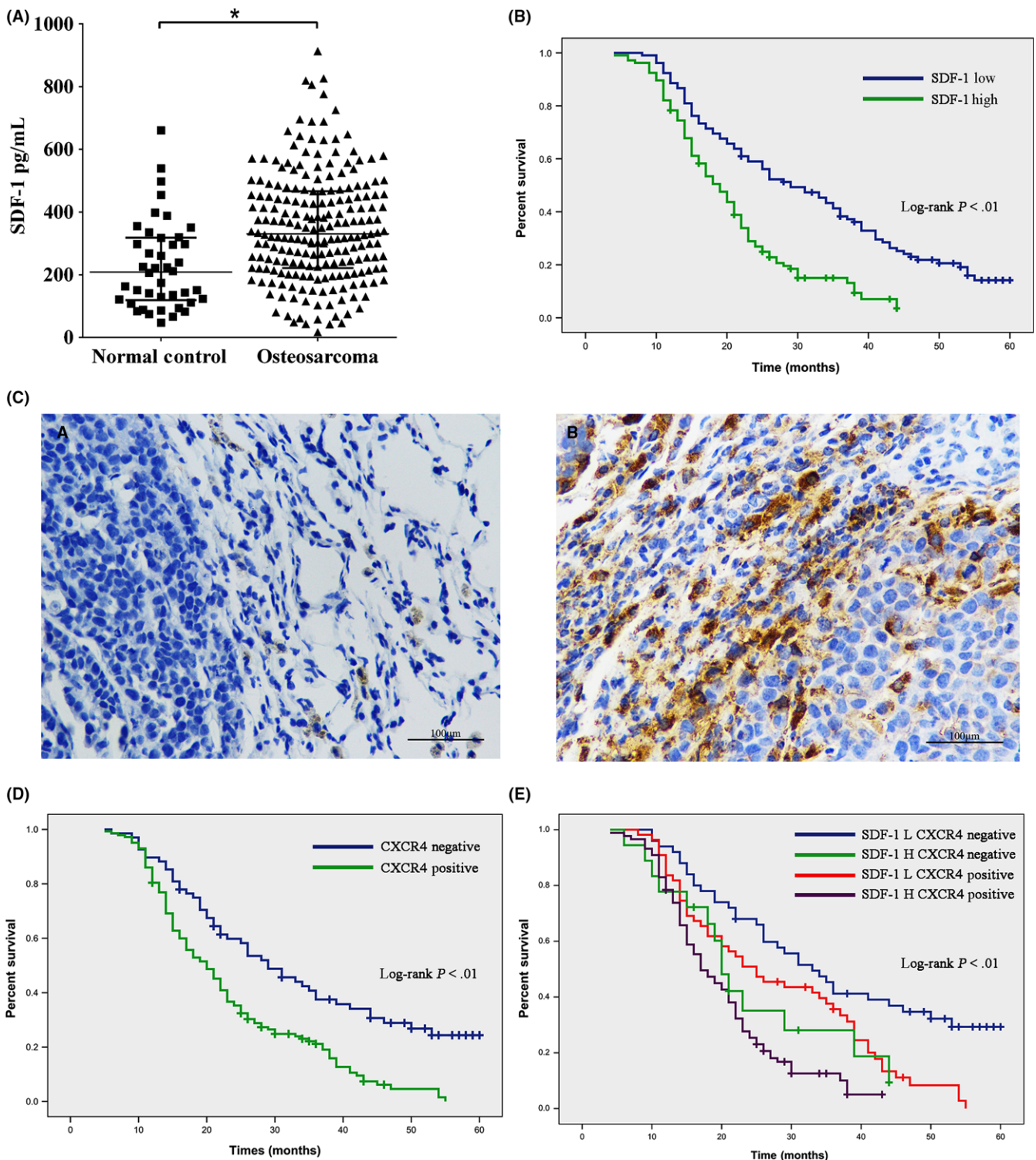
FIGURE 1 A, plasma SDF-1 levels in the osteosarcoma patients (N = 211) significantly elevated than in normal control participants (N = 42) (P < .01). B, Kaplan-Meier curve analysis demonstrated that the patients with high SDF-1 expression (N = 106) had significantly shorter overall survival than those with low SDF-1 expression (N = 105) (P < .01). C, Representative CXCR4 protein expression in osteosarcoma patients examined by IHC (a. negative sample, b. positive sample: a diffuse membrane and cytoplasmic staining of CXCR4 were evident in osteosarcoma tissues). D, Kaplan-Meier survival analysis showed that patients (N = 141) with CXCR4 positive expression were significantly associated with shorter overall survival (P < .01). E. The Kaplan-Meier analysis results of four different groups: SDF-1^{high}/CXCR4 + , SDF-1^{high}/CXCR4−, SDF-1^{low}/CXCR4 + and SDF-1^{low}/CXCR4−. The patients with SDF-1^{high}/CXCR4 + expression pattern had shortest overall survival time (P < .01)

