Knockdown of CXCR7 inhibits proliferation and invasion of osteosarcoma cells through inhibition of the PI3K/Akt and β-arrestin pathways

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Abstract. CXC chemokine receptor 7 (CXCR7) has been implicated in tumor development and metastasis in multiple malignancies. Yet, the function and molecular mechanisms of CXCR7 in human osteosarcoma (OS) are still unclear. The aim of the present study was to investigate the role of CXCR7 in human OS. The expression of CXCR7 was assessed by immunohistochemical assay using a tissue microarray procedure in 45 cases of OS tissues. A loss-of-function approach was used to observe the effects of lentiviral vector-mediated CXCR7 siRNA (Lv-siCXCR7) on biological behaviors including proliferative activities and invasive potential, as indicated by MTT and Transwell assays in OS (MG-63 and U-2 OS) cells. The results showed that the expression of CXCR7 protein in OS tissues was significantly increased compared to that in adjacent non-cancerous tissues (68.9 vs. 53.3%, P=0.033), and was correlated with the distant metastasis of the tumors (P=0.004). Knockdown of CXCR7 suppressed proliferation and invasion of OS cells through decreased expression of PI3K, AKT, β-arrestin, proliferating cell nuclear antigen (PCNA), and matrix metalloproteinase-9 (MMP-9). In addition, the tumor volume in U-2 OS subcutaneous tumor models treated with Lv-siCXCR7 was significantly smaller than the tumor volume in the negative control group (P<0.01). Collectively, our findings indicate that upregulation of CXCR7 expression is correlated with distal metastasis of OS, while knockdown of CXCR7 blocks the development of OS cells through inhibition of the PI3K/AKT and β-arrestin pathways, suggesting that CXCR7 may serve as a potential therapeutic target for the treatment of cancer.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone carcinoma in children and adolescents (1). Due to the high propensity for lung metastasis, survival of OS patients has not improved over the past 30 years (2). A novel therapeutic strategy is needed for intervention in regards to the unfavorable prognosis and ineffective treatment for OS patients. Cancer is a genetic disease and tumors develop through a multi-step process. Single or multiple mutations in genes related to growth control and metastasis form the molecular genetic basis of malignant transformation and tumor progression. Therefore, identification of molecular targets that can be exploited in the clinic to treat metastatic disease is desperately needed. Chemokines and chemokine receptors have been described to act as important targets for the treatment of cancer (3).

Chemokines, a family of small cytokines that bind to specific G-protein-coupled seven-span transmembrane receptors, play an important role in many physiological and pathological processes (4). In addition to their initially revealed function in cell trafficking and adhesion, CXC chemokine receptors (CXCRs) have been reported to participate in the development, progression and metastasis in a variety of malignant tumors (5). CXC chemokine receptor 7 (CXCR7), as the receptor for CXCL12 with higher binding affinity, is overexpressed in glioma and regulates tumor proliferation via the ERK and AKT pathways (6,7). Based on the cellular localization, positive expression of CXCR7, mainly detected in the cytoplasm, mediates anti-apoptotic effects in glioma cells and indicates a poor prognosis in gallbladder cancer (8,9) and renal cell carcinoma (10). These data suggest that CXCR7 is a promising therapeutic target.

Furthermore, the association between CXCR7 expression and clinical and pathological features has been analyzed, indicating that CXCR7 is associated with tumor grade and differentiation. CXCR7 exerts a potential function in tumor progression in pancreatic adenocarcinoma (11) and predicts poor disease-free and disease-specific survival in cervical cancer (12). Some studies have shown that several signaling pathways such as CXCR4 and EGFR assist in CXCR7 signaling transduction promoting tumorigenesis, and that CXCR7 coordinates β-arrestin-2 and K-Ras-dependent pathways involved...
in the development of pancreatic cancer (13). Stromal-derived factor (SDF)-1-mediated CXCR7 signaling has been found to be expressed in bladder and thyroid cancers and to promote cell migration and metastasis (14,15). Thus, targeted inhibition of CXCR7 may provide an effective therapeutic strategy for the treatment of cancer.

However, few studies show that CXCR7 is weakly expressed in malignant hematopoietic cells, and has no enhancing effects on cell proliferation and migration (16). Moreover, it was found that CXCR7 can activate the PI3K/AKT, ERK and STAT3 pathways facilitating the development of bladder cancer (17). To elucidate the function and molecular mechanisms of CXCR7 in human OS, we examined the expression of CXCR7 by immunohistochemical assay using a tissue microarray procedure in OS tissues, and constructed lentiviral vector-mediated Lv-siCXCR7 to observe the effects of CXCR7 on the biological behaviors of OS cells through regulation of the PI3K/AKT and β-arrestin pathways.

