

## Messenger RNA Expression Levels of CXCR4 Correlate with Metastatic Behavior and Outcome in Patients with Osteosarcoma

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**Abstract Purpose:** To determine if osteosarcoma cells express chemokine receptors and if their presence or absence relates to clinical features.

**Experimental Design:** Using fluorescent quantitative real-time PCR, the pattern of 17 chemokine receptors in 3 osteosarcoma cell lines and 68 osteosarcoma patient samples was analyzed.

**Results:** The expression of the chemokine receptors was generally low among the cell lines. In the high-grade osteosarcoma patient samples ( $n = 47$ ), CXCR4 was the most commonly expressed (63%) and its expression level was inversely correlated to overall survival ( $P < 0.0001$ ), event-free survival ( $P < 0.001$ ), and metastasis-free survival (MFS;  $P = 0.002$ ). There was also a correlation between the expression level of CXCR4 and the presence of metastasis at diagnosis ( $P = 0.002$ ). CCR7 was expressed in 43% of the samples and its expression level was inversely correlated with overall survival ( $P = 0.03$ ) and MFS ( $P = 0.007$ ). CCR10 mRNA expression level was inversely correlated with MFS ( $P = 0.009$ ). There was no association between the expression of CXCR4, CCR7, and CCR10. Of the 26 samples studied for stromal cell-derived factor-1 expression, 77% expressed it, but there was no correlation with the clinical variables or CXCR4 expression. Multivariate analysis revealed that mRNA expression level of CXCR4 was the only significant variable for overall survival ( $P = 0.0006$ ), event-free survival ( $P = 0.004$ ), and MFS ( $P = 0.025$ ).

**Conclusions:** These data suggest that CXCR4 could be useful as a prognostic factor and as a predictor of potential metastatic development in osteosarcoma. If further studies confirm that it is relevant to metastases in this disease, it could represent a new therapeutic target.

Chemokines are small molecules that regulate leukocyte trafficking and homing. Their receptors are seven-transmembrane, G-coupled proteins (1–3). Recent data suggest that the interactions between chemokines and their receptors are also critical components in the regulation of tumor progression and metastasis in breast cancer and other tumors (4, 5). The chemokine receptor CXCR4 and its ligand stromal cell-derived factor-1 (SDF-1) have been shown to mediate organ-specific metastasis by creating a chemotactic gradient between the primary tumor site and the metastatic site (4–6). Prostate and breast cancer cells might use the CXCR4/SDF-1 pathway

to localize to bones and develop metastasis (7–9). Many studies also suggest that the CXCR4/SDF-1 pathway is involved in the metastatic process of rhabdomyosarcoma, neuroblastoma, melanoma, glioblastoma, and colon, lung, pancreatic, ovarian, and thyroid carcinomas (10–17). In acute lymphoblastic leukemia, high expression of CXCR4 predicts extramedullary organ infiltration and, in acute myeloid leukemia, is a poor prognosis factor (18, 19). Müller et al. also found that two other chemokine receptors, CCR10 and CCR7, are highly expressed by melanoma and breast cancer cells, respectively (4). CCR7 and its ligand, CCL21, could have a role in lymph node metastasis in breast cancer and other carcinomas (4, 20, 21).

Osteosarcoma is a tumor with a high propensity for metastatic spread, mostly to lungs and bones. Fifteen percent to 20% of osteosarcoma patients have radiologically visible metastasis at diagnosis and 20% to 25% will develop metastasis despite treatment (22). Metastatic osteosarcoma is still associated with a very poor prognosis. The genetic characteristics of the “metastatic phenotype” of the osteosarcoma cells have not been clearly identified and biological prognostic factors in osteosarcoma are lacking (23).

In a study looking at chemokine receptors expression and function in two osteosarcoma cell lines (HOS and MG 63), Paoletti et al. showed that these cell lines released matrix-degrading enzymes in response to chemokine activation (24). Our hypothesis is that osteosarcoma cells might express and

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Received 6/03/04; revised 11/12/04; accepted 1/13/05.

**Grant support:** National Cancer Institute grant CA-83132, Foster Foundation, and National Cancer Institute of Canada with funds from the Terry Fox Run (C. Laverdiere).

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use the CXCR4/SDF-1 pathway and possibly other chemokine-chemokine receptor interactions to migrate to the lungs and bones. In this study, the pattern of expression of chemokine receptors in osteosarcoma was characterized and correlated with relevant clinical variables.

