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ORIGINAL ARTICLE

Expression of CXCL12-CXCR4 in osteosarcoma and its correlation with angiogenesis

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Abstract

Objective The expression of CXCL12 (stromal cell-derived factor-1)-CXCR4 (chemokine receptors-4) in osteosarcoma and its role in angiogenesis were examined.

Methods The expression of CXCR4 and CXCL12 in 40 cases of osteosarcoma was detected by immunohistochemistry and real-time fluorescence quantitative PCR. The expression of CD34 in osteosarcoma was detected by immunohistochemistry. Morphometric image analysis was performed to measure microvessel density (MVD). Additionally, the relationship between CXCL12 and CXCR4 expression and MVD of osteosarcoma and pulmonary metastasis were analyzed.

Results The positive rates of CXCL12 and CXCR4 protein expression in osteosarcoma were 40.0% (16/40) and 60.0% (24/40), respectively. Fluorescence quantitative real-time PCR indicated that the expression level of CXCR4 mRNA in pulmonary metastatic osteosarcoma was higher than that in non-pulmonary metastatic osteosarcoma ($P < 0.01$). The level of MVD in pulmonary metastatic osteosarcoma was higher than that in non-pulmonary metastatic osteosarcoma ($P < 0.01$).

Conclusion The expression level of CXCR4 was significantly associated with pulmonary metastasis and angiogenesis of osteosarcoma.

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Keywords: osteosarcoma; stromal cell-derived factor-1 (CXCL12); chemokine receptors-4 (CXCR4) angiogenesis; pulmonary metastatics

Metastasis is a complex, non-randomized, and multi-step process that involves critical steps such as tumor cell motility, adhesion, invasion, growth, angiogenesis, metastasis-specific organ homing [1], and escape from the immune system [2]. In recent years, many studies have indicated that the biological axis constituted by stromal cell-derived factor-1 (CXCL12) and chemokine receptors-4 (CXCR4) plays an important role in a variety of tumor and organ-specific metastases [3–6]. In this study, we analyzed CXCL12/CXCR4 expression levels in osteosarcoma and their relationship with angiogenesis.

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Materials and Methods

Clinical data

Osteosarcoma primary tumor specimens from 40 patients (22 males, 18 females) were collected. Patients ranged in age from 7 to 45 years, with a mean age of 25.5 years. All samples were collected after obtaining informed consent, with complete clinical data. No patients underwent radiotherapy or chemotherapy before surgery. All cases were confirmed by histology. Among these cases, 16 had lung metastasis and 24 were non-metastatic. Nineteen cases occurred in the femur, 12 in the tibia and fibula, and 9 in the humerus. Histological classification of bone tumors was determined based on WHO classification [7]: 20 cases were in osteoblasts, 10 in cartilage cells, 8 in fibroblasts, and 2 were small cell type.

Immunohistochemistry SP detection

Mouse anti-human CXCL12 monoclonal antibody, mouse anti-human CXCR4 monoclonal antibody, and mouse anti-human CD34 monoclonal antibody were obtained from DAKO Cytomation (Glostrup, Denmark). The S-P kit was purchased from Fuzhou Maixin Company (Fujian, China). Breast cancer specimens were used as positive controls and phosphate-buffered saline as a negative control for the primary antibodies. Immunohistochemical SP staining was conducted according to the kit instructions. Immunohistochemical staining slides were evaluated by two pathologists. Ten fields were counted on each slide at 400× magnification. The percentage of positive cells was determined. Less than 10% were negative, 11%–25% were weakly positive, 25%–50% were middle positive, and 50% were strongly positive. Based on the intensity of staining, no color was considered negative, light brown yellow as weak positive, brown yellow as middle positive, and brown as strong positive. The number of microvascular vessels for 5 fields was counted at 400× magnification and considered the microvessel density (MVD) of each sample.