Materials and methods

Materials. The OS cell lines (MG-63 and U-2 OS) used for the experiments were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Lentiviral-mediated CXCR7 siRNA (Lv-siCXCR7), negative control vector, and virion-packaging elements were purchased from GeneChem (Shanghai, China). Human OS tissues and the corresponding adjacent non-cancerous tissues (ANCT) were collected from patients at the Department of Orthopedic Surgery, Changzheng Hospital. The tissue microarray of OS was made by Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). All of the antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). CXCR7 primer was synthesized by ABI (Framingham, MA, USA).

Drugs and reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA); TRizol reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was purchased from Promega (Madison, WI, USA); SYBR-Green Master Mix was obtained from Takara (Otsu, Japan); and the reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA); SYBR-Green Master Mix was obtained from Takara (Otsu, Japan); and the ECL Plus kit was obtained from GE Healthcare (Piscataway, NJ, USA).

Clinical samples and data. The tissue microarray was prepared for immunohistochemical analysis (IHC). Human OS tissues and the corresponding ANCT were obtained from biopsies in a total of 45 consecutive OS cases admitted to our hospital from January 2005 to December 2011. The baseline characteristics of the patients before neo-adjuvant chemotherapy are summarized in Table I. The study was approved by the Medical Ethics Committee of the Second Military Medical University and written informed consent was obtained from the patients or their parents before sample collection. Two pathologists respectively reviewed all of the cases.

Tissue microarray. The advanced tissue arrayer (ATA-100; Chemicon International, Temecula, CA, USA) was used to create holes in a recipient paraffin block and to acquire cylindrical core tissue biopsies with a diameter of 1 mm from the specific areas of the ‘donor’ block. The tissue core biopsies were transferred to the recipient paraffin block at defined array positions. The tissue microarrays contained tissue samples from 45 formalin-fixed paraffin-embedded cancer specimens with known diagnosis, and the corresponding ANCT from these patients. The block was incubated in an oven at 45°C for 20 min to allow complete embedding of the grafted tissue cylinders in the paraffin of the recipient block, and then stored at 4°C until microtome sectioning.

Immunohistochemical staining. Tissue microarray sections were processed for IHC analysis of CXCR7 protein as follows. Immunohistochemical examinations were carried out on 3-mm sections. For anti-CXCR7 immunohistochemistry, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min. For anti-CXCR7 immunohistochemistry, antigen unmasking was not necessary. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity, and then in blocking serum [0.04% bovine serum albumin (A2153; Sigma-Aldrich, Shanghai, China) and 0.5% normal goat serum (X0907; Dako Corporation, Carpinteria, CA, USA) in phosphate-buffered saline (PBS)] for 30 min at room temperature. Anti-CXCR7 antibody was used at a dilution of 1:200. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked with 0.5% casein and 5% normal serum for 30 min at room temperature. Finally, staining was developed using diaminobenzidine substrate, and the sections were counterstained with hematoxylin. Normal serum or PBS was used to replace the anti-CXCR7 antibody in the negative controls.

Quantification of protein expression. The expression of CXCR7 was semi-quantitatively estimated as the total immunostaining scores, which were calculated as the product of a proportion score and an intensity score. The proportion and intensity of the staining were evaluated independently by two observers. The proportion score reflected the fraction of positive staining cells (0, none; 1, ≤10%; 2, >10 to ≥25%; 3, >25 to 50%; 4, >50%), and the intensity score represented the staining intensity (0, no staining; 1, weak; 2, intermediate; and 3, strong). Finally, a total expression score was given ranging from 0 to 12. Based on the analysis in advance, CXCR7 expression was categorized into two groups: low-level CXCR7 expression (score 0-3) and high-level CXCR7 expression (score 4-12). The scoring was independently assessed by two pathologists.

Cell culture and transfection. OS cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Cells in this medium were placed in a humidified atmosphere containing 5% CO2 at 37°C. Cells were subcultured at a 1:5 dilution in medium containing 300 µg/ml G418 (an aminoglycoside antibody; commonly used stable transfection reagent in molecular genetic testing). On the day of transduction, OS cells were replated at 5×104 cells/well in 24-well plates containing serum-free growth medium with polybrene (5 mg/ml). When
reaching 50% confluency, cells were transfected with recombinant experimental virus or control virus at the optimal MOI (multiplicity of infection) of 50, and cultured at 37°C in 5% CO₂ for 4 h. The supernatant was then discarded and serum containing growth medium was added. At 4 days post-transduction, the transduction efficiency was measured by the frequency of green fluorescent protein (GFP)-positive cells. Positive and stable transfectants were selected and expanded for further study. The Lv-siCXCR7 vector-infected clone and the negative control vector-infected cells were designated as the Lv-siCXCR7 and the NC group.