## Materials and Methods

**Sample collection and clinical data.** All osteosarcoma tumor samples were obtained at Memorial Sloan-Kettering Cancer Center (New York, NY) between February 1998 and December 2000 after obtaining written informed consent according to a biology study approved by the Memorial Hospital Institutional Review Board. A total of 68 samples were analyzed from 52 different patients. All samples were confirmed to have a pathologic diagnosis of osteosarcoma (62 high-grade osteosarcoma samples and 6 low-grade osteosarcoma samples). Patients' medical charts were reviewed retrospectively to collect the clinical information relevant for the analysis.

**Cell lines.** The following cell lines were used as reference cell lines for the chemokine receptor expression analysis and were purchased from the American Type Culture Collection: HOS, U2OS, and SAOS-2 (osteosarcoma); CCRF-CEM (acute T-cell leukemia); NK-92 (natural killer cell lymphoma); MCF-7 and MDA-MB-231 (breast cancer); and Malme-3-M (malignant melanoma and lung metastasis). The cell lines were maintained in the following medium at 37°C in a humid atmosphere of 5% CO<sub>2</sub>/95% air: HOS, U2OS, SAOS-2, CCRF-CEM, MCF-7, and MDA-MB-231 in MEM $\alpha$  medium with 10% fetal bovine serum; NK-92 in MEM $\alpha$  medium with 12.5% fetal bovine serum, 0.1 mmol/L 2-mercaptoethanol, and 100 units/mL recombinant interleukin-2; and Malme-3-M in Leibovitz's L-15 medium with 15% fetal bovine serum. Each medium (except the medium for Malme-3-M cells) contained 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin.

**RNA and DNA preparation of patient samples.** About 20 mg of fresh tumor tissue were frozen in Ultraspec reagent (Biotecx, Houston, TX) and stored at -80°C. Total RNA was isolated using the manufacturer's instructions with RNA resuspended in sterile, DEPC water. To reduce the risk of genomic DNA contamination, mRNA was subsequently isolated from the total RNA suspension using a modified protocol from the Roche mRNA Isolation Kit (Indianapolis, IN). In short, a mixture of biotin-labeled oligo(dT) probe (30 pmol), hybridization buffer, and 300  $\mu$ g streptavidin magnetic particles was separated using a magnet and the supernatant was discarded. The remaining magnetic particles were washed twice (wash buffer); the beads were resuspended in 20  $\mu$ L sterile DEPC water and incubated for 2 minutes at 70°C followed by magnetic separation. The supernatant (containing the mRNA) was immediately reverse transcribed using the Promega Reverse Transcription Kit (Madison, WI). After second strand synthesis, the cDNA was stored at -80°C.

**Quantitative real-time PCR.** The quantitative real-time PCR was done as described previously (25). The conditions were optimized for each set of primers and probe. For example, for CXCR4 gene expression analysis, 5  $\mu$ L cDNA were added to 20  $\mu$ L Master Mix containing 1 $\times$  Invitrogen buffer (Carlsbad, CA), 5.5 mmol/L MgCl<sub>2</sub>, 200  $\mu$ mol/L deoxynucleoside triphosphate, 0.025 unit/ $\mu$ L Platinum Taq DNA polymerase (Invitrogen), 900 nmol/L each of forward and reverse primers, and 100 nmol/L probe. All probes were modified with a 6-carboxyfluorescein fluorophore at the 5' end and a 6-carboxytetramethylrhodamine quencher at the 3' end. All primers and probes for the chemokine receptors and SDF-1 were obtained from IDT (Coralville, IA) based on previous reports (26-28). All reactions were carried out using a Bio-Rad iCycler iQ (Hercules, CA) in 96-well plates. Cycling variables were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 15-second denaturation at 94°C and 1-minute annealing at 60°C (26). Each sample was run in triplicate

for both the target gene and the housekeeping gene. A nontemplate control was included in each amplification reaction to control for contaminating cDNA. For the CXCR4 gene expression analysis only, each sample was run a second time in triplicate and SAOS-2 was used as a negative control as described previously (7).