Real-time fluorescence quantitative PCR

We used the 7500 real-time fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) for SYBR real-time fluorescent quantitative PCR detection to analyze CXCL12 and CXCR4 expression in osteosarcoma; human β-actin [220 base pairs (bp)] was used as an internal reference. The PCR primer sequences and lengths of the amplified fragment were as follows: CXCL12 forward: 5'-CCGCGCTCTGCCTCAGCGACGG GAAG-3', CXCL12 reverse: 5'-CTTGTTTAAAGCTTTCT CCAGGTACT-3' (227 bp); CXCR4 forward: 5'-AGCTGTT GGTGAAAAGGTGGTCTATG-3', CXCR4 reverse: 5'-GC GCTTCTGGTGGCCCTTGGAGTGTG-3' (260 bp); β-actin forward: 5'-CCCAAGGCCAACCGCGAGAAGAT-3', β-actin reverse: 5'-GTCCCGGCCAGCCAGGTCCAG-3' (220 bp).

The reaction conditions for PCR were as follows: 1 cycle at 95 °C for 10 s, 40 cycles at 95 °C for 1 s, 40 cycles at 56 °C for 5 s, and 40 cycles at 72 °C for 35 s. After the reaction, the critical point set in the PCR amplification was determined. The initial fluorescence signal compared to that of the index growth phase at the inflection point corresponding to the number of cycles (threshold cycle, CT) was used as an indirect indicator of the initial concentration of template. The different concentrations of standard template versus the corresponding CT values were plotted to obtain a standard curve.

Statistical analysis

We used SPSS version 10.0 software (SPSS, Inc., Chicago, IL, USA) to analyze the experimental data. A critical value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CXCL12 and CXCR4

CXCL12 protein was mainly located in the cell membrane and/or cytoplasm and showed brown granular staining (Fig. 1). CXCR4 was strongly expressed in osteosarcoma of lung metastasis (Fig. 2) and weakly expressed in osteosarcoma of non-lung metastasis (Fig. 3). The positive rates of CXCL12 and CXCR4 protein expression in osteosarcoma were 40.0% (16/40) and 60.0% (24/40), respectively.

After real-time fluorescence quantitative PCR to detect CXCL12 and CXCR4 expression, we compared CXCL12 and CXCR4 mRNA copy numbers to that of β-actin. Melting curve analysis showed that the CXCR4 PCR melting curve peak was at 86.5 °C. The solution temperature was uniform and the peak was sharp (Fig. 4). The CXCL12 PCR melting curve peak was at 83.5 °C. The solution temperature was uniform and the peak shape was sharp (Fig. 5). All data agreed with the immunohistochemical SP staining results.

Relationship between CXCR4 expression and osteosarcoma lung metastases

In 24 cases of osteosarcoma without pulmonary metastasis, the positive expression of CXCR4 was 41.7%. In 16 patients with lung metastasis of osteosarcoma, CXCR4 expression was 87.5%. CXCR4 expression was significantly different between patients with and without lung distant metastasis ($P = 0.01$) (Table 1). There was no significant correlation with age, tumor size, sex, tumor location, and histological type (Table 2).

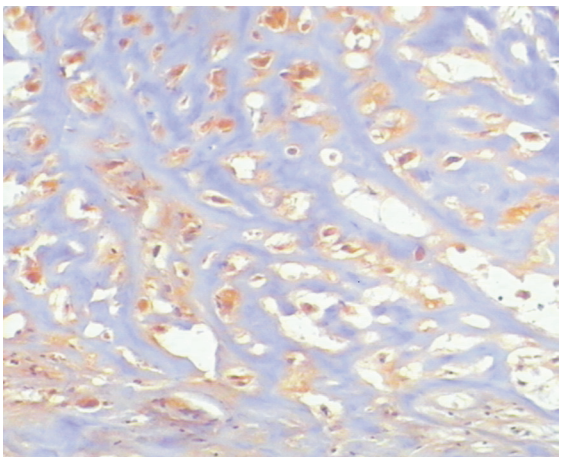

Fig. 1 CXCL12 positive osteosarcoma (×100)