Quantitative real-time PCR. To quantitatively determine the mRNA expression level of CXCR7 in OS cells, real-time PCR was performed. Total RNA was extracted from each clone using TRIzol according to the manufacturer's protocol. Reverse transcription was carried out using M-MLV and cDNA amplification was performed using the SYBR-Green Master Mix kit according to the manufacturer's guidelines. The CXCR7 gene was amplified using a specific oligonucleotide primer, and the β-actin gene was used as an endogenous control. The PCR primer sequences were as follows: CXCR7, 5'-CACCGCATCTCCTCAGACTCAAGAGATCTCTTTTTTGG-3' and 5'-GATCCAAAAAGCATCTCTTCGACTACTCAGATTCAAGAGATC-3'; β-actin, 5'-CAACGGATGTCGAGATGTCAGTCTCTTTTGG-3' and 5'-GATCCAAAAAGCATCTCTTCGACTACTCAGATTCAAGAGATC-3'. Data were analyzed using the comparative Ct method (2-ΔΔCt). Three separate experiments were performed for each clone.

Western blot assay. OS cell lines were harvested and extracted using lysis buffer (Tris-HCl, SDS, mercaptoethanol and glycerol). Cell extracts were boiled for 5 min in loading buffer, and then an equal amount of cell extracts was separated on 15% SDS-PAGE gels. Separated protein were detected in OS (Fig. 1A and B) and ANCT tissues (Fig. 1C and D). Positive CXCR7 immunostaining were detected in OS (Fig. 1A and B) and ANCT tissues. The different levels of positive expression of CXCR7 was used when the probability for ANOVA was statistically significant. Statistical significance was set at P<0.05.

Results

Expression of CXCR7 in OS tissues. The expression of CXCR7 protein was evaluated using IHC staining in OS tissues. The different levels of positive expression of CXCR7 protein were detected in OS (Fig. 1A and B) and ANCT tissues (Fig. 1C and D). Positive CXCR7 immunostaining...
was mainly localized in the cytoplasm of OS tissue cells. According to the CXCR7 immunoreactive intensity, the positive expression of CXCR7 in OS tissues was significantly increased compared with that in ANCT (P=0.033) (Table II).

### Correlation of CXCR7 expression with clinicopathological parameters

The association of CXCR7 expression with various clinical and pathological factors was analyzed. As shown in Table I, increased expression of CXCR7 in OS tissues was closely correlated with the distant metastasis of OS (P=0.004). However, no significant association was found between CXCR7 expression and other factors including age, gender, histology and Ennecking staging of the tumor (all P>0.05).

### Effect of CXCR7 knockdown on the expression of PI3K, AKT and β-arrestin

First, lentiviruses of different multiplicity of infections (MOIs) were used to transfect OS cell lines (MG-63 and U-2 OS), and the transfection efficiency of Lv-siCXCR7 (MOI=50) was >65%. After Lv-siCXCR7 was transfected into the OS cell lines for 24 h, the expression levels of CXCR7 mRNA (Fig. 2A and B) and protein (Fig. 2C and D), p-PI3K, and p-AKT and β-arrestin proteins (Fig. 2E and F) were detected by real-time PCR and western blot assays, indicating a reduced expression of CXCR7, p-PI3K, p-AKT and β-arrestin in the Lv-siCXCR7 group when compared with the levels in the NC group.

### Effect of CXCR7 knockdown on cell proliferation

Deregulated cell proliferation is a hallmark of cancer. To verify the effect of CXCR7 on tumor growth in OS cells, we examined cell proliferative activities by MTT assay. The results showed that knockdown of CXCR7 diminished the proliferative activities of OS cells in a time-dependent manner compared to the NC group (Fig. 3A and B). In addition, the expression level of PCNA protein, examined by western blot assay (Fig. 3C and D), was found to be significantly downregulated in the Lv-siCXCR7 group compared with the NC group.

### Table I. Correlation of CXCR7 expression with clinicopathological factors of the OS patients.

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CXCR7, CXC chemokine receptor 7; OS, osteosarcoma.

### Table II. Expression of CXCR7 protein in OS tissues.

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OS, osteosarcoma; ANCT, adjacent non-cancer tissue; CXCR7, CXC chemokine receptor 7.
Effect of CXCR7 knockdown on cell invasion. To determine the effect of CXCR7 on cell invasion, a Transwell assay was performed. The invasive potential of tumor cells in the Transwell assay was determined by the ability of cells to invade a matrix barrier containing laminin and type IV collagen, the major components of the basement membrane.
Representative micrographs of the Transwell filters are shown in Fig. 4A and B. The invasive potential of the OS cells was apparently weakened in the Lv-siCXCR7 group compared to the NC group (P<0.01). The expression level of MMP-9 protein examined by western blot assay was downregulated in the Lv-siCXCR7 group when compared with the level in the NC group. CXCR7, CXC chemokine receptor 7; Lv-siCXCR7, lentiviral vector-mediated CXCR7 siRNA; MMP-9, metalloproteinase-9; NC, negative control.

