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Original Article

CXCR4 promotes metastases to the lung of osteosarcoma xenografts in mice

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Abstract: Objectives: To investigate the effect of CXCR4 on the metastasis of osteosarcoma cells. Methods: The in vitro invasion and migration of osteosarcoma MG-63 cells was assessed by transwell assay. Bioluminescence was applied to evaluate the metastasis of osteosarcoma cells in a mouse xenograft model. Changes in the expression of the metastasis-related cytokines vascular endothelial growth factor (VEGF), matrix metallopeptidase 9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were assessed by western blotting. Results: A significantly ($p<0.05$) higher invasion ability was observed in MG-63 cells with CXCR4 overexpression by transwell assay. A similar size of orthotopic osteosarcoma but more pulmonary tumors were found in mice injected with CXCR4-overexpressing MG-63 cells than the control. Moreover, the expressions of VEGF and MMP-9 in the groups with CXCR4 overexpression were significantly ($p<0.05$) higher than those in the control; however, the opposite results were found for TIMP-1 in cultured MG-63 cells and in mouse orthotopic osteosarcoma and pulmonary tumors. Conclusions: CXCR4 up-regulates the expression of VEGF and MMP-9 but down-regulates TIMP-1, thereby promoting lung metastases of human osteosarcoma cells. Our findings suggest that CXCR4 is a potential target for the treatment of human osteosarcoma.

Keywords: CXCR4, osteosarcoma, pulmonary metastasis, VEGF, TIMP-1

Introduction

Osteosarcoma (OS) is a bone malignant tumor in children and teenagers. OS is the most common pediatric bone tumor, accounting for 5% of all pediatric tumors. OS derives from stromal cells that evolve into tumor osteoid tissue and bone tissue in the cartilage phase. OS is characterized by aggressiveness, high malignancy, and early pulmonary metastasis [1, 2]. Due to a lack of efficient methods for the early detection of metastases and effective drugs targeting micro-metastases, the prognosis of OS patients is very poor with a 5-year survival rate of approximately 20% [3-5]. It is thus of high importance to elucidate the molecular mechanisms and identify biomarkers for the early diagnosis and targeted therapy of OS metastasis.

Cancer cell metastasis is an extremely complex process including deregulated cell proliferation, as well as the initiation, promotion, progression and invasion of metastatic cells. Numerous factors, such as matrix metallopeptidase 9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and vascular endothelial growth factor (VEGF), have been shown to promote the metastasis of cancer cells including OS [6-8]. Chemokine/chemokine-receptor system has been thoroughly established to play a pivotal role in cancer cell metastasis. Mounting evidence has shown that C-X-C motif chemokine 12 and its receptor CXCR4 play a crucial role in the invasion and metastasis of several types of cancers [9, 10]. A recent study demonstrated that the inhibition of CXCR4 with a CXCR4 antibody reduces the metastatic spread of OS cells to the lung of intratibial human osteosarcoma xenografts in mice probably by inhibiting the AKT and ERK signaling pathways [3]. However, the exact role of CXCR4 in the metastasis of OS is not fully understood.

In this study, to explore CXCR4 as a therapy target for osteosarcoma, we assessed the overexpression of CXCR4 in the invasion and metasta-

sis of OS cells both in cell culture and mouse xenograft models, as well as the underlying molecular mechanisms.

Materials and methods

Cell culture

Osteosarcoma MG-63 cells were purchased from the Global Bioresource Center (ATCC, USA) and were grown in Dulbecco's modified Eagle medium (DMEM) and F-12K supplemented with 10% fetal bovine serum (FBS) (OriCell, Australia) and 100 μg/mL penicillin/streptomycin in a humidified incubator under 5% CO_2 at 37°C. Cell culture medium was replaced with fresh medium every two days.