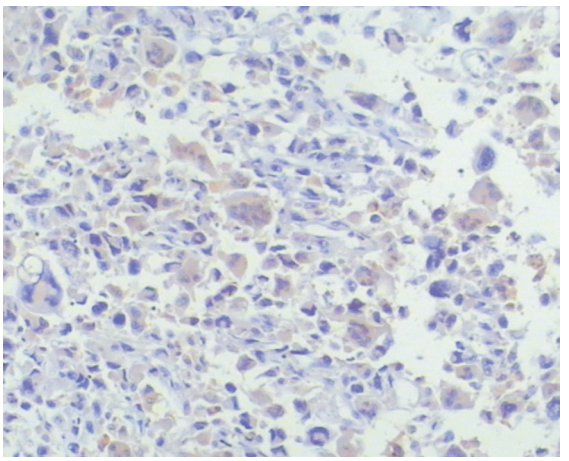

Fig. 2 CXCR4 strongly positive osteosarcoma (×100)

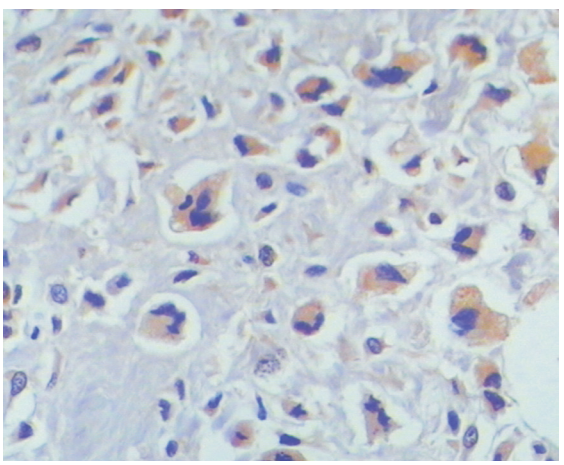

Fig. 3 CXCR4 weakly positive osteosarcoma (×100)

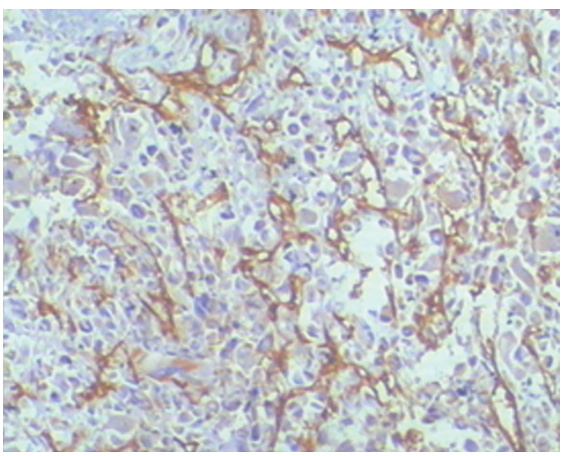

Fig. 6 CD34 positive microvascular endothelial cells (×100)

