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Strong expression of CXCL12 is associated with a favorable outcome in osteosarcoma

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Hematogenous spread determines the outcome of osteosarcoma (OS) patients, but the pathogenesis of developing metastatic disease is still unclear. Chemokines are critical regulators of cell trafficking and adhesion, and have been reported to be aberrantly expressed and to correlate with an unfavorable prognosis and metastatic spread in several malignant tumors. The chemokine receptors CXCR4 and CXCR7 together with their common ligand CXCL12 form one of the most important chemokine axes in this context. To investigate a potential role of these chemokines in OSs, we analyzed their expression in a series of 223 well-characterized and pretherapeutic OS samples. Interestingly, we found the expression of CXCL12 and CXCR4 to correlate with a better long-term outcome and with a lower prevalence of metastases. These findings suggest a distinct role of CXCR4/CXCR7/CXCL12 signaling in the tumors of bone, as has also been previously described in acute leukemia. As many malignant tumors metastasize to bone, and tumor cells are thought to be directed to bone in response to CXCL12, OS cells expressing both CXCL12 and the corresponding receptors might be detained at their site of origin. The disruption of CXCR4/CXCR7/CXCL12 signaling could therefore be crucial in OSs for the migration of tumor cells from bone into circulation and for developing systemic disease.
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Osteosarcomas (OSs) are highly aggressive neoplasms generally affecting the metaphyses of long bones in children and adolescents.[1] As is seen in many other malignant tumors hematogenous spread primarily determines the patient's prognosis and explains why intense neoadjuvant and adjuvant chemotherapy protocols in addition to radical surgery benefit the outcome of patients significantly when compared with surgery alone.[2] Although 5-year survival rates of up to 50–70% can be achieved by multimodal therapy, a large group of patients are still left with a poor prognosis due to lack of effective treatment options.[3] Predicting the clinical course of OS patients can be achieved by assessing the response to chemotherapy histologically or, as our group recently proposed, by determining distinct chromosomal alterations already at the time of initial biopsy.[4,5] The identification of patients that may not respond to first-line chemotherapy or will be at higher risk of developing metastases is required for a more precise treatment stratification. However, the pathogenesis of systemic spread in OS has not been elucidated.

The chemokine receptors CXCR4, CXCR7 and their common ligand CXCL12 are major regulators

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of cell trafficking and adhesion, and are thought to mediate hematogenous metastases in several types of cancer. Consequently, they were proposed as potential biomarkers of tumor behavior and as therapeutic targets.[6] CXCL12 is known to be produced by osteoblasts and to act as a chemoattractant for CXCR4- and CXCR7-expressing cells.[7,8] Interestingly, CXCL12 is also expressed in several OS cell lines that were shown to adhere to CXCR4-positive prostate cancer cells *in vitro* and, therefore, were used as a model to explain the predilection of prostate cancer to metastasize to bone.[8] The CXCL12 receptor, CXCR4, is expressed by various types of human cancers or by distinct sub-populations of cancer cells including breast cancer, non-small-cell lung cancer, ovarian cancer and also OS. In most normal tissue types, however, CXCR4 is generally not expressed.[9–11] CXCR7 (RDC-1) was identified in 2005 as a novel decoy receptor for CXCL12 interacting with CXCR4–CXCL12 signaling.[12,13] Furthermore, CXCR7 was shown to contribute to neoangiogenesis in prostate cancer and to promote tumor cell proliferation in breast cancer and adenocarcinoma of the lung.[14,15]

The aim of our study was to investigate the role of CXCR4, CXCR7 and CXCL12 signaling in OS and in the context of systemic spread. For this purpose, we analyzed a well-characterized series of 223 OS samples that were obtained exclusively from patients before neoadjuvant treatment (diagnostic biopsies).