Establishment of CXCR4-overexpressing OS cells

CXCR4 protein-encoding cDNA was obtained from MG-63 cells by RT-PCR amplification using primers for CXCR4 (sense: 5'-ATGGAGGGGATCAGTATATACA-3' and anti-sense: 5'-TTAGCTGGAGTGAAAACTTGA-3'). PCR products were digested with XhoI and BamHI restriction endonucleases (Fermentas, Canada) and inserted into the corresponding sites of the vector pcDNA-3.1(+) digested with the same restriction enzymes. Constructed recombinant plasmid pcDNA-3.1(+)-CXCR4 was purified and verified by DNA sequencing (Sangon, Shanghai, China). OS MG-63 cells were transfected with pcDNA-3.1(+)-CXCR4 or empty vector pcDNA-3.1(+) with Lipofectamine 2000 (Invitrogen, USA) for 24 h, followed by the detection of protein expression level by western blotting.

Transwell assay

Matrigel (BD, USA) was diluted using DMEM medium with a ratio of 1:8, 100 μL of which was used to cover the upper chamber of the Transwell plate, followed by incubation at 37°C for 30 min. OS cells were collected and suspended by serum-free DMEM medium containing 20% bovine serum albumin (BSA) to 5×10^5/mL. Then, a 100-μL cell suspension was added into each upper well, while 600 μL medium with 20% FBS was added to the lower wells followed by incubation in a 5% CO_2 incubator at 37°C for 24 h. Transwells were washed by PBS twice, fixed with methanol for 30 min and stained with crystal violet dye. Cells without invasion on membranes were cleared with cotton swabs and washed by PBS three times. Five visions were chosen randomly under 400× microscopy to count the cell number of the well surface. The mean value was used to represent the invasion ability of OS cells.

Real-time RT-PCR

Total RNA from OS cells was extracted using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. cDNA was obtained by reverse transcription using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. The expression of CXCR4 in OS cells was detected by Real-Time PCR using the SYBR Green method on a Corbett Rotor-gene 3000 real-time thermal cycler (Corbett Research, Concorde, Australia). The PCR reaction conditions were 95°C for 2 min followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 7 min. The relative expression levels are presented as $2^{-\Delta Ct}$. The PCR primers were as follows: β-actin (sense: 5'-TGGGCATGGGTCAGAAGGA-3' and anti-sense: 5'-AAGCATTTGCGGTGGACGA-3'); CXCR4 (sense: 5'-CCCCATCCTCTATGCT-3' and anti-sense: 5'-ATGTCCACCTCGCTTT-3').

Establishment of a mouse tumor xenograft model

BALB/c nude mice (n=12) of 5 weeks old with a weight of 21 ± 3 g were obtained from laboratory animal center of Fujian Medical University. The mice were anesthetized with 1% sodium pentobarbital and fixed in a mouse holder to expose the anus. Ten-microliter cell suspensions (5×10^5 MG-63 cells) were injected into the medullar cavity of the left tibia of individual mice. The mice were sacrificed after one month. The volume of the tumors was calculated as follows: $V=a\times b^2/2$ (a, long diameter; b, short diameter). Then, CXCR4 overexpression and expression of metastasis-related cytokines in the tumors were assessed by immunoblotting. The experimental procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the experimental animal ethics committee of Fujian Medical University.