SDF-1 decreased the apoptosis rate while inhibition of CXCR4 by AMD3100 or CXCR4 siRNA induced MG-63 apoptosis.

3.4 | miR-613 downregulates CXCR4 to reduce proliferation and induce apoptosis in osteosarcoma

Compared with human fetal osteoblastic hFOB 1.19 cell, the expression level of miR-613 significantly decreased in MG-63 cell line (Figure 3A). By using Targetscan programs, we predicted CXCR4 was the target gene of miR-613 (Figure 3B). To confirm it, luciferase reporter assay showed that miR-613 downregulated CXCR4 by targeting 3′UTR of CXCR4 gene while miR-613 had no effect on mutated CXCR4 gene (Figure 3C). Furthermore, to determine whether miR-613 play a role in osteosarcoma proliferation and apoptosis, we transfected MG-63 with miR-613 mimics, miR-613 inhibitor or CXCR4 plasmid. RT-PCR and western blot indicated that CXCR4 mRNA and protein were significantly downregulated by miR-613 mimics while upregulated by miR-613 inhibitor respectively (Figure 3D-E). Similarity, miR-613 mimics could decrease MG-63 proliferation, cell migration and induce apoptosis while miR-613 inhibitor increase MG-63 proliferation (Figure 3F), cell migration (Figures 3G and S1E) and reduce apoptosis (Figure 3H-J).

3.5 | Inhibition of CXCR4 by miR-613 plays an important role in development of lung metastases in osteosarcoma

Osteosarcoma cells ($5*10^5$) were injected into nude mice which were treated for 14 days of miR-613 agomir or antagomir. At day 18, mice which were shown in Figure 4A were sacrificed and organs were harvested. Compared with control group, smaller in situ osteosarcoma cancer was detected in agomir-treated nude mice while larger osteosarcoma cancer was detected in antagomir-treated nude mice (Figure 4B-C, $P < .05$). To find out whether the SDF-1 contribute to the osteosarcoma cancer growth, plasma SDF-1 level was measured in animal models. The results showed that there was no significant difference between the groups (Figure S1F). The level of miR-613 in agomir was significantly increased while decreased in antagomir group, which proved the agomir and antagomir worked (Figure 4 D, $P < .05$). As predicted, the CXCR4 protein expression elevated in antagomir group and reduced in agomir group respectively (Figures 4E and S1G, $P < .05$). Pearson correlation analysis showed the CXCR4 protein levels were inversely correlated with miR-613(the percentage of Integrated Density of CXCR4 to GAPDH was positively correlated with the ΔCt value) (Figure S1H, $P < .05$) .In Figure 4F, lung tissues were shown. The weight of lung tissues was significantly larger in antagomir group than in control and agomir group (Figure S1I, $P < .05$). Surprisingly, there was no statistical difference in lung weigh between control and agomir group (Figure S1I, $P = .12$). While compared with control group, nude mice treated with agomir showed fewer metastatic nodules in lung tissue while more in antagomir-treated group (Figure 4G, $P < .05$). HE staining demonstrated that more metastatic area in antagomir-treated group while less in agomir-treated group (Figure 4H, $P < .05$).