Effect of CXCR7 knockdown on xenograft tumor growth. Our in vitro experiments demonstrated an inhibitory effect of CXCR7 knockdown on OS growth. Therefore, we further...
investigated the effect of CXCR7 on U-2 OS xenograft tumor growth in vivo. The mean volume of the tumors in the experimental mice before treatment was 68.32±14.25 mm<sup>3</sup>. During the entire tumor growth period, the tumor growth activity was determined. The tumors treated with Lv-siCXCR7 grew substantially slower compared to the NC group (Fig. 5A). When the tumors were harvested, the average weight and volume of the tumors in Lv-siCXCR7 group were significantly reduced when compared with these parameters in the NC group (Fig. 5B and C), suggesting that CXCR7 knockout blocked the growth of OS cells.

Discussion

CXCR7, a chemokine receptor, has been previously demonstrated to have an impact on growth, apoptosis, invasion/metastasis and prognosis in many types of cancers. CXCR7 was found to be differentially expressed in pancreatic cancer, while knockdown of CXCR7 resulted in decreased migration and invasion (18). The expression of CXCR7 was found to be upregulated in renal cell (10) and human hepatocellular carcinoma (19), and human brain tumors (20). Overexpression of CXCR7 was found to be associated with metastasis and poor survival of patients with hepatocellular carcinoma, indicating its potential function as a therapeutic target (21). CXCR4 and CXCR7 are frequently co-expressed in human cancer tissues and cell lines (12,13). Given the demonstrated importance of CXCR7 in tumor pathogenesis, we further examined the expression of CXCR7 in human OS tissues. The present study showed that CXCR7 was markedly upregulated in the cytoplasm of cells in the OS tissues when compared to the cells in the adjacent non-tumor tissues, and was positively correlated with distant metastasis of OS, suggesting that CXCR7 may represent a new biomarker involved in the development of OS.

Furthermore, the function of CXCR-7 in cancer warrants further research. Some studies have shown that CXCR-7 and IL-8 can contribute to tumor proliferation and metastasis in renal cell carcinoma (22). Activation of CXCR7 on tumor blood vessels facilitates the progression of colon cancer with lung metastasis (23). High expression of CXCR7 also leads to progression, metastasis and angiogenesis in brain tumors, and regulates the transendothelial migration in lymphoma (24,25). To confirm the function of CXCR7 in cancer, our present study showed that knockdown of CXCR7 suppressed the proliferation and invasion of OS cells. Likewise, the findings of Zheng et al support our study, indicating that targeted inhibition of the CXCR7 axis suppresses proliferation, invasion and angiogenesis in hepatocellular carcinoma (26), suggesting that CXCR7 may be a critical therapeutic target for the treatment of cancer.

In response to the regulatory mechanisms of CXCR7 in cancer, a few studies have shown that MAPK, ERK and β-arrestin-linked signaling pathways are recruited and activated by the CXCR4-CXCR7 complex implicated in cell migration (27). The PI3K/AKT pathway mediates the CXCR7 signaling regulating cell survival and prognosis in head and neck cell carcinoma (28). CXCR7 promotes proliferation and motility in bladder cancer through activation of the AKT and ERK pathways (17,29). Therefore, further study needs to be carried out to investigate the regulatory mechanisms of CXCR7 in OS cells. Our present findings indicated that knockdown of CXCR7 downregulated the expression of p-PI3K, p-AKT and β-arrestin in OS cells, suggesting that CXCR7 may be involved in the progression of OS via regulation of the PI3K/AKT and β-arrestin pathways.

PCNA is a nuclear protein that is expressed in proliferating cells and may be required for maintaining cell proliferation, and may be used as a marker for cell proliferation of colon cancer (30). MMP-9 is thought to be a key enzyme involved in the degradation of type IV collagen, and a high level of MMP-9 in tissues is associated with tumor invasion and metastasis (31). It has been reported that the AKT and β-arrestin pathways promote growth and invasion of tumor cells via regulation of PCNA and MMP-9 expression (32). In the present study, it was found that knockdown of CXCR7 also downregulated the expression of PCNA and MMP-9 in OS cells, suggesting that CXCR7 may promote the progression of OS through AKT and β-arrestin pathway-mediated regulation of PCNA and MMP-2 expression.

In conclusion, our findings indicate that upregulation of CXCR7 expression is correlated with distant metastasis of OS, and knockdown of CXCR7 inhibits the growth and invasion of OS cells through inhibition of the PI3K/AKT and β-arrestin pathways, suggesting that CXCR7 may serve as a potential therapeutic target for the treatment of cancer.

References


