Clinical Significance of CXC Chemokine Receptor-4 and c-Met in Childhood Rhabdomyosarcoma

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Abstract

Purpose: The CXC chemokine receptor-4 (CXCR4) /stromal-derived factor-1 and c-Met/hepatocyte growth factor axes promote the metastatic potential of rhabdomyosarcoma cell lines in experimental models, but no data are available on their role in rhabdomyosarcoma tumors. The expressions of CXCR4 and c-Met were evaluated in primary tumors and isolated tumor cells in marrow, and were correlated with clinicopathologic variables and survival.

Experimental Design: Forty patients with recently diagnosed rhabdomyosarcoma were retrospectively enrolled. CXCR4 and c-Met expression was investigated in primary tumors by immunohistochemistry, in isolated marrow-infiltrating tumor cells using double-label immunocytochemistry. Results were expressed as the mean percentage of immunostained tumor cells.

Results: CXCR4 and c-Met were expressed in ≥5% of tumor cells from 40 of 40 tumors, with 14 of 40 cases showing ≥50% of immunostained tumor cells (high expression). High CXCR4 expression correlated with alveolar histology (P = 0.006), unfavorable primary site (P = 0.009), advanced group (P < 0.001), marrow involvement (P = 0.007), and shorter overall survival and event-free survival (P < 0.001); high c-Met expression correlated with alveolar histology (P = 0.005), advanced group (P = 0.04), and marrow involvement (P = 0.02). In patients with a positive diagnosis for isolated tumor cells in marrow (n = 16), a significant enrichment in the percentage of CXCR4-positive (P = 0.001) and c-Met-positive (P = 0.003) tumor cells was shown in marrow aspirates compared with the corresponding primary tumors.

Conclusions: CXCR4 and c-Met are widely expressed in both rhabdomyosarcoma subtypes and, at higher levels, in isolated marrow-infiltrating tumor cells. High levels of expression are associated with unfavorable clinical features, tumor marrow involvement and, only for CXCR4, poor outcome. In rhabdomyosarcoma, CXCR4 and c-Met represent novel exploitable targets for disease-directed therapy.

Rhabdomyosarcoma is the most common soft tissue sarcoma among children and adolescents, accounting for 5% of all malignant tumors in this age range (1). There are two major rhabdomyosarcoma histologic subtypes: embryonal and alveolar, the latter being associated with PAX3-FKHR or PAX7-FKHR gene fusions and with a worse prognosis (2). Treatment is allocated according to disease staging, which depends on a multifactorial process including age at diagnosis, primary site and size, histology, and postsurgical group (3, 4). However, although multimodal treatment has substantially increased overall survival in patients with localized low-risk and medium-risk rhabdomyosarcoma to >80%, the outcome for 15% of patients diagnosed with metastatic disease remains poor at ~25% and has not significantly improved over the last 15 years, in spite of more intensive treatment (5). Similarly, the outcome for patients with localized alveolar rhabdomyosarcoma and regional lymph node involvement remains poor (6). Therefore, the development of novel therapeutic strategies is urgently needed.

Despite extensive knowledge of genetic alterations responsible for cancer initiation and local progression, our understanding of the molecular determinants for tumor invasion and metastatic spread is still limited. The series of coordinated sequential steps that a tumor cell must go through in order to
successfully colonize a distant site has been previously described (7). More recently, several genes necessary for the successful execution of distinct steps of this metastatic cascade have been identified in different tumor types (reviewed in refs. 8, 9). In rhabdomyosarcoma, it has been reported that CXC chemokine receptor-4 (CXCR4) and c-Met are expressed in cell lines and tumors (10–16), and in experimental models promote invasive tumor growth (10, 12, 13, 15, 16) and migration of tumor cells to bone marrow in response to the secretion of the corresponding ligands—CXCL12 (stromal-derived factor-1) and hepatocyte growth factor (HGF), respectively—by marrow stromal cells, facilitating their establishment and expansion at this site (13, 16).

The availability of a clinical series of patients with rhabdomyosarcoma, prospectively investigated for marrow involvement by conventional cytomorphology and more sensitive immunocytology, prompted us to retrospectively analyze the expression of CXCR4 and c-Met in primary tumors and in isolated tumor cells detected at diagnosis in marrow aspirates from the same patients. Our findings show that (a) CXCR4 and c-Met are expressed in primary tumors and isolated marrow-infiltrating tumor cells, and (b) high levels of expression for CXCR4 and c-Met are associated with unfavorable clinical features and, only for CXCR4, poor outcome.

Materials and Methods

Patients. Forty patients, 24 males and 16 females, ages 2 to 180 months (median, 58), with recently diagnosed rhabdomyosarcoma admitted between 1995 and 2004 in the Divisions of Oncology at Bambino Gesù Children’s Hospital and at the Department of Pediatrics of La Sapienza University were retrospectively included in this study. Selection criteria were (a) the availability of immunocytologic data on marrow involvement at diagnosis, and (b) the availability of paraffin-embedded primary tumor tissue and marrow cytospins, both obtained at diagnosis, for immunohistochemical and immunocytologic analyses.