3.6 | The expression of miR-613 and its prognostic value in osteosarcoma

The tissue expression level of miR-613 was examined in osteosarcoma patients and healthy controls using RT-PCR. As shown in Figure 5A, significantly lower expression of miR-613 was observed in the osteosarcoma patients than in controls, especially in CXCR4 positive group($P < .05$). According to the median value of miR-613 expression, the osteosarcoma patients were divided into high and low miR-613 expression. Kaplan-Meier curve analysis showed that the patients with low miR-613 expression had significantly shorter overall survival time than those with high miR-613 expression ($P < .01$, Figure 5B).

4 | DISCUSSION

Osteosarcoma is a highly aggressive tumor and usually arise in long bones. Studies have demonstrated that osteosarcoma may be involved in genetic and molecular alterations which affect osteoblast differentiation.[14,15] SDF-1/CXCR4 axis was found to play important roles in almost all malignancies including osteosarcoma.[16,17] miRNAs play critical roles in regulating cell processes and may function as oncogenes or tumor suppressors to regulate gene and protein expression. Research data suggested that several miRNAs were involved in the pathogenesis of osteosarcoma and had the potential for development in disease diagnostics and therapeutics.[18] In this study, we confirmed that SDF-1/CXCR4 axis played important roles in osteosarcoma and identified miR-613 targeted 3′UTR of CXCR4 mRNA, which also involved in the pathogenesis of osteosarcoma including lung metastases.

In line with earlier reports, we also observed an increase in SDF-1 and CXCR4 expressions in osteosarcoma patients.[16,17,19] Although different studies revealed that both SDF-1 and CXCR4 were prognostic markers for osteosarcoma respectively, there is no study evaluating the combination role of SDF-1 and CXCR4 in prognostic value. Our data showed that the SDF-1^{high}/CXCR4 + group had the shortest overall survival time as previously expected.