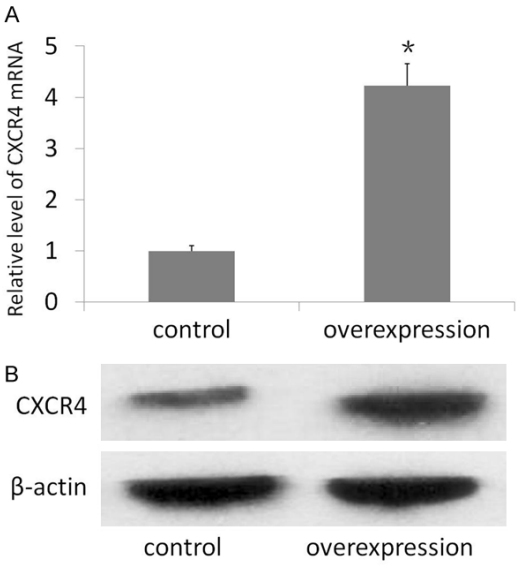

Figure 1. CXCR4 overexpression in OS MG-63 cells. A. MG-63 cells were transfected with plasmid pcDNA-3.1(+)-CXCR4 (overexpression) or empty vector pcDNA-3.1(+) (control) for 48 hrs. Total RNAs were extracted for real time RT-PCR analysis of the mRNA levels of CXCR4 with β-Actin as an internal control. *p<0.05 vs control. B. Protein expression of CXCR4 in MG-63 cells was detected by western blotting with β-actin as a loading control.

Western blotting

Cells were lysed in RIPA lysis buffer (Invitrogen, USA) on ice for 30 min, followed by centrifugation and quantification. An equal amount of proteins was separated by SDS-PAGE (10%) gel electrophoresis and transferred onto a PVDF membrane. The membrane was subsequently incubated with 5% non-fat milk intris buffered saline(TBS) for 1 h to block nonspecific binding sites, a primary antibody (diluted 1:500) overnight at 4°C and then an appropriate peroxidase-conjugated secondary antibody (diluted 1:4,000) (Abcam, UK) for 2 h. After the final washing, signals were developed by an ECL detection system and the images were quantitated and assessed by E-Gel Imager (Thermo Fisher, USA). The used antibodies were VEGF, MMP-9, tissue inhibitor of TIMP-1 and CXCR4; all these materials were purchased from Abcam (UK).

In vivo bioluminescence assay

Mice were intraperitoneally injected with 200 μL of 15 mg/mL luciferin for 8 min before being anesthetized with isoflurane. Imaging was performed using a MIIS imaging system (Molecular Devices, USA). After 5 seconds of exposure, the total flux of the region of interest was recorded as photons/second for each mouse.

Statistical analysis

Data are expressed as the mean ± SD (standard deviation) of triplicates. Statistical significances of differences between two groups were assessed using t test; Statistical significances of differences between multiple groups were assessed using a one-way ANOVA test. All data were analyzed using SPSS 19.0 software. A p value <0.05 was considered to be statistically significant.

Results

Overexpression of CXCR4 promotes the invasion of MG-63 cells

To explore CXCR4 as a therapy target for osteosarcoma, we assessed the ectopic overexpression of CXCR4 in the invasion and metastasis of OS MG-63 cells. Real-time RT-PCR showed that transfection of MG-63 cells with plasmid pcDNA-3.1(+)-CXCR4 led to significant upregulation of the mRNA level of CXCR4 compared to that transfected with empty vector pcDNA-3.1(+) (**Figure 1A**). Similarly, western blot analysis demonstrated that the expression of CXCR4 in MG-63 cells transfected with CXCR4 was higher than that in control (**Figure 1B**). To assess CXCR4 overexpression in the invasion of MG-63 cells, we performed a Transwell assay with these cells transfected with either pcDNA-3.1(+)-CXCR4 or pcDNA-3.1(+). We found that MG-63 cells with CXCR4 overexpression had higher invasion ability than control (p=0.02, **Figure 2**).