**Real-time PCR data analysis.** To determine the relative quantitation of gene expression, the comparative C<sub>T</sub> (threshold cycle) method was used (25). This method uses arithmetic formulas to determine relative quantitation. To normalize the samples for varying cDNA quantities, a housekeeping gene ( *$\beta$ -actin*) is run concurrently with the gene of interest (e.g., CXCR4). For each sample, C<sub>T</sub> is determined for both the gene of interest and the housekeeping gene, which normalizes for cDNA quantity. Subtracting the C<sub>T</sub> of the housekeeping gene from the C<sub>T</sub> of the gene of interest yields the  $\Delta$ C<sub>T</sub>. For each run, identical calculations were made for a known positive control reference cell line (calibrator sample). CCRF-CEM was used for CXCR1, CXCR2, CXCR4, CXCR5, CCR4, CCR5, CCR6, CCR7, and CCR9 (17); NK-92 for CXCR3, CCR1, CCR2, CCR3, CCR8, CCR11, and XCR1; Malme-3-M for CCR10 (4); and MCF-7 for SDF-1 (28). The  $\Delta$ C<sub>T</sub> of the reference cell line was then subtracted from the  $\Delta$ C<sub>T</sub> of the gene of interest, yielding the  $\Delta\Delta$ C<sub>T</sub>, accounting for the exponential amplification of PCR. The reference cell line for each gene was arbitrarily assigned an expression of one. The expression of the gene of interest therefore represents the fold difference expression relative to the reference cell line. It was considered negative if under a threshold of 1% of the reference cell line and positive if  $\geq$ 1%.

**Statistical analysis.** Statistical analysis of the data was done using the Splus (MathSoft, Inc., Seattle, WA) and SPSS (SPSS, Inc., Chicago, IL) software packages. The gene expression data were analyzed qualitatively and quantitatively. The clinical variables studied were age at diagnosis, gender, histologic osteosarcoma subtype, site of the primary disease, metastatic status at diagnosis, presence or absence of metastatic disease (at any time during the evolution of the disease), site of metastatic disease, chemotherapy response, and type of samples (Table 1). Overall survival was defined as the time from diagnosis until the date of either the last follow-up or death. The duration of event-free survival (EFS) was defined, for the patients for whom we had a sample from the biopsy at diagnosis or at the definitive surgery, as the time from diagnosis until the date of relapse or death. If these patients had metastatic disease at diagnosis, the event was considered at time 0. For patients for whom we had a first chronological sample from a metastatic site, an event was either a second or a third relapse or death. For the metastasis-free survival (MFS) analysis, the duration was defined as the time from diagnosis until the occurrence of metastasis. Patients with metastatic disease at diagnosis were excluded from this analysis.

Log-rank or univariate Cox model was used to test the correlation between the relative level of expression of each gene of interest and the survival outcomes. Multivariate Cox regression analysis was used to determine the independent variables for the survival analysis.

Fisher exact test was used to correlate the qualitative expression of the genes of interest with the clinical variables. Survival curves were generated using the Kaplan-Meier method. Mann-Whitney tests were used to compare the median expression of a gene of interest between clinical groups (e.g., high-grade versus low-grade samples). Spearman rank correlation test was used to determine the correlation between genes and the presence of metastatic disease as well as correlative values between the expression of genes of interest. In all analyses, values were considered significant if  $P$ s < 0.05.

## Results

**Pattern of chemokine receptors in osteosarcoma cell lines.** The expression of 17 chemokine receptors in three osteosarcoma cell lines was studied. The overall results showed low levels