As a regulator of spindle microtubule, CXCR4 plays an essential role in mitosis. In addition, CXCR4 is an inhibitor of apoptosis. Previous researches have demonstrated that SDF-1/CXCR4 axis mediated osteosarcoma cells proliferation and migration and effects could be abrogated by CXCR4 inhibitor.[20,21] Liao et al[20] found that mouse osteosarcoma cell line LM8, but not Dunn cells, expressed high CXCR4 protein levels. And CXCR4 played a key role in LM8 cell survival, apoptosis and migration. Consistent with these results, the in vitro study found that SDF-1 significantly increased human osteosarcoma cell line MG-63 cell proliferation and migration while decreased apoptosis. This promoting effect could be specificity reduced by CXCR4 inhibitor AMD3100 and CXCR4 siRNA.
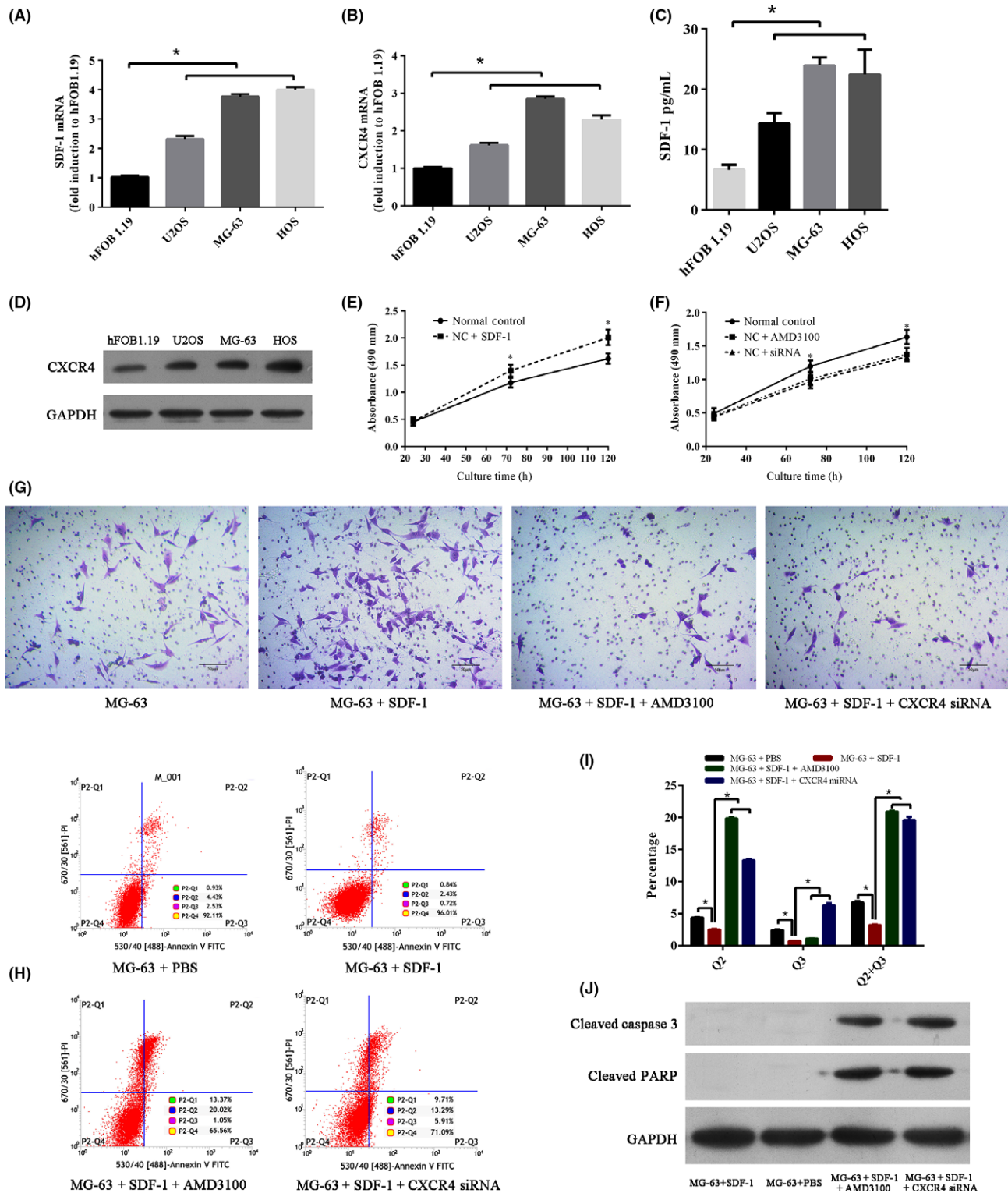
FIGURE 2   The SDF-1(A) and CXCR4(B) mRNA expression level in different cell lines: hFOB 1.19, U2OS, MG-63 and HOS. SDF-1(A) and CXCR4(B) mRNA expression level significantly increased in osteosarcoma cell lines (U2OS, MG-63 and HOS, $P < .01$). C, ELISA results indicted SDF-1 protein expression level in osteosarcoma cells supernatant were significantly higher than in hFOB 1.19 cell supernatant ($P < .01$). D, Western blot results showed that CXCR4 protein expression in osteosarcoma cells were significantly higher that in hFOB 1.19 cell. E. Addition of SDF-1 (0.1 μg/mL) could increase MG-63 cell proliferation significantly ($P < .01$). F. CXCR4 specific pharmaceutical inhibitor AMD3100 and genetic inhibitor CXCR4 siRNA