Thirty-one patients were part of the series of 37 patients with nonmetastatic disease described in a previous report (17). Primary site was the orbit in 3 patients, head and neck parameningeal in 12 patients, head and neck nonparameningeal in 3 patients, genitourinary bladder or prostate in 9 patients, genitourinary bladder or prostate in 5 patients, extremity in 1 patient, and others in 7 patients. Patients were grouped according to the Intergroup Rhabdomyosarcoma Study postaural group (18) using conventional techniques of imaging and marrow examination; and assigned, as group I (n = 3), group II (n = 2), group III (n = 28), or group IV (n = 7). Histologic subtype was embryonal in 20 patients and alveolar in 20 patients. Molecular analysis of gene fusions associated with alveolar histology was carried out at diagnosis using a previously described reverse transcription-PCR assay for PAX3-FKHR and PAX7-FKIR (19): 13 of 20 (65%) alveolar tumors were PAX3-FKIR–positive, 3 tumors (15%) were PAX7-FKIR–positive, and 4 tumors (20%) were fusion-negative. Treatment in patients with localized disease was given according to the RMS88 or RMS96 protocol of the Italian Association of Pediatric Hematology/Oncology (AIEOP; ref. 20), and in patients with metastatic disease according to the MMT-98 protocol of the International Society of Pediatric Oncology (SIOP; ref. 21). Institutional written informed consent was obtained from the patient’s parents or legal guardians. The study underwent ethical review and approval according to local institutional guidelines.

Cell lines. Human rhabdomyosarcoma embryonal cell lines RD, RD18, and CCA were kindly provided by P.L. Lollini (22–24); human rhabdomyosarcoma alveolar cell lines Rh4 and Rh30 were kindly provided by D.N. Shapiro (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT) and P.L. Lollini (Department of Experimental Pathology, University of Bologna, Bologna, Italy; ref. 25). All cell lines were grown in DMEM supplemented with 10% FCS (Sigma), 1% L-glutamine, and 1% streptomycin/penicillin. The CCA cell line was incubated in 7% CO2, whereas the remaining cell lines were incubated in 5% CO2.

Immunocytologic analysis of cell lines. Multiple cytospins were obtained from each cell line according to standard protocols and processed for immunohistochemistry as previously described (17). Primary antibodies were a goat polyclonal antibody against human CXCR4 (ab1671, Novus Biologicals) and a rabbit polyclonal antibody against human c-Met (C-12, Santa Cruz Biotechnology). Secondary antibody was a swine biotinylated polyclonal antibody against goat/rabbit/mouse IgG (Dako). Sections of well-characterized breast carcinoma and osteosarcoma were used as positive controls for CXCR4 and c-Met, respectively; cytospins incubated with isotype-matched control immunoglobulins were used as negative controls. Three separate counts (each including >5,000 tumor cells) were carried out and the results were expressed as a percentage. Cytospins were considered positive when >20% of tumor cells were unequivocally immunostained.

Real-time reverse transcription-PCR analysis of cell lines. Total RNA was isolated and reverse-transcribed from each cell line as previously described (26). Quantitative real-time PCR was carried out to detect β-actin expression that was used to normalize the amount of cDNA for each sample. β-Actin primers were 5’CTTCTAACACCCCCGCA3’ and 5’ACCCCTCTGATGATGGGCA3’. Equal amounts of cDNA from each sample were amplified using the following primers to detect CXCR4 5’GTGCCGCGACCTCTCTTGTG3’ and 5’ATGACATGGACGCTTCTG3’; and c-Met 5’CCGAGGAGAATGATCGCAAAGA3’ and 5’CCGAGGACCTCTTCACCTC3’. Reaction linearity was checked by running serial dilutions of cDNA from CCA cells (for CXCR4) and RD18 cells (for c-Met) taken as positive controls. Two independent experiments were carried out in triplicate using the ABI Prism 7000 cycler (Applied Biosystems) with the SYBR green fluorochrome. Results were analyzed by using the ABI Prism 7000 SDS software (Applied Biosystems).

Immunohistochemical analysis of primary tumors. Immunohistochemistry of sections from formalin-fixed paraffin-embedded primary tumors was carried out as previously described (17). Primary and secondary antibodies were the same as that used for the analysis of cell lines. Sections of well-characterized breast carcinomas and osteosarcomas were used as positive controls for CXCR4 and c-Met, respectively; sections incubated with isotype-matched control immunoglobulins were used as negative controls. In order to compare the immunohistochemical findings between primary tumors and marrow aspirates (see subsequent paragraph), each primary tumor had additional serial sections prepared and stained for myogenin. The percentage of myogenin-positive tumor cells was determined in tumor tissue, which ranged from 25% to 100%. Immunohistochemical scoring was carried out by two independent investigators (F. Dimedi-Camassi and S. Uccini) who were blinded to the clinical features and outcomes, and was expressed as the mean percentage of immunostained tumor cells. Because immunostaining was not always uniformly distributed, 15 fields were randomly selected for each case, three separate counts (each comprising >5,000 tumor cells) were done by each investigator, and a final mean value obtained for each tumor. For statistical purposes, the percentage of immunostained tumor cells was treated as a continuous variable.