**Table 1.** Clinical characteristics of the high-grade osteosarcoma patients (total number of patients = 47)

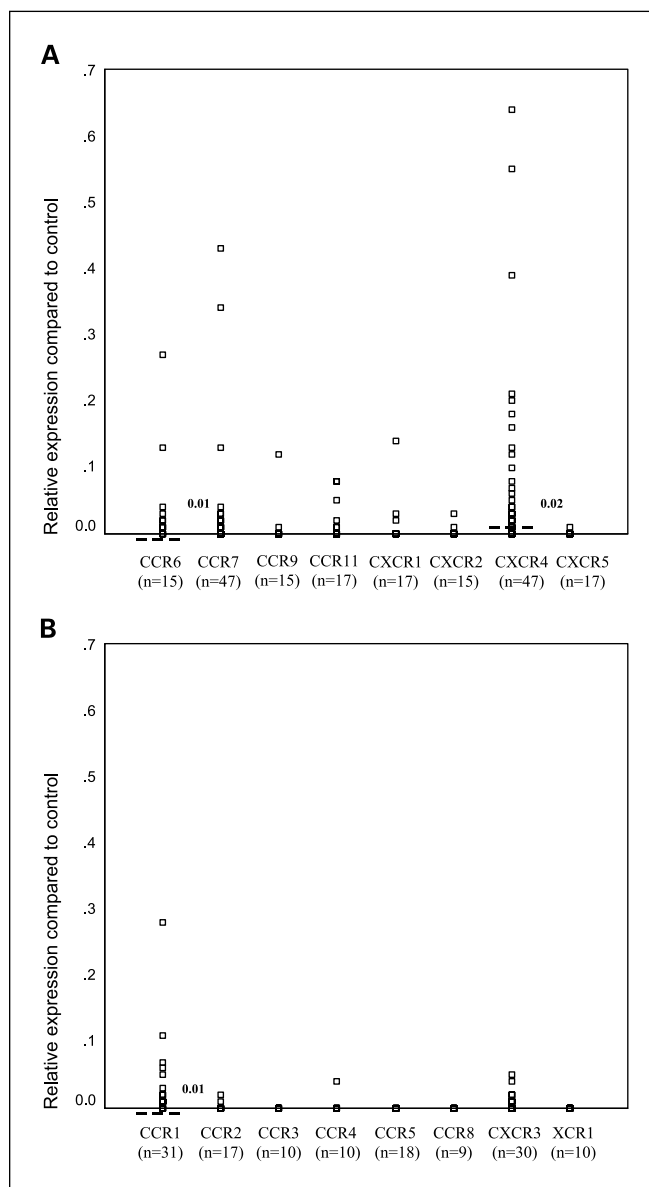
Age at diagnosis (median), y	16 (4-77)
Gender	
Female/male	22/25
Duration of follow-up (median), mo	41 (1-329)
Site of primary disease [ <i>n</i> (%)]	
Extremity	40 (85)
Pelvis	4 (9)
Other	3 (6)
Histologic subtype [ <i>n</i> (%)]	
Osteoblastic	17 (36)
Chondroblastic	12 (25)
Others	13 (28)
Unavailable	5 (11)
Metastatic status at diagnosis [ <i>n</i> (%)]	
Nonmetastatic	33 (70)
Metastatic	14 (30)
Metastatic disease (at any time during the evolution of the disease) [ <i>n</i> (%)]	
Absent	20 (43)
Present	27 (57)
Site of metastatic disease [ <i>n</i> (%)]	
None	20 (43)
Lung	10 (21)
Bone	2 (4)
Lung and bone	9 (19)
Lung and/or bone and others	6 (13)
Type of samples (timing in the disease evolution) [ <i>n</i> (%)]	
Primary biopsy (diagnosis)	16 (34)
Definitive surgery	16 (34)
First relapse	6 (13)
More than one relapse	9 (19)
Chemotherapy response [ <i>n</i> (%)]	
Huvos I and II	23 (49)
Huvos III and IV	13 (28)
Unavailable/nonapplicable	11 (23)
Survival [ <i>n</i> (%)]	
Alive	29 (62)
Dead	18 (38)

of mRNA expression for all the chemokine receptors in the three osteosarcoma cell lines (data not shown). Among the CXC receptors, CXCR3 and CXCR5 were expressed at low levels in the three cell lines (median value of 0.01 for CXCR3 and median value of 0.02 for CXCR5). CXCR4 was expressed only by U2OS and HOS cells (median value of 0.02). The expression of the other CXC receptors was considered negative (median values of <0.001) for all the cell lines. Among the CC receptors, CCR6 and CCR11 were expressed at low levels in the three cell lines (median values of 0.02 and 0.03). CCR1, CCR2, CCR5, CCR7, CCR9, and CCR10 were expressed at very low levels (median values of <0.01) in all the cell lines and no mRNA expression was present at detectable levels for CCR3, CCR8, and XCR1.

**Expression of chemokine receptors in osteosarcoma samples.** A total of 68 osteosarcoma samples were analyzed: 62 high-grade osteosarcoma and 6 low-grade osteosarcoma. The 62 high-

grade osteosarcoma samples belonged to a total of 47 patients. For 34 patients, we had a single sample. From the remaining 13 patients, we obtained more than one sample at different times during their illness. For all patients, the results from the first sample obtained were used in the analysis of survival or EFS. Table 1 summarizes the clinical characteristics of these patients.