Materials and methods

Tissue Samples and Patients' Characteristics

All tissue samples were obtained from the archives of the Bone Tumor Reference Center at the University Hospital Basel and the Clinical Cooperation Group OS at the Helmholtz Zentrum Muenchen and comprised cases that were diagnosed between 1974 and 2010. Only samples from patients without prior treatment were included in the study ($n = 223$). Full patients' characteristics are presented in Table 1.

Table 1 Patients' characteristics

Parameter	*Number (*n*)*
Sex	208/223 (93%)[a]
Male	106
Female	102
Age at diagnosis	210/223 (94%)[a]
Average	22.9 years
Median	16.3 years
Range	4–88 years
Localization	212/223 (95%)[a]
Femur	103
Tibia	50
Humerus	17
Fibula	11
Pelvis	11
Other	20
Metastases (lung)[b]	202/223 (91%)[a]
Yes (total)	89
Yes (at initial diagnosis)	24
No	113
Time to metastases average	15.6 months
Time to metastases median	12.4 months
Time to metastases range	0–79 months
Follow-up	214/223 (96%)[a]
Average	65 months
Median	41.7 months
Range	0–286.7 months
Survival	214/223 (96%)[a]
Died	79
Alive	135
Response to chemotherapy[c]	146/223 (66%)[a]
Good	87
Poor	59

[a]X/223 (Y%) = data available in X cases (Y% of the total number of cases).
[b]There were no patients included with metastases to other organs that did not also have metastases to the lungs.
[c]Good response $\leq 10\%$ vital tumor cells in the resection specimen, poor response $\geq 10\%$ vital tumor cells.

Tissue Microarray Construction

Tissue samples were fixed in buffered 4% formalin, decalcified using EDTA if required, embedded in paraffin, and used to construct tissue microarrays. Briefly, hematoxylin–eosin-stained sections were made from each selected primary block (donor blocks) to define representative tissue regions. Tissue cylinders (0.6 mm in diameter) were then punched from the respective regions of the donor block with the use of a custom-made precision instrument (Beecher Instruments, Silver Spring, USA). The number of punches per patient ranged from 1 to 8 (average 3.5, median 4.0). When more than one punch was obtained, the additional punches were taken from different representative regions of the tumor. Tissue cylinders were transferred to two 25×35 mm paraffin blocks (recipient blocks) to assemble the tissue microarrays. The resulting blocks were cut into 3-μm sections that were transferred to glass slides by use of the Paraffin Sectioning Aid System (Instrumedics, Hackensack, USA). Subsequently, sections were used for immunohistochemistry.

Immunohistochemistry

To ensure proper immunoreactivity of tumor samples, immunohistochemistry for vimentin was performed according to routine protocols (Ventana BenchMark XT, Roche, Basel, Switzerland; CC1 pretreatment; prediluted antibody, clone V9, incubation 32 minutes at 37°C; DAB chromogen). Immunohistochemistry for CXCR4 was also conducted using the Ventana BenchMark XT system

(CC1 pretreatment; antibody from Epitomics, Roche, clone UMB2, dilution 1:500, incubation 32 min at 37°C; DAB chromogen), whereas the immunoreactions for CXCR7 (microwave pretreatment in citrate buffer for 5 min at 70°C; antibody from Proteintech, Lucerne, Switzerland, polyclonal, dilution 1:800, incubation over night at 4°C; DAB chromogen) and CXCL12 (microwave pretreatment in EDTA for 20 min at 100°C; antibody from R&D Systems, Abingdon, UK, clone 79018, dilution 1:300, incubation over night at 4°C; DAB chromogen) were performed using indirect immunoperoxidase procedures according to the manufacturer's instructions (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Optimal dilutions were predetermined in our laboratory.

Evaluation of Immunohistochemistry

Immunoreactivity for each protein was scored semiquantitatively by evaluating the number of positive cells over the total number of cells. Additionally, an intensity scale ranging from 0 for no, 1+ for weak and 2+ for strong staining was applied. All cellular compartments were evaluated separately including nuclear, cytoplasmic and membranous positivity. In cases with more than one punch per tumor the average expression was determined for further analyses. Punches that were not completely enclosed on the sections or showed artefacts due to sectioning were excluded from the analysis.