Immunocytologic analysis of bone marrow aspirates. At diagnosis, on the day of surgery or biopsy, four marrow aspirates were obtained from the iliac crests of each patient: mononuclear cells were separated by Ficoll-Paque (Amersham Biosciences Europe GmbH) density-gradient centrifugation, and multiple cytospins were prepared according to standard protocols and fixed in cold absolute acetone for 5 min. A portion of the cytopsins, upon patient presentation, was immediately (prospectively) analyzed for tumor involvement using single-label immunocytochemistry for MyoD1 and myogenin. The remaining cytopsins were stored at -80°C until double-label immunocytochemistry for myogenin and CXCR4 or c-Met was retrospectively done.
Single-label immunocytology for myogenin and MyoD1 was carried out as previously described (17). Primary antibodies were monoclonal antibodies against myogenin and MyoD1 (Dako). The Rh30 cell line was used as a positive control, whereas in negative controls, isotype-matched immunoglobulins were used as the primary antibody. Five separate visual counts on different cytospins were done, each of which included ≥500,000 cells, and only nuclear immunostaining was scored positive. The sensitivity of the assay was measured as previously reported (17) and the detection limit was shown to be 1:50,000. The specificity was determined by testing normal marrow samples obtained from 10 healthy volunteer marrow donors and marrow samples obtained from 25 children with neuroblastoma for the presence of myogenin-positive or MyoD1-positive cells (17). All samples were found to be negative.

Double-label immunocytology was carried out by combining indirect immunofluorescence for CXCR4 or c-Met, with indirect avidin-biotin immunoperoxidase for myogenin. In the first staining step, cytospins were pretreated with 10% normal chicken serum (Santa Cruz Biotechnology) for 10 min at room temperature and, after quick rinsing in TBS, incubated with goat polyclonal antibody against human CXCR4 (Novus Biologicals) or rabbit polyclonal antibody against human c-Met (Santa Cruz Biotechnology). Secondary antibodies were chicken anti-goat IgG-FITC or anti-rabbit IgG-FITC (Santa Cruz Biotechnology). The procedures in the second staining step were all performed in a dark chamber. Cytospins were pretreated with 10% normal mouse serum (Santa Cruz Biotechnology) for 10 min at room temperature and, after quick rinsing in TBS, incubated with monoclonal antibody against myogenin (Dako) for 30 min at room temperature. Indirect avidin-biotin immunoperoxidase staining was carried out under standard conditions (Vectastain, Vector Laboratories). Cytospins were mounted in 90% glycerol in TBS and kept at 4°C in the dark until reading, which was done by combining bright-field illumination (for myogenin) and epifluorescence illumination (for CXCR4 and c-Met).

In each staining step, appropriate positive and negative controls were assessed (as described in previous sections). Immunocytologic scoring of the percentage of tumor cells (showing nuclear immunostaining for myogenin) positive for CXCR4 or c-Met was done by two independent investigators (H.P. McDowell and C. Dominici) who were blinded to other findings. Five separate visual counts on different cytospins were done, each of which included ≥500,000 cells.

Categorization of clinicopathologic variables and statistical analysis. Clinicopathologic variables were categorized as follows: age at diagnosis, favorable (12-120 months) versus unfavorable (<12 or >120 months); sex, male versus female; histologic subtype, embryonal versus alveolar; primary site, favorable (orbit and neuroblastoma) or unfavorable (head and neck para- meningeval and nonparameningeval, genitourinary bladder or prostate, extremity, and others); size of primary, ≤5 cm versus >5 cm; group, I and II versus III and IV; and marrow involvement, no involvement versus involvement.

Clinical end point was survival. Both overall survival (OS) and event-free survival (EFS) were considered. OS was calculated from the date of histologic diagnosis until time of last follow-up or death from any cause. EFS was calculated from the date of histologic diagnosis until time of disease progression (i.e., volumetric increase of any preexisting lesion and/or appearance of any new lesion), relapse, second malignancy, or death from any cause; patients not experiencing an event of interest were censored at the time of last contact.

Associations between immunostaining for CXCR4 or c-Met in primary tumors versus marrow aspirates, or between immunostaining for CXCR4 or c-Met in primary tumors and clinicopathologic variables were analyzed using two-sided Student’s t test and one-way ANOVA test. The effect on OS and EFS of immunostaining for CXCR4 or c-Met and of clinicopathologic variables was evaluated using the Kaplan-Meier method (27), and the log-rank test was used for comparing survival. The software package SPSS for Windows (SPSS Inc.) was used.

Results

Patient status and outcome. As of May 2007, median follow-up for the 40 patients was 65.5 months (range, 9-142), with 25 patients disease-free and 15 dead of disease. Of the 15 patients which died of disease, 1 patient with localized disease never achieved complete remission and progressed 24 months after diagnosis; 9 patients with localized disease achieved complete remission but recurred with local relapse (9 and 25 months after diagnosis), regional relapse (after 14 and 19 months), or distant relapse (after 2, 6, 8, 8, and 9 months), as defined elsewhere (28); and 5 patients with metastatic disease never achieved complete remission and progressed 5, 6, 7, 18, and 43 months after diagnosis. Marrow involvement was shown by immunocytologic analysis for myogenin and MyoD1 in 16 of the 40 patients: 10 of 33 (30%) with localized disease and 6 of 7 (86%) with metastatic disease.

Expression of CXCR4 and c-Met mRNAs and proteins in cell lines. Initially, in order to relate the immunohistochemical and immunocytologic findings obtained in this study to functional results previously reported in preclinical rhabdomyosarcoma models (12, 13, 15), CXCR4 and c-Met expressions were analyzed in five rhabdomyosarcoma cell lines by both real-time reverse transcription-PCR and immunocytochemistry (Table 1). Noticeable levels of mRNA expression for CXCR4 were detected in three of five cell lines (Rh30, CCA, and RD), and for c-Met in five of five cell lines. However, positive immunostaining (>20% of stained tumor cells) was observed in two of five cell lines for both CXCR4 (Rh30 and CCA) and c-Met (Rh30 and RD18). These results are in agreement with