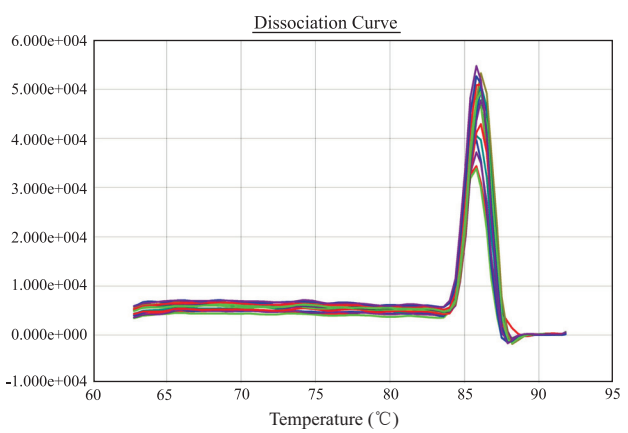

Fig. 4 CXCR4 melting curve

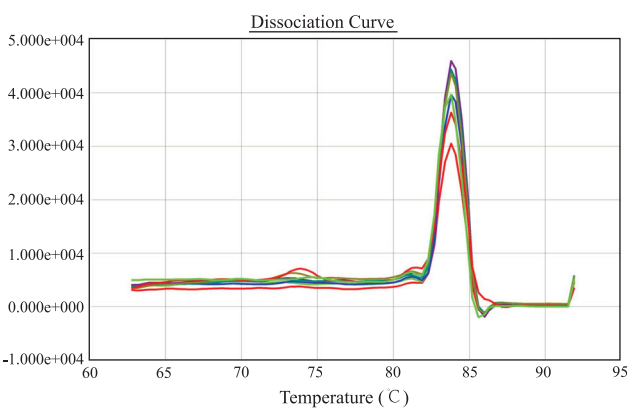

Fig. 5 CXCL12 melting curve

Table 1 Expression of CXCR4 in osteosarcoma (*n*)

Groups	*n*	CXCR4 ($P < 0.05$)	
		Negative	Positive (%)
No metastasis of osteosarcoma	24	14	10 (41.7)
Metastasis of osteosarcoma	16	2	14 (87.5)

Table 3 Relationship between MVD and pulmonary metastasis of osteosarcoma

Groups	*n*	MVD ($P < 0.01$)
No metastasis of osteosarcoma	24	64 ± 6.9
Osteosarcoma pulmonary metastasis	16	55 ± 7.0

Relationship between MVD expression and osteosarcoma lung metastases

CD34 was mainly localized in microvascular endothelial cells, according to immunohistochemical staining (Fig. 6). The shape of microvessels in osteosarcoma tissues was irregular and the distribution was uneven. The vascular around the tumor edge was dense, tufted, and sprouting. The microvessels were unevenly distributed. We found that MVD of patients with osteosarcoma lung metastasis was significantly higher than that in patients without metastasis ($P < 0.01$) (Table 3).

Discussion

Osteosarcoma is the most common malignant bone tumor in children and adolescents [8]. It may show local invasive growth or metastasis. The lung is the most common metastatic site for osteosarcoma [9]. Despite the use of chemotherapy and surgery, 40%–50% of patients experience lung metastases and the 5-year survival rate is only approximately 28% [10].

Muller *et al* [11] first reported human breast cancer cell lines with high expression of chemokine receptor CXCR4 and CCR7 in 2001. Primary breast cancer cells also highly express CXCR4 and CCR7. In breast cancer metastasis sites such as the lymph nodes, lung, liver, and bone marrow, the ligands CXCL12 and CCL21 (6Ckine) are highly expressed. Proteins not from skin and muscle but from lung and liver tissue show significant chemotaxis to breast cancer cells. Muller *et al* verified the hypothesis that tumor cells use chemokines and determined the relationship between cancer and chemokines. To clarify the mechanism of tumor metastasis to search new drugs with anti-metastatic mechanisms, Abu-Khalaf *et al* [12] conducted breast cancer microarray analysis of CXCR4 protein expression and cellular localization in tumor cells. They found that CXCR4 expression in the membrane and cytoplasm was associated with a low survival rate of patients. Another study [13] found that CXCR4 is highly expressed in gastric lymph nodes and

Table 2 Relationship between CXCR4 expression and clinical pathological factors of osteosarcoma (*n*)

Clinical pathological factors	*n*	CXCR4 expression		
		Negative	Positive	*P*
Gender				0.798
Male	22	10	12	
Female	18	8	10	
Age (year)				0.948
≤ 18	19	8	11	
> 18	21	8	13	
Tumor diameter (CM)				0.845
≤ 10	17	7	10	
> 10	23	9	14	
Tumor location				0.898
Femur	19	8	11	
Tibia and fibula	12	5	7	
Humerus	9	3	6	
Organization credit (WHO)				1.000
Osteoblast type	20	8	12	
Cartilage cell type	10	4	6	
Small cell type	2	1	1	
Fibroblast type	8	3	5	

lymphatic vessels. CXCR4 can induce chemotaxis and invasion of tumor cells. CXCR4+ cancer cell lines exhibit significant dose-dependent chemotaxis to CXCL12. High expression of CXCR4 may determine the direction and location of lymph node metastasis. CXCR4 expression detected by endoscopic biopsy may help predict lymph node metastasis and lymph node dissection to determine the surgery range.