Statistical Analyses

Survival analyses were carried out using the Kaplan–Meier and Log-rank (Mantel-Cox) test. The differences in protein expression between patients with and without metastases were determined using the Mann–Whitney Test. Spearman's correlation was used to calculate the correlation between CXCR4, CXCR7 and CXCL12 expression. For multivariate survival time analysis a Cox regression model was applied. Only *P*-values <0.05 were considered statistically significant. All analyses were conducted using GraphPad Prism 5.0d (La Jolla, CA, USA) and SPSS 19 (IBM Corporation, Armonk, NY, USA).

Results

Expression of Vimentin

All but four cases showed strong and consistent immunoreactivity for vimentin. The four negative cases were excluded from the evaluation leaving a total of 219 OS cases for further analysis.

Expression of CXCR4, CXCR7 and CXCL12

All immunoreactions demonstrated a predominantly mixed cytoplasmic and membranous staining pattern. As a substantial fraction of cases showed strong (intensity 2+) and constant (> 90% positive tumor cells) immunoreactivity, only those cases were considered positive for the respective protein. Consequently, tumor samples with incomplete (<90% positive tumor cells), weak or total lack of immunostaining (intensity 1+ and 0) was regarded as negative (Figure 1, Table 2). In total, CXCR4 was evaluable in 159/219 (73%), CXCR7 in 198/219 (90%) and CXCL12 in 204/219 (93%) cases. Drop out of samples was mainly due to cutting artefacts and/or lack of sufficient amounts of tumor tissue per punch.

Correlation of Protein Expression and Patient's Survival

The 10-year survival rate (10-YSR) differed between CXCR4-positive and -negative cases (68 *vs* 57%, $P=0.1325$) but was nearly identical between CXCR7-positive and -negative tumors (57 *vs* 61%, $P=0.8158$). Both the comparisons did not reach statistical significance. The 10-YSR between CXCL12-positive and -negative cases, in contrast, differed more distinctively (63 *vs* 39%, $P=0.0068$, Figure 2) resulting in a statistically significant correlation between CXCL12 positivity and a favorable outcome. Combining CXCR4 and CXCL12 positivity yielded 10-YSR of 71% in positive and 56% in negative cases ($P=0.0675$).

Correlation of Protein Expression and Systemic Spread

The expression of CXCL12 was associated with a significantly lower prevalence of metastases ($P=0.02$, Table 3). In case of CXCR4 and CXCR7 expression, no statistically significant correlations could be determined ($P=0.65$ and $P=0.12$, respectively, Table 3).

Correlation of Protein Expression and Response to Chemotherapy

There were no statistically significant correlations between the expression of CXCR4, CXCR7 and CXCL12, and the response to chemotherapy ($P=0.13$, 0.35 and 0.08, respectively).

Correlation Between CXCR4, CXCR7 and CXCL12 Expression and Multivariate Survival Time Analysis

CXCL12 positivity showed a moderate positive correlation with CXCR7 ($r=0.31$) and CXCR4