Table 1. Expression of CXCR4 and c-Met mRNAs and proteins in rhabdomyosarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CXCR4 mRNA</th>
<th>CXCR4 protein (% positive cells)</th>
<th>c-Met mRNA</th>
<th>c-Met protein (% positive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD</td>
<td>56.35 (+:9.48)</td>
<td>12 (+:0.27)</td>
<td>29.77 (+:4.84)</td>
<td>11 (+:0.33)</td>
</tr>
<tr>
<td>RD18</td>
<td>4.34 (+:0.85)</td>
<td>0</td>
<td>100.00 (+:0)</td>
<td>51 (+:1.64)</td>
</tr>
<tr>
<td>CCA</td>
<td>100.00 (+:0)</td>
<td>45 (+:0.91)</td>
<td>13.40 (+:2.78)</td>
<td>2 (+:0.07)</td>
</tr>
<tr>
<td>Rh4</td>
<td>1.63 (+:0.45)</td>
<td>0</td>
<td>38.85 (+:2.82)</td>
<td>18 (+:0.49)</td>
</tr>
<tr>
<td>Rh30</td>
<td>197.66 (+:4.21)</td>
<td>84 (+:1.56)</td>
<td>142.69 (+:12.53)</td>
<td>76 (+:1.98)</td>
</tr>
</tbody>
</table>

Note: Expression for mRNA was measured by real-time reverse transcription-PCR and quantified in relationship to the levels found in CCA cells (for CXCR4) and RD18 cells (for c-Met) taken as reference (see Materials and Methods); results were expressed as relative expression level. Expression for protein was determined by immunocytochemistry on cytospins and results were expressed as a percentage (see Materials and Methods). SDs are reported in parentheses.
Expression of CXCR4 and c-Met proteins in primary tumors. Immunohistochemical analysis of CXCR4 and c-Met expression was carried out on paraffin sections obtained from 40 recently diagnosed primary rhabdomyosarcomas. No immunostaining for either CXCR4 or c-Met was detected in surrounding normal striated muscle fibers. A percentage (≥5%) of the tumor cells unequivocally positive for both CXCR4 and c-Met was shown in all cases, with no obvious relationship to the degree of myogenic differentiation or to other morphologic features (Fig. 1A and B). Staining for CXCR4 was diffuse and was found at the cytoplasmic level, but in 5 of 40 cases, a concomitant nuclear staining was also recognized. Staining for c-Met was diffuse and always confined to the cytoplasm. In order to categorize the percentages of immunostained tumor cells found in each primary tumor into two groups (low or high levels of expression), a cut point was chosen on the basis of the frequency distribution of these percentages in the 40 patients, which was bimodal for both proteins (data not shown). The cut point of ≥50% of tumor cells unequivocally positive was selected for both proteins, being the value that better discriminated the two peaks. High levels of expression for either protein was observed in 14 of 40 cases, in 4 of 20 embryonal tumors, and in 10 of 20 alveolar tumors. Among the alveolar tumors, high CXCR4 expression was found in 8 of 13 (62%) PAX3-FKHR–positive cases and in 2 of 4 (50%) fusion-negative cases, but in none of the 3 PAX7-FKHR–positive cases. Likewise, high c-Met expression was found in 7 of 13 (54%) PAX3-FKHR–positive cases and in 3 of 4 (75%) fusion-negative cases, but in none of the 3 PAX7-FKHR–positive cases.

Expression of CXCR4 and c-Met proteins in isolated tumor cells detected in marrow aspirates. Two types of immunocytologic analysis were carried out at different times on marrow cytospins obtained at diagnosis. A portion of the cytospins were immediately (prospectively) analyzed, upon patient presentation, to assess for tumor involvement. The remaining portion was stored until the second analysis, which was aimed at evaluating the percentage of tumor cells positive for either CXCR4 or c-Met, was retrospectively done.

Overt tumor involvement (i.e., detectable by conventional cytomorphology on bilateral trephines and/or marrow aspirates) was found in 3 of 7 (43%) patients with metastatic disease (all with alveolar histology), but in none of the 33 patients with localized rhabdomyosarcoma (groups I-III). However, single-label immunocytochemistry for myogenin and MyoD1 detected isolated tumor cells (i.e., cells with nuclear staining for one or both proteins) in 6 of 7 (86%) patients with metastatic rhabdomyosarcoma (all with alveolar histology) and in 10 of 33 (30%) patients with localized disease (5 with embryonal and 5 with alveolar histology). In the latter, tumor cells represented a minimal percentage of marrow mononuclear cells, ranging from 7:100,000 to 3:10,000 (median, 1:10,000) of the total, quite below the threshold of 1:1,000 tumor cells conventionally regarded as the detection limit for light microscopy (29). In the former, however, tumor cells ranged from 4:10,000 to 2:100 (median, 2:1,000), spanning through the same detection limit.