All the samples ( $N = 47$ ) were analyzed for CXCR4, CCR7, and CCR10. We based this decision on the data from studies in other solid tumors showing a high expression of these chemokine receptors (4, 7, 9–11). Because of limited quantities of cDNA to perform the analysis, subsets were analyzed for the other chemokine receptors: CCR1 ( $n = 31$ ); CCR2 ( $n = 17$ ); CCR3 and CCR4 ( $n = 10$ ); CCR5 ( $n = 18$ ); CCR6 ( $n = 15$ ); CCR8 ( $n = 9$ ); CCR9 ( $n = 15$ ); CCR11



**Fig. 1.** A, relative expression of chemokine receptors in osteosarcoma patient samples compared with control (CCRF-CEM; median, range). B, relative expression of chemokine receptors in osteosarcoma patient samples compared with control (NK-92; median, range).

( $n = 17$ ); CXCR1 ( $n = 17$ ), CXCR2 ( $n = 15$ ); CXCR3 ( $n = 30$ ); CXCR5 ( $n = 17$ ); and XCR1 ( $n = 10$ ). The results are shown in Fig. 1A and B.

Among the 47 samples analyzed for CXCR4, CCR7, and CCR10, CXCR4 is the most frequently and highly expressed (63% of the samples; median value of 0.02; range 0-0.64). CCR7 is expressed by 43% of the samples and its expression is heterogeneous (median value 0; range 0-0.43). CCR10 expression is also heterogeneous (median value 0; range 0-0.32) and 26% of the samples expressed it (data not shown). There is no significant correlation between the expression of both CXCR4 and CCR7 ( $P = 0.24$ ) or CXCR4 and CCR10 ( $P = 0.07$ ). However, there is a significant correlation between CCR7 and CCR10 expression ( $r = 0.41$ ;  $P = 0.005$ ).

Six low-grade osteosarcoma samples were also analyzed for CXCR4 expression only. The median CXCR4 expression was 0 (range 0-0.07) for low-grade samples compared with 0.02 (range 0-0.64) for the 47 high-grade osteosarcoma samples ( $P = 0.06$ ; Fig. 2).

Seven patients had "paired samples," including the first biopsy and the definitive surgery samples (after chemotherapy and resection or amputation). Only CXCR4 expression analysis was measured. The median expression of CXCR4 for the first biopsy group is 0.02 (range 0-0.21) compared with 0 (range 0-0.1) for the definitive surgery group ( $P = 0.18$ ; data not shown).

Pulmonary metastasis and bone metastasis were analyzed. Most of these samples were not included in the 47 patient samples analyzed for survival because they were paired with earlier samples. Nine pulmonary metastasis samples and six bone metastasis samples were analyzed for CXCR4 expression only. There was no significant difference between the median CXCR4 expression in the primary tumor samples compared with metastatic samples ( $P = 0.52$ ; Fig. 3).

**SDF-1 gene expression.** Twenty-six of the 62 high-grade osteosarcoma samples were studied for SDF-1 expression. Seventy-seven percent of these samples expressed SDF-1

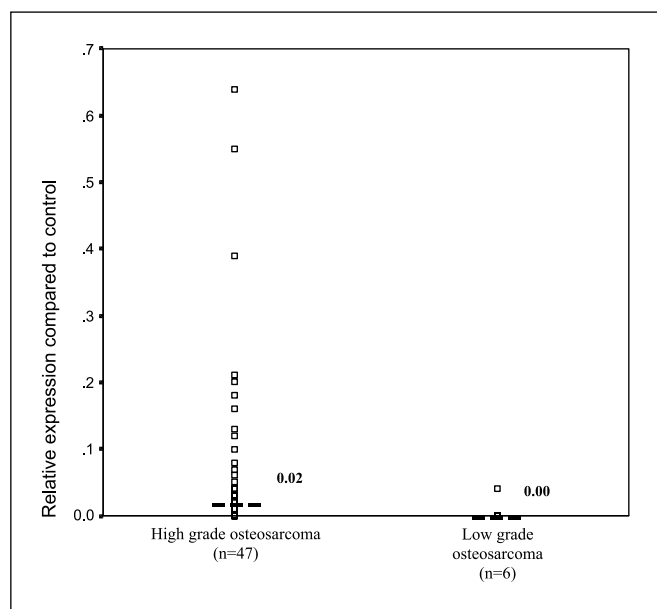


Fig. 2. Expression of CXCR4 in high-grade osteosarcoma compared with low-grade osteosarcoma samples. Median, range ( $P = 0.06$ , Mann-Whitney test).