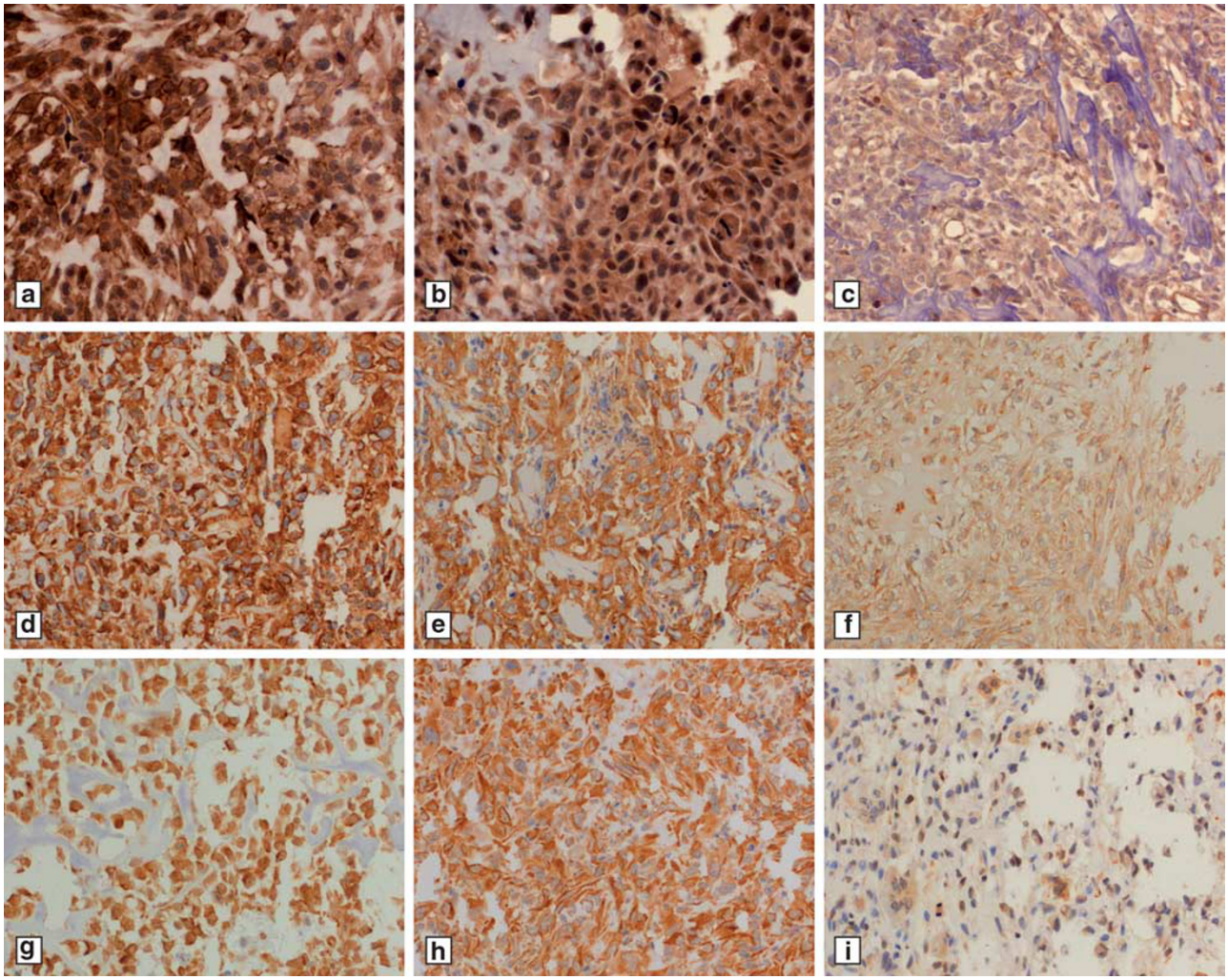

Figure 1 Immunohistochemistry for CXCR4 (**a–c**), CXCR7 (**d–f**) and CXCL12 (**g–i**) showed strong and constant membranous (**a**, **d**, **h**) and cytoplasmic (**b**, **e**, **g**) staining in positive cases. Weak (intensity 1+) and inconsistent staining was considered negative (**c**, **f**, **i**). All pictures ×400.