Double-label immunocytochemistry combining indirect immunofluorescence for CXCR4 or c-Met, with indirect avidin-biotin immunoperoxidase for myogenin was done with the aim of (a) determining whether and to what extent isolated tumor cells found in marrow aspirates (identified by nuclear staining for myogenin) expressed CXCR4 and c-Met, and (b) comparing in each patient, the levels of expression of CXCR4 and c-Met (assessed as the mean percentage of immunostained tumor cells) detected in the primary tumor with the corresponding levels in marrow. In order to validate the comparison between results obtained on the same marrow aspirates but at different times, cytospins from all 40 patients were analyzed, irrespective of their marrow status at diagnosis. The extent of tumor involvement assessed retrospectively was, in all cases, consistent with the findings observed at diagnosis: no case changed category (from positive to negative, or vice versa) and, in each patient, the percentages of tumor cells immunostained for myogenin were substantially comparable in the two analyses (data not shown). In the 16 cases in which marrow was confirmed to be positive for tumor involvement, a significant enrichment in the percentage of CXCR4-positive and c-Met–positive tumor cells found in individual marrow aspirates compared with the corresponding primary tumors was shown in 14 and 11 cases, respectively (Fig. 2A and B). Overall, in these 16 cases considered as a group, the mean percentages of
CXCR4-positive and c-Met–positive tumor cells in marrow aspirates were significantly higher than the corresponding percentages determined in primary tumors: 77% versus 59% for CXCR4 ($P = 0.001$), and 62% versus 50% for c-Met ($P = 0.003$). Interestingly, in the 11 alveolar cases with tumor marrow involvement, a higher mean percentage of CXCR4-positive tumor cells was found in association with PAX3-FKHR fusion status: 88% in the seven PAX3-FKHR–positive cases versus 69% in the remaining four cases, all fusion-negative ($P = 0.03$).

**Associations between expression of CXCR4 and c-Met in primary tumors and clinicopathologic variables.** The associations between the expression of CXCR4 and c-Met in primary tumors, and clinicopathologic features were investigated in the 40 patients (Table 2). Cases were grouped into subsets according to each variable (see Categorization of clinicopathologic variables and statistical analysis in Materials and Methods), and the mean percentages of CXCR4-positive and c-Met–positive tumor cells of each subset were compared by using two-sided Student’s $t$ test or one-way ANOVA test. Higher mean levels of CXCR4 expression were found to be significantly associated with alveolar subtype ($P = 0.006$), unfavorable primary site ($P = 0.009$), size of primary tumor $>5$ cm ($P = 0.006$), advanced group ($P < 0.001$), and marrow involvement ($P = 0.007$). Higher mean levels of c-Met expression were found to be significantly associated with alveolar subtype ($P = 0.005$), advanced group ($P = 0.04$), and marrow involvement ($P = 0.02$). Interestingly, the association between higher mean levels of CXCR4 and marrow involvement was still significant when the analysis was limited to the 33 patients with localized disease (groups I-III; $P = 0.02$), although this was no longer true for c-Met. In alveolar tumors, despite a trend for either protein to be expressed at higher levels in PAX3-FKHR–positive cases compared with PAX7-FKHR–positive or fusion-negative cases (60% versus 50% for CXCR4, and 54% versus 41% for c-Met), no significant association was identified.

**Effect of clinicopathologic variables (including CXCR4 and c-Met expression in primary tumors) on survival.** The effects of clinicopathologic variables, including the expression of CXCR4 and c-Met in primary tumors, on OS and EFS were analyzed in the 40 patients by using Kaplan-Meier method, and log-rank test was used for comparing survivals (Table 3). Only the advanced group and marrow involvement were significantly associated with decreased OS and EFS.

### Table 2. Associations between CXCR4 and c-Met protein expression in primary tumors and clinicopathologic variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
<th>Patients (no.)</th>
<th>CXCR4 (mean %) $P$</th>
<th>c-Met (mean %) $P$</th>
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</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>12-120</td>
<td>26</td>
<td>43</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>&lt;12 or &gt;120</td>
<td>14</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>16</td>
<td>52</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>24</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>Embryonal</td>
<td>20</td>
<td>36</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Alveolar</td>
<td>20</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Primary site</td>
<td>Favorable*</td>
<td>7</td>
<td>25</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>Unfavorable †</td>
<td>33</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Primary size (cm)</td>
<td>≤5</td>
<td>17</td>
<td>35</td>
<td>0.006</td>
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<tr>
<td></td>
<td>&gt;5</td>
<td>23</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>I and II</td>
<td>5</td>
<td>20</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>III</td>
<td>28</td>
<td>45</td>
<td></td>
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<tr>
<td></td>
<td>IV</td>
<td>7</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
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<td>24</td>
<td>38</td>
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<tr>
<td></td>
<td>Positive</td>
<td>16</td>
<td>59</td>
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</table>

NOTE: Patients were grouped into subsets according to the levels of each variable and the mean percentages of CXCR4-positive and c-Met–positive tumor cells of each subset were compared by using two-sided Student’s $t$ test, except for the variable “group” for which one-way ANOVA test was used.

Abbreviation. n.s., not significant.

*Orbit and genitourinary nonbladder or prostate.
†Head and neck parameningeal and nonparameningeal, genitourinary bladder or prostate, extremity, and others.
associated with reduced OS and EFS in this series (Table 3). High levels of CXCR4 expression (≥50% of tumor cells unequivocally positive) were significantly associated with shorter OS and EFS ($P < 0.001$ for both), whereas no such significant association was shown for c-Met. OS probability was 84% in 26 patients with low levels of CXCR4 expression compared with 21% in 14 patients with high levels. Similarly, EFS probability was 84% in patients with low levels of CXCR4 expression compared with 21% in patients with high levels. High levels of CXCR4 expression were significantly associated with shorter OS and EFS even when the analysis was limited to the 33 patients with group I to III disease (OS probability, 84% versus 25%; $P = 0.001$; EFS, 84% versus 25%; $P = 0.001$) or to the 28 patients with group III disease (OS probability, 80% versus 25%; $P = 0.001$; EFS, 80% versus 25%; $P = 0.003$). Multiple regression analysis was not attempted because of the limited number of cases.