CXCR4 overexpression increases the metastasis of osteosarcoma cells in mouse xenografts

To assess the role of CXCR4 overexpression in OS metastasis in mice, an in vivo bioluminescence assay was performed to monitor the metastatic status of mice bearing MG-63 tumor xenografts. Bioluminescence was observed on the area near the left leg and upper region of the mice (**Figure 3A**). There was no significant difference between the number of photons on the left leg near the location of injection (orthotopic xenografts) in mice injected with MG-63 cells transfected with empty vector pcDNA-3.1(+) (control group) and those injected with

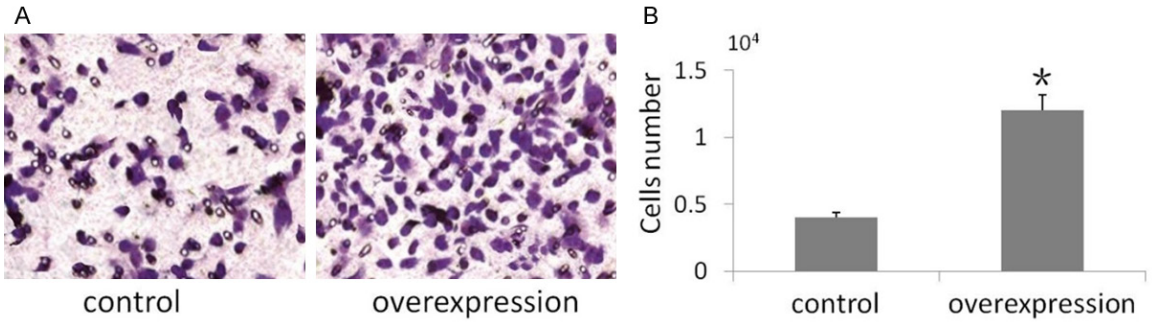

Figure 2. Overexpression of CXCR4 resulted in enhanced invasion of MG-63 cells. (A) MG-63 cells were transfected with plasmid pcDNA-3.1(+)-CXCR4 (overexpression) or empty vector pcDNA-3.1(+) (control) for 24 hrs, followed by assessment of invasion by transwell assay (magnification ×200). (B) Quantification of the invaded cells of (A). *P<0.05.