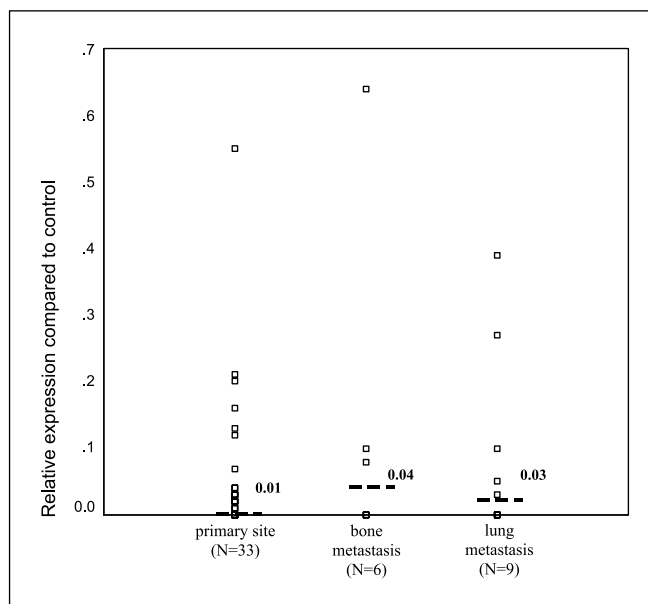


Fig. 3. Expression of CXCR4 in primary sites and metastatic samples. Median, range ( $P = 0.52$ ).

(median 0.36; range 0-9.06; data not shown). From these 26 patients, 16 (62%) expressed both SDF-1 and CXCR4, 4 (15%) expressed only SDF-1, 5 (19%) expressed only CXCR4, and 1 (4%) expressed neither SDF-1 or CXCR4. There was no correlation between SDF-1 expression and CXCR4 expression among these osteosarcoma patient samples ( $P = 0.27$ ).

**Clinical correlations and survival.** The correlation between the expression of the chemokine receptors and clinical variables of relevance in osteosarcoma were analyzed. CXCR4, CCR7, and CCR10 were significantly associated with some of the clinical variables and survival outcome. Univariate analysis revealed that the levels of CXCR4 expression are highly inversely correlated with overall survival ( $P < 0.0001$ ), EFS ( $P < 0.001$ ), and MFS ( $P = 0.002$ ). CXCR4 level of expression also correlated significantly with the presence of metastasis at diagnosis ( $P = 0.002$ ). The patients with tumors expressing CXCR4 have a worse overall survival and EFS ( $P = 0.02$  for both; Figs. 4 and 5). Other clinical variables (age at diagnosis, sex, histologic osteosarcoma subtype, Huvos grade, site of primary disease, site of metastatic disease, and site of samples) did not show significant correlation with CXCR4 expression.

CCR10 expression is also significantly inversely correlated with MFS ( $p = 0.009$ ) but not with overall survival ( $P = 0.89$ ) or EFS ( $P = 0.29$ ). A trend toward an inverse correlation of CCR10 expression with overall survival ( $P = 0.06$ ) but not with EFS ( $P = 0.16$ ) was observed. No other clinical variables were significantly associated with CCR10 expression.

For CCR7 expression, the level was also inversely correlated with overall survival ( $P = 0.03$ ) and MFS ( $P = 0.007$ ). However, qualitative analysis of CCR7 expression did not show any significant correlation with overall survival ( $P = 0.79$ ) or EFS ( $P = 0.89$ ). SDF-1 expression was not significantly associated with any of the clinical variables or survival outcomes.