dramatically reduced MG-63 proliferation induced by SDF-1($P < .01$). G, AMD3100 and CXCR4 siRNA significantly decreased MG-63 cell migration which could be promoted by SDF-1($P < .01$). H&I. Flow cytometry results indicted that SDF-1 decreased the apoptosis rate (Q2 and Q3) while inhibition of CXCR4 by AMD3100 or CXCR4 siRNA induced MG-63 apoptosis ($P < .01$). J. Cleaved Caspase 3 and Cleaved PARP significantly increased in MG-63 cell treated with AMD3100 and CXCR4 miRNA
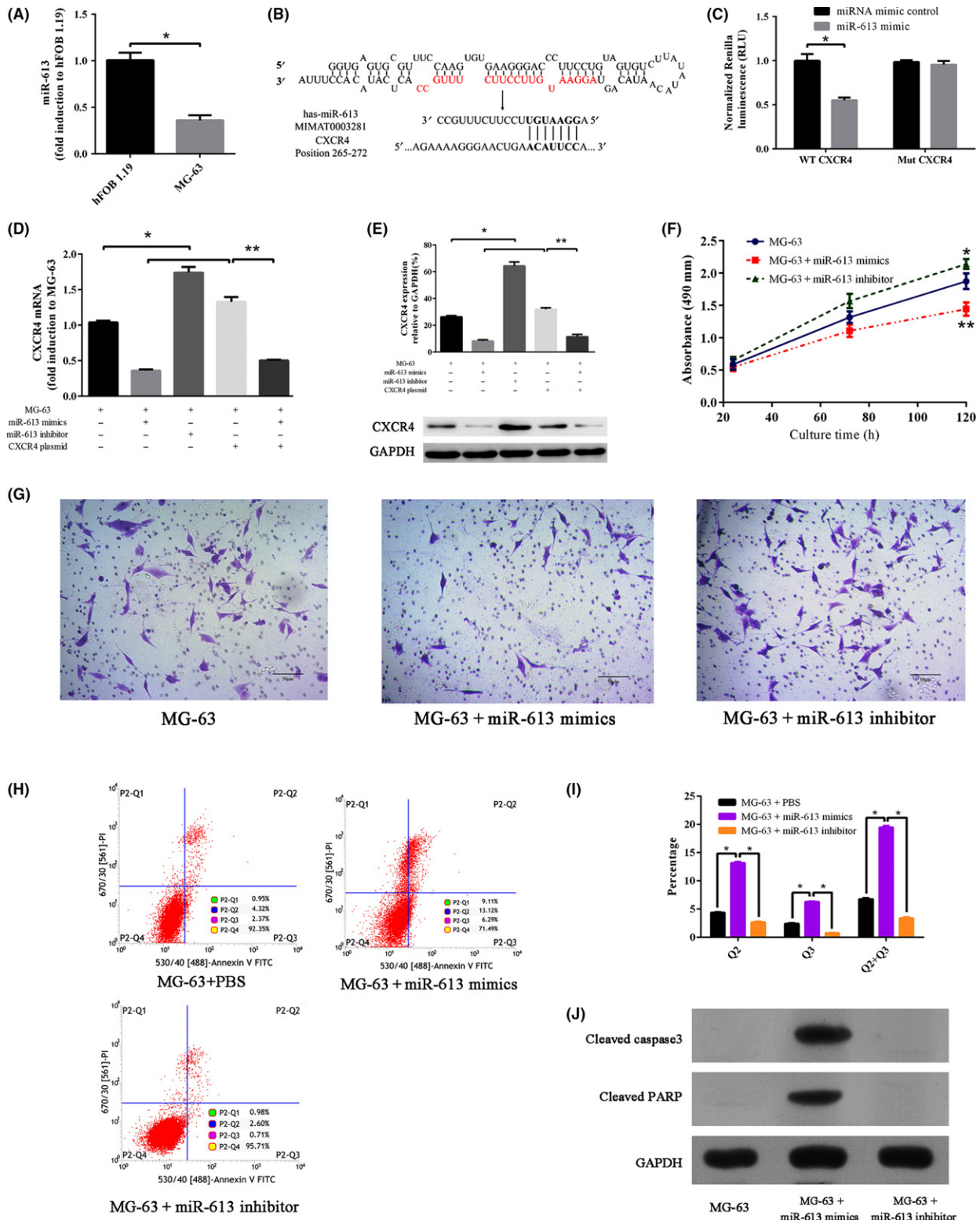
FIGURE 3 A, The expression level of miR-613 significantly lower in MG-63 cell line ($P < .01$). B, Targetscan programs predicted CXCR4 was the target gene of miR-613. C, Luciferase reporter assay demonstrated that miR-613 downregulated wild type CXCR4($P < .01$) while had no effect on mutated CXCR4 gene ($P > .05$). CXCR mRNA (D) and protein (E) levels were dramatically downregulated by miR-613 mimics and upregulated by miR-613 inhibitor ($P < .01$). F, miR-613 mimics decreased MG-63 proliferation while miR-613 inhibitor increased MG-63 proliferation significantly ($P < .01$). G, miR-613 mimics decreased MG-63 migration ($P < .01$) while miR-613 inhibitor increased MG-63 migration ($P < .01$) significantly. H&I, Flow cytometry results showed that miR-613 mimics increased the apoptosis rate (Q2 and Q3) ($P < .01$) while miR-613 decreased the apoptosis rate ($P < .01$). J, Cleaved Caspase 3 and Cleaved PARP significantly increased in MG-63 cell treated with miR-613 mimics ($P < .01$) while had no significantly different in MG-63 cell treated with miR-613 inhibitor