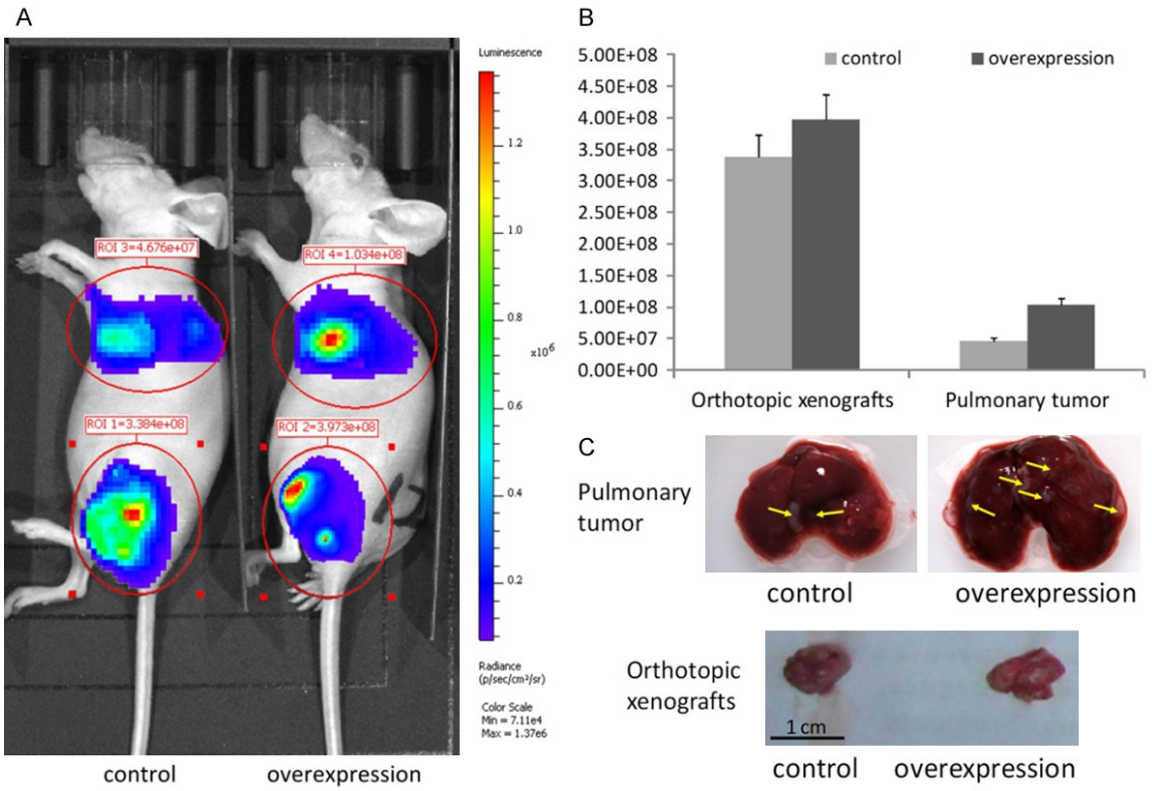

Figure 3. CXCR4 overexpression increased the lung metastasis of osteosarcoma cells in mouse xenografts. A. In vivo bioluminescence assay for tumors from orthotopic osteosarcoma in mice. Luciferase intensity represents the degree of tumor progression, red color represents high invasion and blue represents low invasion. B. Luciferase intensity of orthotopic xenografts of osteosarcoma and pulmonary tumors. C. Orthotopic xenografts of osteosarcoma and pulmonary tumors.

CXCR4 overexpressing MG-63 cells (overexpression group) (p>0.05). However, the number of photons on the lung in the CXCR4-overexpressing group was significantly higher than that in the control group (p<0.01, **Figure 3C**). After tumor removal, we found obvious tumors on both the left legs and lung in the control group and CXCR4-overexpression group (**Figure 3B**). Consistent with the bioluminescence assay, there were no significant differences between the size of OS in the left legs in the control and CXCR4 overexpression group (p< 0.05); however, significantly larger tumors were found in the lungs of the CXCR4-overexpressing

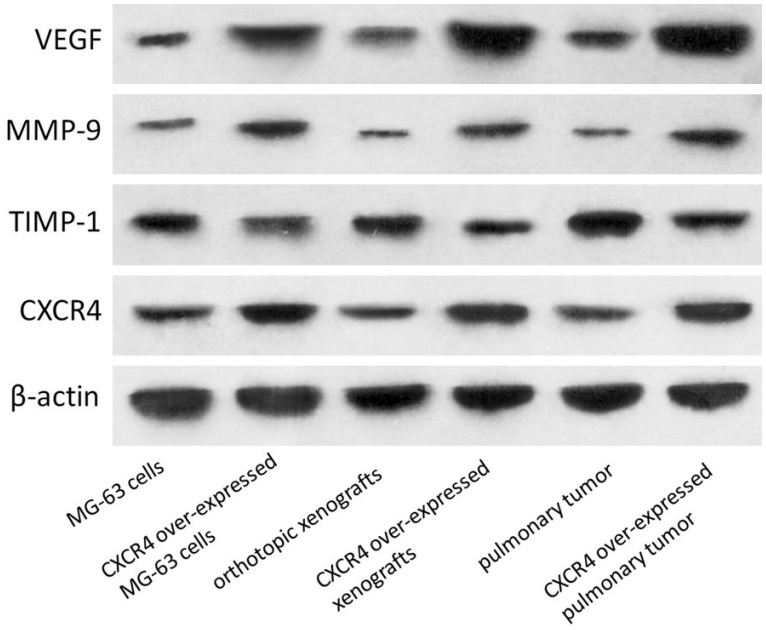

Figure 4. CXCR4 overexpression increased VEGF and MMP-9 but reduced TIMP-1 expression. Group MG-63 cells and CXCR4 over-expressed MG-63 cells were in vitro experiments; group orthotopic xenografts, CXCR4 over-expressed orthotopic xenografts, pulmonary tumor and CXCR4 over-expressed pulmonary tumor were in vitvo experiments.

group compared to the control group ($p<0.05$, **Figure 3C**).