**Discussion**

For the first time, to our knowledge, we provide evidence in a clinical series that CXCR4 and c-Met are widely expressed in primary rhabdomyosarcoma tumors and at significantly higher levels in isolated marrow-infiltrating tumor cells, and that their expression is associated with unfavorable clinical features, tumor marrow involvement and, only for CXCR4, poor outcome.

The development of metastases is the leading cause of death from solid tumors, yet, studies have only recently focused on the molecular mechanisms involved in how tumor cells colonize a distant site (reviewed in refs. 8, 9).

Table 3. Associations between clinicopathologic variables (including CXCR4 and c-Met expression in primary tumors) and survival

<table>
<thead>
<tr>
<th>Variables Levels</th>
<th>Patients (no.)</th>
<th>OS</th>
<th>EFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Events (no.)</td>
<td>Survival %</td>
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<tr>
<td>Age (mo) 12-120</td>
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<td>8</td>
<td>69</td>
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<td>&lt;12 or &gt;120</td>
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<td>Sex Female</td>
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<td>Male</td>
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<td>58</td>
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<td>Histology Embryonal</td>
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<td>Alveolar</td>
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<td>Primary site</td>
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<td>0</td>
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<tr>
<td>Unfavorable†</td>
<td>33</td>
<td>14</td>
<td>57</td>
</tr>
<tr>
<td>Primary size (cm) ≤5</td>
<td>17</td>
<td>3</td>
<td>82</td>
</tr>
<tr>
<td>&gt;5</td>
<td>23</td>
<td>11</td>
<td>51</td>
</tr>
<tr>
<td>Group I and II</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>28</td>
<td>9</td>
<td>68</td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Negative</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
<td>9</td>
<td>42</td>
</tr>
<tr>
<td>CXCR4 &lt;50% positive cells</td>
<td>26</td>
<td>3</td>
<td>89</td>
</tr>
<tr>
<td>≥50% positive cells</td>
<td>14</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>c-Met &lt;50% positive cells</td>
<td>26</td>
<td>8</td>
<td>69</td>
</tr>
<tr>
<td>≥50% positive cells</td>
<td>14</td>
<td>6</td>
<td>54</td>
</tr>
</tbody>
</table>

NOTE: The effect on OS and EFS of the expression of CXCR4 and c-Met in primary tumors as well as of additional clinicopathologic variables was analyzed by using the Kaplan-Meier method, and log-rank test was used for comparing survivals.

Abbreviation. n.s., not significant.

*Orbit and genitourinary nonbladder or prostate.

† Head and neck parameningeal and nonparameningeal, genitourinary bladder or prostate, extremity, and others.

Initial studies suggested that tumor cells constituting a metastatic lesion are the progeny of rare cells in the primary tumor that stochastically express many, if not all, of the genes needed for a successful execution of the metastatic program (30). This model implies that, in primary tumors, cells with metastatic potential are extremely rare and only arise at the late stages of tumor progression (31). However, more recent studies on gene expression profiling in breast cancer have challenged this model by identifying a set of genes whose expression in primary tumors significantly correlates with the likelihood of subsequent metastatic spread (32). Similar results have been independently reported in other tumor types (33). Although there is not yet a general agreement on the best model of the metastatic process, these findings implicate that the presence in primary tumors of cells with metastatic potential, harboring gene activities involved in the execution of the metastatic cascade, may not be as rare and late as initially thought (34).

The CXCL12/CXCR4 axis plays a pivotal role in regulating the homing of CXCR4-positive hematopoietic stem/progenitor cells and CXCR4-positive tissue-committed stem/progenitor cells during normal development, tissue repair, and regeneration (35). Constitutive secretion of CXCL12 by bone marrow stromal and endothelial cells is the major source for this chemoattractant in postnatal life, and circulating CXCR4-positive cells migrate along a CXCL12 gradient to marrow, where stromal cells create viable cellular niches in which hematopoietic stem/progenitor cells and tissue-committed stem/progenitor cells can survive and proliferate (35). A growing burden of evidence shows, however, that the CXCL12/CXCR4 axis can also be operating in tumor cells as a
mechanism of organ-specific recruitment to sites with high constitutive CXCL12 expression such as marrow, liver, lung, and lymph nodes (36–38). Expression for CXCR4 by tumor cells has been reported in many epithelial, mesenchymal, and hematopoietic tumor types, and its activation by CXCL12 in CXCR4-positive tumor cells has been shown to stimulate in vitro motility, migration, and invasion (36–38). Subsequently, the causative role of the CXCL12/CXCR4 axis in promoting metastasis and tumor growth at distant sites has been shown in in vivo experimental models (39, 40). Furthermore, in some clinical studies, the levels of CXCR4 expression in primary tumors have been shown to be associated with increased propensity to metastasize and/or decreased survival (41–44). However, the observation that CXCR4-positive tumor types do not always share an identical pattern of metastatic spread shows that additional pathways must be involved in metastatic development.