Table 2 CXCR4, CXCR7 and CXCL12 expression

	Positive	*Negative*	*Total*
CXCR4	78	81	159
CXCR7	167	31	198
CXCL12	158	46	204

($r=0.38$) expression, CXCR4 expression correlated weakly with CXCR7 positivity ($r=0.17$). Multivariate survival time analysis for metastases, response to chemotherapy and CXCL12 expression showed a highly significant effect only of metastases with poorer outcome (HR (95%CI): 59.81 (8.01–446.72; $P<0.0001$)). Testing solely response to chemotherapy, and CXCL12 expression resulted in a significant effect only of response to therapy with clinical outcome (HR (95%CI): 3.39 (1.71–6.74; $P<0.0001$)).

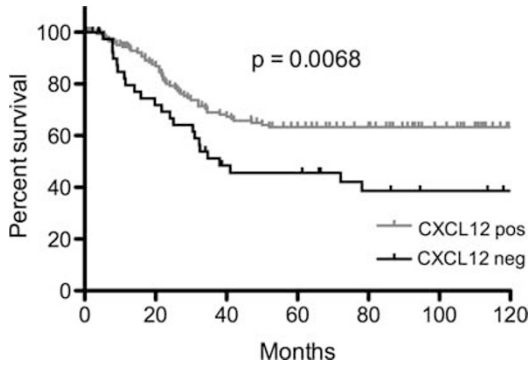

Figure 2 Kaplan–Meier curves comparing 10-year survival of osteosarcomas with and without CXCL12 expression.

Discussion

OS is a rare disease with an estimated incidence of 4–5 per million population.[1] In addition to its rarity, current treatment protocols make research on OSs

Table 3 CXCR4, CXCR7 and CXCL12 expression in cases with and without metastases

	Metastases	*No metastases*	*Total*	P-*value*
CXCR4 positive	29	42	71	
CXCR4 negative	33	41	74	0.65
CXCR4 total	62	83	145	
CXCR7 positive	65	86	151	
CXCR7 negative	16	11	27	0.12
CXCR7 total	81	97	178	
CXCL12 positive	56	86	142	
CXCL12 negative	25	17	42	0.02
CXCL12 total	81	103	184	

even more difficult because resection specimens have generally undergone neoadjuvant chemotherapy that can hamper molecular analyses. Therefore, only the usually scarce residual tissue from the initial diagnostic biopsies can be used for further investigations. To ensure optimal utilization of these valuable samples, tissue microarrays were constructed with a total of 223 well-characterized OSs. As the identification of novel biomarkers seems to be essential for a better treatment stratification, and for recognizing patients at high risk for systemic spread or chemoresistance, tissue microarrays represent an important tool for the analysis of large numbers of samples under the same technical conditions.

The chemokine receptors CXCR4, CXCR7 and their ligand CXCL12 have been shown to be aberrantly expressed in several malignant tumors and to be correlated with hematogenous spread and patient's prognosis.[8,9,14,15] Metastases to the bone are thought to be strongly influenced by CXCL12 signaling, as CXCL12 is known to be abundantly expressed by osteoblasts and bone marrow stromal cells.[7,16] In fact, also in several OS cell lines CXCL12 expression has been reported that was utilized in experimental models to demonstrate their adhesion to CXCR4-positive prostate cancer cells. The authors concluded the CXCR4/CXCL12 axis to decisively contribute to the predilection of prostate cancer to metastasize to bone.[8] A recent study, furthermore, demonstrated that ursolic acid can downregulate CXCR4 expression and inhibit distant organ metastasis in a transgenic mouse model of prostate cancer.[17] Interestingly, CXCR4 is known to be also expressed in several OS cell lines and has been reported to critically influence cell migration via CXCR4/CXCL12 interaction.[10,11] CXCR7 is another receptor for CXCL12 with at least partial decoy function that is thought to help moderate CXCR4/CXCL12 signaling. Although its precise function is still awaiting further elucidation, several studies indicate an important impact of CXCR7 on proliferation, vascularization and metastatic potential of breast, lung and prostate cancer cells.[14,15]