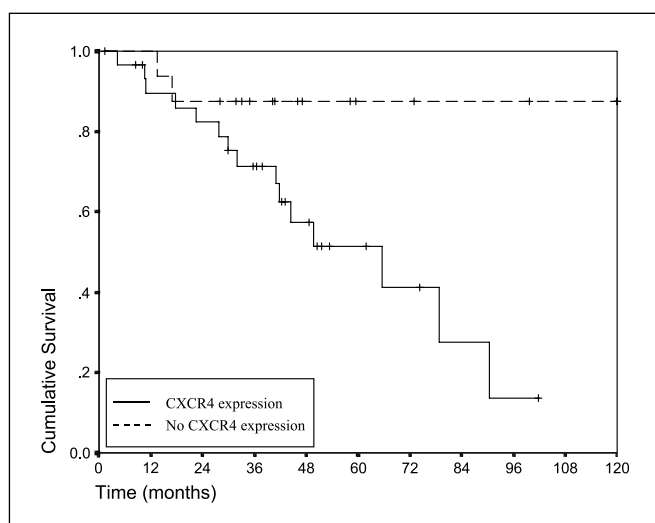


Fig. 4. Kaplan-Meier overall survival curve according to CXCR4 expression ( $P = 0.02$ , log-rank test).

Multivariate analysis revealed that the level of CXCR4 expression was the only significant independent variable for overall survival ( $P = 0.0006$ ), EFS ( $P = 0.004$ ), and MFS ( $P = 0.025$ ).

## Discussion

Recent studies suggest that chemokines and their receptors, particularly the CXCR4/SDF-1 pathway, play an important role in determining the metastatic destination of tumor cells (4–7). This study is the first to our knowledge to look at chemokine receptors expression in osteosarcoma patient samples. Our results showed that CXCR4 is frequently expressed in osteosarcoma and that its level of expression correlates with the presence of metastatic disease at diagnosis, MFS, and overall survival.

Experimental metastasis models of breast cancer and melanoma have shown that CXCR4 expression by tumor cells enhance the pulmonary metastatic potential (4, 6). Prostate cancer cells also express functional CXCR4 and showed chemotactic responses to SDF-1 across bone marrow monolayers (7). These results suggest that the CXCR4/SDF-1 pathway could have a role in the development of bone metastasis (7, 9). Only a few studies have however looked at the correlation between CXCR4 expression and clinical outcome. In one study of 43 prostate cancer samples, CXCR4 overexpression was a significant predictor for distant metastasis (29). Another study in neuroblastoma primary tumors showed that a higher grade of expression of CXCR4 measured by immunohistochemistry correlated with the presence of bone marrow and bone metastases (30).

Two recent studies evaluated the expression of chemokine receptors by flow cytometry in osteosarcoma cell lines (24, 31) and reported results quite similar to those obtained in this study using real-time PCR. Perissinotto et al. showed in a migration assay that the osteosarcoma cells expressing CXCR4 migrated in response to SDF-1. They also showed in their osteosarcoma mouse model that neutralization of the CXCR4/SDF-1 axis prevents lung metastases development (31). Many other studies looked at chemokine receptors expression in normal bone cells, osteoblasts, and osteoclasts (32–35). Osteoclasts expressed

mainly CCR1 but also CCR3, CXCR4, and CX3CR1 (32–34). In osteoblasts, CXCR3 and CXCR5 are the main chemokine receptors expressed (35). The pattern of expression of chemokine receptors observed in the osteosarcoma samples corresponds to both osteoblast and osteoclast patterns.

As immunochemistry with anti-CXCR4 antibody was not done in our study, we could not confirm the expression of the protein. Immunohistochemistry in formalin-fixed, paraffin-embedded osteosarcoma tissue is often not reliable. Because of their bone component, these tumor samples must be decalcified to perform immunochemistry. As mentioned previously, Perissinotto et al. showed that osteosarcoma cell lines expressed CXCR4 protein (fluorescence-activated cell sorting analysis). Existing data in breast and prostate cancer cells also suggest that mRNA expression of CXCR4 and its protein expression are likely to be concordant (4, 7, 10, 11). Osteosarcoma tumors are usually rich in stromal cells like fibroblasts, macrophages, endothelial cells, and lymphocytes (22); leukocytes and epithelial cells have been shown to express CXCR4 and other chemokine receptors (1, 2). Recent studies using metastatic models have indeed shown that interactions between the primary tumor cells and the stromal cells are critical for invasion into the tissues (36). Furthermore, other studies suggested that the metastatic potential correlates with the expression of chemokine receptors in nontumoral stromal cells rather than in primary tumor cells (37). This hypothesis will have to be verified in osteosarcoma tumors. Recent data also suggest that autocrine and paracrine pathways maintain the growth of the cancer cells and facilitate their migration to their metastatic sites (36, 38). In osteosarcoma cell lines, the release of matrix metalloproteinases following chemokine activation has been described (24). A recent study using melanoma cell lines reported a stimulation of matrix metalloproteinase-1 activities following SDF-1 exposition and a secondary up-regulation of CXCR4 (39). Other studies also suggest a regulation of CXCR4 in an autocrine manner by angiogenic factors like vascular endothelial growth factor (40). Recent studies using rhabdomyosarcoma and Ewing's sarcoma cells showed that CXCR4