Another axis modulating motility, migration, and invasion in normal and pathologic conditions is represented by HGF and its receptor tyrosine kinase c-Met. Activation of c-Met induces a series of coordinated and rate-limiting events, often referred to as "invasive growth", that occur physiologically in normal tissue patterning as well as in establishment and maintenance of normal organ architecture (45). Initially, cells acquire the ability to dissociate from their neighbors, move through surrounding tissues, and reach the circulation. Once in the bloodstream, survival is sustained by c-Met–induced anchorage-independent growth ability and protection from apoptosis. Finally, cells extravasate through surrounding tissues, survive in the new environment, proliferate, and eventually undergo terminal differentiation. Moreover, c-Met activation also has a critical role in the process of epithelial-mesenchymal transition that occurs in embryonic development, and which describes the differentiation switch through which epithelial cells acquire a migratory phenotype (46). Recent data supports epithelial-mesenchymal transition, at least in epithelial tumors, as an early step in the progression of tumor cells to metastatic competence (47). Deregulated c-Met activation subsequent to activating mutations, autocrine stimulation, or overexpression has been described in many epithelial, mesenchymal, and hematopoietic tumor types, and has been shown to promote disaggregation of tumor cells, infiltration of basement membrane and extracellular matrix, colonization of distant sites, and establishment of metastasis (45, 48, 49). In rhabdomyosarcoma, it has been shown that disruption of c-Met signaling in combination with loss of Ink4a/Arf function in Ink4a/Arf<sup>−/−</sup> mice transgenic for HGF induces the development of highly invasive and multicentric malignant tumors closely resembling embryonal rhabdomyosarcoma (50). Clinically, deregulated c-Met expression (more often overexpression) in human tumors usually correlates with poor outcome (48, 49).

Recently, the concomitant actions of the CXCL12/CXCR4 and HGF/c-Met axes, combined with the activity of a third axis formed by leukemia-inhibitory factor (LIF), and its receptor LIF-R, have been reported in a murine model to promote and direct the homing of tissue-committed stem/progenitor cells (which also includes a population expressing early markers of striated muscle differentiation) from bone marrow to distant sites (51). These findings suggest that multiple axes promoting cell migration and invasion may also be simultaneously active in human tumors.

In rhabdomyosarcoma experimental models, the expression of functionally active CXCR4 and c-Met has been reported in continuous cell lines (10, 12, 13, 15, 16), and has been associated with in vitro invasive growth (10, 12, 13, 15, 16) and in vitro migration to marrow in a CXCR12-dependent and HGF-dependent manner (13, 15). Rhabdomyosarcoma tumors have also been investigated and the great majority of them have been found to express CXCR4 (14, 16) or c-Met (10, 11, 15), but no systematic analysis of the expression of these two genes in a clinical series of rhabdomyosarcoma primary tumors has been done with regard to clinicopathologic variables, marrow involvement, and outcome. The present study shows that CXCR4 and c-Met are widely expressed in primary rhabdomyosarcoma tumors and, at significantly higher levels, by isolated marrow-infiltrating tumor cells. This latter finding demonstrates the clinical relevance of previous experimental results showing that CXCR4-positive and/or c-Met–positive tumor cells are particularly prone to migrate to and survive in the bone marrow (13). We also show that the levels of CXCR4 and c-Met expression in primary tumors are associated with unfavorable clinical features and, only for CXCR4, a shorter OS and EFS. Taken together with the previous experimental results (10, 12, 13, 15, 16), these findings strongly implicate the causative role of CXCR4 and c-Met expression in invasive growth and metastatic spread which occurs in patients with rhabdomyosarcoma.

The identification of isolated CXCR4- and c-Met–positive tumor cells in marrow requires some further consideration. First, in this study, the expression of CXCR4 and c-Met by isolated tumor cells infiltrating the marrow was done at diagnosis and—in patients with otherwise localized disease and in three patients with metastatic disease but cytomorphologically negative marrow—in a setting of "occult" disease, i.e., when tumor cells had not yet proliferated into microscopically recognizable cell nests. The early timing of sampling enabled us to assess the role of CXCR4 and c-Met at a stage when molecular mechanisms are likely to be of pivotal importance in directing the pattern of metastatic spread (36), but concomitantly, it prevented us from evaluating the in vivo role of the HGF/c-Met axis in supporting tumor cell survival after exposure to cytotoxic drugs or radiotherapy, a role that has been described in in vitro experiments (13). It could be speculated that CXCR4 is predominant in directing rhabdomyosarcoma cells to marrow but that, under chemotherapy, the ratio of isolated marrow-infiltrating tumor cells may change in favor of c-Met–positive cells. Second, these findings extend our previous observations on the occurrence and significance of occult marrow involvement in patients with rhabdomyosarcoma (17). As previously described for other solid tumors in children (52, 53) and adults (54, 55), the presence at diagnosis of occult marrow disease in rhabdomyosarcoma is predictive of overt metastases at distant sites and poor outcome (17). Nonetheless, as also described in this study, in the majority of cases, isolated tumor cells detected in marrow may not represent actual metastatic tumor cells, which harbor the molecular machinery needed to execute the complete metastatic cascade in the marrow microenvironment. The characteristics of marrow sinusoids, together with the secretion by marrow endothelial and stromal cells of CXCL12, HGF, LIF, and other chemokine and growth factors, provide a unique microenvironment which is able to successfully chemotact and concentrate different types of circulating normal and...
tumor cells. Marrow may thus represent a transitory permissive microenvironment for circulating CXCR4-positive, c-Met-positive, and LIF-R-positive tissue-committed stem/progenitor cells (51) as well as CXCR4-positive and c-Met-positive tumor cells (this study) to stop and survive in a state of quiescence, the latter eventually proceeding to colonize other more steadily permissive sites and give rise to overt metastasis. The observation in this study that, out of 13 patients with occult marrow involvement, only 2 of 10 patients with otherwise localized disease and 1 of 3 patients with metastatic disease subsequently developed overt marrow and/or bone metastases, seems consistent with this view of bone marrow as a possible transitory reservoir for circulating tumor cells expressing the appropriate cell surface receptors.