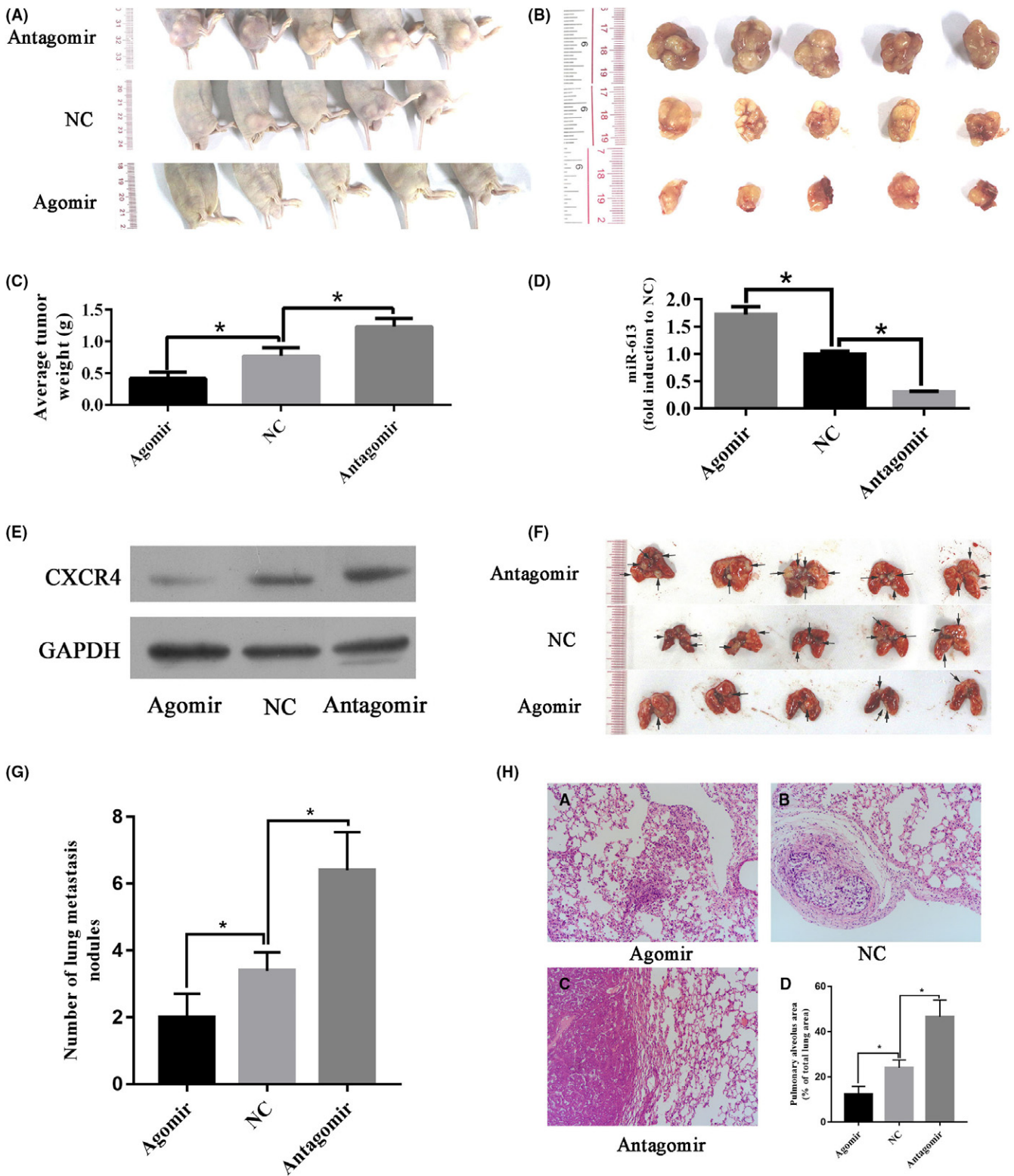
FIGURE 4 A, Nude mice injected with osteosarcoma cells were treated for miR-613 agomir, negative control and miR-613 antagomir (representative mice were presented, N = 5/group). B, in situ osteosarcoma cancer was isolated and representative cancer tissues were presented (N = 5/group). C, smaller weight in situ osteosarcoma cancer was detected in agomir-treated nude mice while larger in antagomir-treated nude mice (N = 5/group) (P < .01). D, RT-PCR results showed miR-613 in agomir was significantly increased while decreased in antagomir group (P < .01). E, Western blot results showed that the CXCR4 protein expression elevated in antagomir group and reduced in agomir group (N = 5/group). F, Representative lung tissues were presented. Less metastases (white nodules) was observed in agomir group while more metastases in antagomir-treated group (N = 5/group). G, HE staining results of lung tissues showed that more nodules and massive substitution in antagomir-treated group while less in agomir-treated group (N = 5/group)
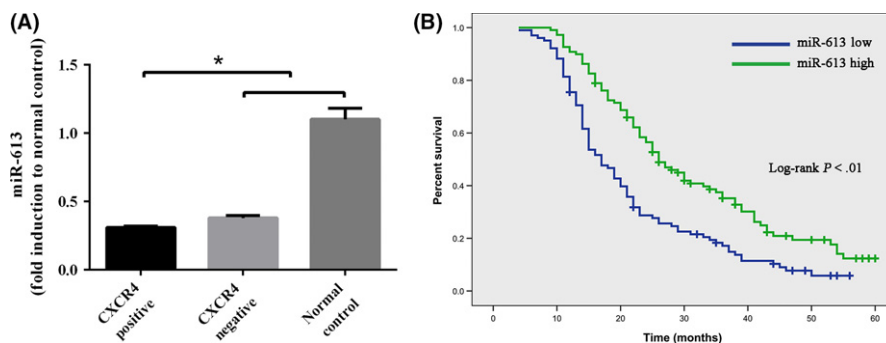
FIGURE 5 A, Compared with healthy participants (N = 42), miR-613 expression level in the plasma of osteosarcoma patients (N = 211) significantly decreased (P < .01), especially in CXCR4 positive group (N = 143) (P < .01). B, Kaplan-Meier curve analysis demonstrated that the patients with low miR-613 expression (N = 102) had significantly shorter overall survival time (P < .01)