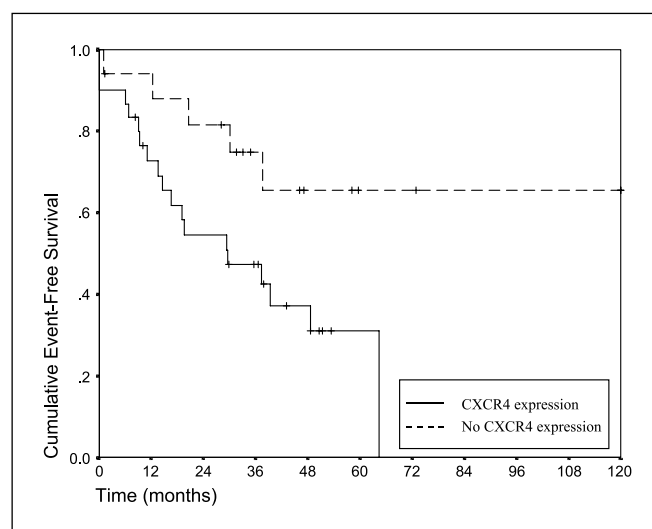


Fig. 5. Kaplan-Meier EFS curve according to CXCR4 expression ( $P = 0.02$ , log-rank test).

expression is up-regulated by *PAX3-FKHR* gene fusion and *EWS/FLI1*, respectively (10, 41). Signal transduction following CXCR4 stimulation may also lead to the activation of G proteins, extracellular signal-regulated kinase-2, and phosphatidylinositol 3-kinase that can be relevant in osteosarcoma pathogenesis (37).

One of the limits of this study is the heterogeneity of the osteosarcoma samples analyzed, which represented a convenience sample. Thirty-four percent of the samples were obtained at the first biopsy ( $n = 16$ ), 34% at the definitive surgery (after preoperative surgery;  $n = 16$ ), and 32% obtained either at the first relapse ( $n = 13$ ) or at a subsequent relapse ( $n = 19$ ). Because the prognosis of relapsing patients is usually very poor, the high number of relapsing patients in our sample set is biasing the group toward a decreased patient survival.

Another limit of this study is that we did not test all the osteosarcoma samples for all the chemokine receptors. We introduced a selection bias by analyzing in a larger number of samples 3 of the 17 chemokine receptors (CXCR4, CCR7, and CCR10). We based our decisions on the previous studies done in other solid tumors rather than on the results we obtained in osteosarcoma cell lines because of the low levels

of mRNA expression for all the chemokine receptors (4, 7, 9, 10).

SDF-1 expression could also be analyzed only in a subset of samples. Our results do not suggest any correlation between CXCR4 and SDF-1 expression. The number of samples analyzed was not sufficient to compare the SDF-1 expression in primary tumors versus metastatic sites.

Antagonists of CXCR4 have been developed recently (42–44). *In vivo* studies in breast cancer, melanoma, and pancreatic cancer showed that neutralizing antibodies to CXCR4 markedly reduced metastasis (4, 6, 43, 44). In human studies, these antibodies are well tolerated (44). Clinical studies are ongoing for the use of anti-CXCR4 antibodies in the treatment of HIV infection (45). Other strategies to target CXCR4 include RNA interference. One recent study using small interfering RNA has shown a down-regulation of CXCR4 expression in breast cancer cells (46).

In conclusion, these data suggest that CXCR4 could be useful as a predictor of potential metastatic development in osteosarcoma. CXCR4 represents a new therapeutic target in osteosarcoma. Validation in a larger and more uniform group of osteosarcoma patient samples is needed.

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*Clin Cancer Res* 2005;11:2561-2567.

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