Our results have shown substantial levels of CXCR4 and c-Met expression in the majority of rhabdomyosarcoma tumors analyzed, with higher levels of expression associated with alveolar histology. The molecular determinants for the expression of these two genes during myogenic differentiation have been previously investigated, and it has been shown that both CXCR4 and c-Met genes are downstream targets of PAX3-FKHR, and probably, of wild-type PAX3 and/or PAX7 (11, 14). Accordingly, CXCR4 and c-Met are expressed in striated muscle progenitor cells and regulate their migration during the embryonal development of skeletal muscles (56). Therefore, it is possible that the high levels of CXCR4 and c-Met expression could be related to a retained developmentally regulated transcriptional accessibility of these two genes in the genome of rhabdomyosarcoma cells. Based on the observation that both CXCR4 and c-Met genes are downstream targets of PAX3-FKHR (11, 14), we investigated the possible associations in alveolar tumors between gene fusion status and CXCR4 and c-Met expression. However, despite a trend for either protein to be expressed at higher levels in PAX3-FKHR-positive tumors compared with PAX7-FKHR-positive or fusion-negative tumors, no significant association was identified, probably also because of the limited number of patients investigated. Another reason for high levels of CXCR4 and c-Met in rhabdomyosarcoma primary tumors can be represented by the diffuse state of hypoxia as reported in a meta-analysis of the gene expression data sets available for rhabdomyosarcoma (57). Hypoxia-inducible factor-1α, a key mediator of the cellular response to hypoxia, is known to induce the expression of a series of genes involved in cell survival, angiogenesis, and invasion, including CXCR4 (58) and c-Met (59), thus enabling tumor cells to egress from hypoxic areas and migrate to distant, more favorable microenvironments. Further studies evaluating the expression and role of hypoxia-inducible factor-1α in rhabdomyosarcoma may clarify whether and to what extent hypoxia can contribute to rhabdomyosarcoma metastatic spread.

The present study has several limitations. The number of patients investigated was limited, with small numbers represented from each clinical group, and they were selected and analyzed retrospectively. Moreover, CXCR4 expression was strongly associated with alveolar histology, unfavorable primary site, size of primary tumor (>5 cm), and advanced group—all well-established indicators of poor outcome in patients with rhabdomyosarcoma. A larger, prospective study using multiple regression analysis would be needed to confirm the role of CXCR4 and c-Met in rhabdomyosarcoma, and to evaluate whether CXCR4 is an independent indicator of outcome. In addition, quantitative assessment of protein expression levels by immunohistochemistry or immunocytoLOGY has obvious limits when compared with molecular assessment of RNA expression. Moreover, the evaluation of myogenin staining in isolated tumor cells detected in marrow aspirates was done by using immunoperoxidase and false-positive reactions cannot be totally ruled out due to the technical limitations of this technique. Yet, in this study, the morphologic identification in primary tumors of immunostained tumor cells allowed us to exclude the possible confounding effect of the expression of the same proteins by stromal and inflammatory cells; likewise, double-label immunocytoLOGY enabled us to carry out the selective analysis of CXCR4 and c-Met expression among isolated marrow-infiltrating tumor cells. Finally, only two of the several receptors potentially involved in regulating tumor cell migration and metastasis have been evaluated in this series. In 2007, when this study was already completed, the role of the LIF/LIF-R axis in promoting invasive growth and directing tumor cells to marrow was reported in a rhabdomyosarcoma murine model (60). However, because several additional pathways active in promoting organ-specific metastatic spread have already been described in other cancer types (9, 36), it is highly improbable that the CXCL12/CXCR4, HGF/c-Met, and LIF/LIF-R axes are the only players in rhabdomyosarcoma.

Occult or overt marrow involvement at diagnosis is frequent in patients with localized or metastatic rhabdomyosarcoma, respectively, and in both settings, is associated with a significantly poorer outcome (5, 17, 61). Here, we have provided clinical evidence supporting the role of CXCR4 and c-Met in promoting tumor spread to this site, and have shown that tumor cells belonging to both alveolar and embryonal subtypes express significant levels of the two proteins in primary tumors as well as in marrow. These findings, together with the availability of selective inhibitors of CXCR4 and c-Met, the efficacy of which in reducing tumor invasion and metastasis in animal models has already been shown (62, 63), validate the previous suggestions that CXCR4 and c-Met are novel therapeutic targets in rhabdomyosarcoma. Both the observation in experimental models that such inhibition is inadequate to eradicate established metastases (62, 63), and the presence of CXCR4-positive and c-Met-positive tumor cells in the marrow of patients with otherwise localized rhabdomyosarcoma as shown in this study, would suggest that anti-CXCR4 and c-Met treatment should be administered as early as possible in the course of disease. Hypothetically, this treatment would not only inhibit tumor spread to marrow but, as a result, would also reduce the possible spread from marrow to other more permissive sites and the subsequent emergence of overt metastasis at these sites. The present therapeutic strategy, including up-front window studies for patients with poor-risk rhabdomyosarcoma (64), affords an appropriate setting in which these new agents may be included and evaluated alongside cytotoxic agents.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

Acknowledgments

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Clinical Significance of CXC Chemokine Receptor-4 and c-Met in Childhood Rhabdomyosarcoma

Francesca Diomedi-Camassei, Heather P. McDowell, Maria A. De Ioris, et al.

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