Since the discovery of the first miRNA in 1993,[22] more than 1000 miRNAs have been found in the human genome.[23,24] They have the potential to regulate various critical biological processes including proliferation, migration and apoptosis of different kind of cancer cells.[25,26] Previous studies have indicated that many miRNAs, such as miR-19a,[27] miR-610,[28] miR-22,[29] played an important role in the pathogenesis of osteosarcoma. miR-613 has been studied in pancreatic cancer,[30] bladder cancer,[31] colorectal cancer[32] and so on. The researches suggested that miR-613 acted a tumor suppressive role in pancreatic cancer, bladder cancer and colorectal cancer by regulating lncRNA HOTAIR, SPHK1 and FMNL2 respectively. Based on our previous study and Targetscan programs, we predicted CXCR4 was the target gene of miR-613. As expected, our results demonstrated that the expression of miR-613 was down-regulated in osteosarcoma tissues and cancer lines, which was also confirmed by Li et al[33] Low level of miR-613 was positively correlated with shorter overall survival in osteosarcoma patients. Up-regulation of miR-613 inhibited osteosarcoma cancer cells proliferation and migration. In addition, overexpression of miR-613 significantly induced apoptosis of osteosarcoma cancer cells. Bioinformatics analysis and luciferase reporter assay indicated that CXCR4 was a novel a target of miR-613 in osteosarcoma cancer cells.