In this study, the positive rates of CXCL12 and CXCR4 protein expression in osteosarcoma were 40.0% (16/40) and 60.0% (24/40), respectively. Real-time PCR confirmed these results. CXCR4 protein levels in patients with osteosarcoma lung metastases were significantly higher than those in patients without metastases ($P < 0.05$).

From the perspective of embryology and histology, generalized angiogenesis can be divided into vasculogenesis and angiogenesis. The former refers to endothelial mesoderm-derived precursor cells (endothelial progenitor cell, EPC) or vascular stem cells (angioblasts; also known as hemangioblastoma or hemangioblasts). Through differentiation and the cluster period, the original vascular network forms during repeated remodeling formation. The angiogenesis process occurs when endothelial cells in pre-existing mature tissue proliferate and migrate by sprouting or intussusceptions in new blood vessels. The concept of angiogenesis had a new breakthrough in 1997. Asahara *et al* [14] detected endothelial precursor cells in adult peripheral blood, indicating that angiogenesis in adults not only occurred during capillary endothelial

cell proliferation and migration, but also contributed angioblasts as vascular endothelial precursors. The formation of new blood vessels involves three steps: angiogenesis, which includes the formation of pre-mature somatic mesoderm tissues in the blood vessel; second, the cell enters the vascular tissue formed from the initial capillary network; third, trimming and transformation into a functional cycle network. Vascular endothelial growth factors such as vascular endothelial growth factor (VEGF) play important roles in the angiogenesis process.

Microvessel counting is commonly conducted to evaluate tumor angiogenesis. Polyclonal anti-CD34 antibody shows high sensitivity for labeling endothelial cells. Our study confirmed that CD34 was localized in microvascular endothelial cells. Microvessels in osteosarcoma exhibit irregular shapes and uneven distributions. The vascular around the tumor edge was dense, tufted, and sprouting as well as unevenly distributed. We found that MVD of patients with osteosarcoma lung metastasis was significantly higher than that of patients without metastasis ($P < 0.01$).

Recent studies [15] showed that CXCL12 and CXCR4 play important roles in solid tumor growth, metastasis, angiogenesis. Neovascularization is necessary for solid tumor growth. Generally, angiogenesis-promoting chemokines promote tumor formation and inhibiting these chemokines could be anti-tumorigenic. In the VEGF-CXCL12/CXCR4 chain, CXCL12 increases the expression of VEGF and VEGF increases CXCL12 expression. This forms an amplification circuit that is significantly affected by hypoxia. Salvucci *et al* [16] found that VEGF promotes the expression of CXCL12 in endothelial cells, as well as blocks CXCL12/CXCR4 function and has an anti-angiogenic effect. Koshiba *et al* [17] found that CXCL12 and the ligands of CXCR4 can promote the formation of blood vessels in the tumor and the migration of tumor cells, leading to rapid tumor growth. We found that MVD expression in the CXCL12-negative group was significantly higher than that in the positive group. This indicates that CXCL12 protein expression and MVD are significantly negatively correlated, which is inconsistent with the results of Salvucci. Cui *et al* [18] found that CXCR4-CXCL12 induced angiogenesis and lymph node metastases of pancreatic cancer and that adjacent tissues and pancreatic lymph nodes expressed moderate levels of CXCL12 protein. We hypothesize that the VEGF -CXCL12/CXCR4 adjusted chain is related not only to the tumor tissue but also the microenvironment.

Our study confirmed that CXCL12/CXCR4 may play important roles in the progression of osteosarcoma. These results should be confirmed in an animal model of osteosarcoma and *in vivo* and *in vitro* experiments are needed to further verify the CXCL12/CXCR4 mechanism in osteosarcoma.

Institutional review board statement

This case report was reviewed and approved by the Shandong Cancer Hospital & Institute Institutional Review Board.

Informed consent statement

The patients involved in this study gave written informed consent authorizing the use and disclosure of protected health information.

Conflict of interest

The authors indicated no potential conflicts of interest.

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