The role and function of CXCR4, CXCR7 and their common ligand CXCL12 in OSs has been analyzed in several smaller studies using different techniques. Although CXCR7 expression has been shown to occur in OS cell lines, studies investigating this receptor on OS tissue samples and correlations to clinicopathological parameters like systemic spread and patient's prognosis have not been reported yet.[18] The expression of CXCR4, however, has been analyzed at the protein and mRNA level by several groups. Oda *et al*[19] reported CXCR4 to be less commonly expressed in a series of 30 OSs compared with the corresponding lung metastases of the same patients using immunohistochemistry (33 *vs* 67%). Although they were not able to demonstrate a correlation between protein expression and patient's survival, they concluded CXCR4 expression to be associated with metastatic progression. A more recent study by Lin *et al*[20] showed immunohistochemical CXCR4 expression in 39/56 (70%) OS samples to correlate with a shorter survival and also with metastatic progression. In our study, we found 78/159 (49%) CXCR4-positive cases regarding only strong and constant immunoreactivity as positive. Interestingly, CXCR4 positivity showed a trend towards a favorable long-term outcome in our series (10-YSR 68 *vs* 57% in negative cases, $P=0.1325$). Our results are, however, not easy to compare with the previous reports as both the studies used different antibodies and did not describe what kind of decalcification was conducted, which can potentially alter the immunoreactivity of tissue samples.[19,20] Moreover, while Oda *et al*[19] did not define the cellular compartments evaluated, Lin *et al*[20] investigated only nuclear and cytoplasmic positivity. As functional chemokine receptors would be expected on the surface of cells, we explicitly also analyzed membranous staining in our series. Although we detected nuclear CXCR4 immunoreactivity in a smaller subset of cases (18/159, 11%), we were not able to demonstrate a correlation to patient's survival or hematogenous spread (data not shown). Concerning the transcriptional level, our group and others reported CXCR4 mRNA to be expressed even more constantly in microdissected OS tissue samples and cell lines, suggesting this chemokine receptor to be frequently expressed in OSs.[10,21,22] CXCL12 has only once been investigated immunohistochemically in a larger series of OSs and was found to be positive in just 4/113 (4%) of cases.[23] In contrast, we detected strong and constant immunoreactivity in 158/204 (78%) of cases in our series. Li *et al*[20] applied the same antibody used in our study, and although they did not state what kind of decalcification was performed or which cellular compartments were evaluated, this marked difference cannot be explained unequivocally. In our series, however, we found CXCL12 positivity to correlate with a better long-term survival (63 *vs* 39%, $P=0.0068$) and with a lower prevalence of metastases ($P=0.02$). The multivariate survival time analysis did not suggest CXCL12 expression to represent an independent prognostic factor in our

series but confirmed the occurrence of metastatic spread and the response to chemotherapy as the crucial prognostic factors in OS. Nevertheless, CXCL12 negativity was correlated with metastatic spread and might therefore indirectly contribute to the strong prognostic value of metastatic disease.

As most previous studies have found the expression of CXCR4 to correlate with an unfavorable outcome and systemic spread, our findings appear rather unexpected. However, in tumors originating in the bone marrow, CXCR4/CXCL12 interaction may help detain the neoplastic cells from systemic spread. In acute lymphoid and acute myeloid leukemia, CXCR4 and its ligand are thought to be responsible for the retention of leukemic cells in the bone marrow.[24,25] Consequently, the disruption of CXCR4/CXCL12 signaling is thought to be a prerequisite for these cells to egress from bone marrow into circulation.[26,27] OSs, however, are tumors of bone and are most likely derived from mesenchymal stem cells differentiating towards an osteoblastic lineage. As osteoblasts and OS cells have been shown to express CXCR4, CXCR7 and CXCL12, this specific chemokine axis could have a similar effect in OSs and prevent tumor cells from entering the circulation.[7,18,28] Certainly, these chemokine receptors and their common ligand are not the sole regulators of hematogenous spread but could have crucial impact on the development of metastatic disease.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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