CXCR4 is a well-studied G-protein coupled chemokine receptor and commonly expressed on lots of cell types including osteosarcoma cells.[34] The binding of SDF-1 to CXCR4 results in divergent downstream signaling pathways that mediate cell proliferation, migration and apoptosis.[35] Many experiments have proven that the expression level of SDF-1 and CXCR4 significantly increased in a wide variety of malignancies.[9] High level of CXCR4 has been shown to correlate with the presence of metastatic disease in different cancers, such as breast,[36] prostate,[37] lung[38] and melanoma.[39] Although the SDF-1/CXCR4 axis appears to affect metastasis in numerous cancers, its mechanism in osteosarcoma remains unclear. Perissinotto's study showed that high SDF-1 concentration contributed to the osteosarcoma metastatic development into the lung and inhibiting the SDF-1/CXCR4 pathway by small-molecule inhibitors or anti-CXCR4 antibodies, T134 peptide, might prevent the dissemination of osteosarcoma cells.[17] Liao et al[20] found that blocking CXCR4 by AMD3100 could reduce primary tumor growth and lung metastasis. And this effect was regulated through the JNK and AKT pathways, not the p38 or Erk1/2. Until now, no study explored the role of miRNA in lung metastasis in osteosarcoma. This study found that miR-613 directly downregulated CXCR4 gene expression. Consequently, miR-613 reduced cancer proliferation and increased apoptosis. To further study the role miR-613 in lung metastasis in osteosarcoma, osteosarcoma cells were injected into nude mice treated with miR-613 agomir or antagomir in vivo. The results showed that overexpression of miR-613 could significantly decrease the tumor size and the development of lung metastasis. As many studies demonstrated that each miRNA likely performs a different type of regulatory function and miRNA targets cloud vary widely from one miRNA to the others. Moreover, a single miRNA target relationship could vary in different tissues or different diseases. In this present study, we found that miR-613 was a direct target of CXCR4. It should be more potential targets of miR-613 expect CXCR4 which also contribute the pathogenesis of osteosarcoma. More studies should be conducted to explore the role of miR-613 in the mechanism of osteosarcoma in detail.

In conclusion, our results strongly suggested that SDF-1/CXCR4 pathway played an impartment role in the pathogenesis of osteosarcoma. miR-613 directly targets CXCR4 and has a role in proliferation, migration and apoptosis in osteosarcoma cells. Inhibition of CXCR4 by overexpression of miR-613 prevented lung metastasis, which could represent the starting point for therapeutic strategies in osteosarcoma treatment.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Haitao Long http://orcid.org/0000-0001-5084-8569

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