CXCR4 overexpression promotes the expression of VEGF and MMP-9 but reduces TIMP-1 expression

To explore the underlying mechanisms of the enhancement of OS metastasis by CXCR4, we analyzed the protein expression of VEGF, MMP-9, TIMP-1 and CXCR4 by western blotting in cultured MG-63 cells, mouse orthotopic xenografts of osteosarcomas and pulmonary metastatic tumors, respectively. The expression of VEGF and MMP-9 in MG-63 cells with CXCR4 overexpression were higher than those in the control. However, TIMP-1 in MG-63 cells with CXCR4 overexpression was significantly lower than that in the control. Similar, VEGF, MMP-9 and TIMP-1 protein expression patterns were found in orthotopic xenografts and pulmonary tumors (**Figure 4**).

Discussion

Patients with OS usually have a low 5-year survival rate predominantly due to metastasis. Metastasis is an extremely complicated multistep process, and its mechanism has not yet been completely elucidated. Cytokines and their receptors have crucial roles in the process of tumor metastasis [11-13]. Mueller and coworkers initially discovered the important role of the CXCR12-CXCR4 homing axis in tumor metastasis [14]. They found that CXCR4-expressing breast cancer cells preferentially spread to the lung, which expresses abundant CXCR12 [14]. Studies have shown that CXCR4 promotes the development and progression of various cancers, including hematopoietic, epithelial and mesenchymal cancers [15-20]. Fuchs and coworkers recently found that metastasis to the lung of intratibial human OS xenografts in mice was suppressed by CXCR4 antibody treatment [3]. These advances suggest an important role for the CXCR4-CXCR12 signaling axis in the metastasis of OS and CXCR4-CXCR12 as a novel target for OS treatment.

The present study investigated the effect of CXCR4 overexpression on the metastasis of MG-63 cells and osteosarcoma xenografts in a mouse model. CXCR4 overexpression enhanced the invasion of MG-63 cells. The size and luciferase intensity of orthotopic xenografts with CXCR4 overexpression were larger and higher than normal orthotopic xenografts but were not significantly different. Consistent with the results from a previous study [3], significantly higher luciferase intensity and more pulmonary tumors were observed in orthotopic xenografts with CXCR4 overexpression, suggesting that CXCR4 preferentially promotes orthotopic OS metastasis to the lungs.

VEGF is a vascular endothelial cell specific growth factor and plays a crucial role in the induction of new blood vessels in the body [8, 21]. MMP-9 is a member of MMPs that degrade a variety of proteins in the extracellular matrix and destroy the histologic barrier in the process of tumor cell invasion and metastasis [22, 23]. TIMP-1, an inhibitor of metalloproteinase, specifically inhibits the activity of MMP-9,

thereby preventing tumor invasion and metastasis [24, 25]. We analyzed the changes in the expression of those metastasis-related cytokines after CXCR4 overexpression both in vitro and in vivo. Significantly higher expressions of VEGF and MMP-9 and a lower expression of TIMP-1 were observed in MG-63 cells with CXCR4 overexpression. The same expression pattern was found in orthotopic xenografts and pulmonary tumors. These results suggest that CXCR4 may promote OS tumor metastasis at least in part by enhancing the expression of VEGF and MMP-9 while suppressing TIMP-1 expression. However, the molecular mechanism by which the CXCR4-CXCR12 signaling axis regulates these cytokines warrants further investigation. In conclusion, the present results demonstrated that CXCR4 promotes metastases to the lung of human osteosarcoma xenografts in mice, likely through regulating the expression of metastasis-related cytokines such as VEGF, MMP-9 and TIMP-1. Our findings suggest that the development of CXCR4-blocking reagents is a promising approach for the treatment of metastasizing OS in combination with current treatment modes.

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Disclosure of conflict of interest

None.

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