Tumor-Cell Homing to Lymph Nodes and Bone Marrow and CXCR4 Expression in Esophageal Cancer

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Background: The chemokine and bone marrow-homing receptor CXCR4 has been implicated in metastatic dissemination of various cancers. We investigated CXCR4 expression in esophageal cancer specimens and its association with survival, lymph node microinvolvement, and bone marrow micrometastasis. Methods: We analyzed frozen tumor specimens from 136 patients with completely resected esophageal cancer for CXCR4 expression by immunohistochemistry. Lymph node microinvolvement and bone marrow micrometastasis were assessed by immunohistochemistry with monoclonal antibodies Ber-EP4 (against epithelial cell adhesion molecule) and pancytokeratin A45-B/B3 (against several cytokeratins), respectively. Associations between CXCR4 expression and clinicopathologic features, including tumor stage, histologic grade, lymph node metastasis and microinvolvement, bone marrow micrometastasis, and survival, were investigated with Fisher’s test, log-rank test, and Cox multivariable analysis. All statistical tests were two-sided. Results: CXCR4 protein was expressed in 75 (55%) of 136 esophageal tumors examined. CXCR4 expression was statistically significantly associated with reduced median overall and disease-specific survival, compared with CXCR4 nonexpression (P<.001; log-rank test). The median overall survival of patients with CXCR4-positive tumors was 20 months and with CXCR4-negative tumors, 76 months (difference = 56 months, 95% confidence interval [CI] = 4 to 108 months; P<.001). The median disease-specific survival of patients with CXCR4-positive tumors was 25 months and with CXCR4-negative tumors was 97 months (difference = 72 months, 95% CI = 34 to 110 months; P<.001). CXCR4 expression was statistically significantly associated with increased lymph node microinvolvement (P<.001) and with increased bone marrow micrometastasis (P<.001). In multivariable analysis, CXCR4 expression, compared with its nonexpression, was identified as the independent variable that was most strongly associated with reduced disease-specific survival (relative risk [RR] of death = 2.03, 95% CI = 1.20 to 3.41; P = .008) and overall survival (RR of death = 2.18, 95% CI = 1.33 to 3.59; P = .002). Conclusion: CXCR4 expression was associated with poor clinical outcome in esophageal cancer patients. CXCR4 may have a role in early metastatic spread because its expression was associated with micrometastases to both the lymph nodes and bone marrow. Thus, CXCR4 should be explored further as a target for adjuvant therapy for micrometastatic disease.

Esophageal carcinoma is one of the most aggressive tumors, with a highly malignant potential for lymph node metastasis and vascular invasion, even at the time of diagnosis (1). Although postoperative mortality has declined and rates of complete resection have improved considerably, reported 5-year survival rates are only 20% to 36% after curative surgery (1). The presence of micrometastases in regional lymph nodes or peripheral organs is a strong prognostic factor for cancer-related death from diseases such as esophageal cancer, but some patients will relapse with metastatic disease despite the absence of detectable metastases at the time of primary surgery.

Metastasis involves several sequential steps and is a highly organized and organ-selective process. Early metastatic relapse after complete resection of an apparently localized tumor indicates that disseminated tumor cells or micrometastases, undetectable by current routine methods, may have been present at the time of surgery (2,3). The first phase of the metastatic cascade consists of loss of tumor cell adhesion, induction of cell motility, and local tumor cell invasion (4). These steps are followed by dissemination to regional lymph nodes or circulation through the blood and then by homing to secondary organs, in which tumor cells may reside as viable cells in a dormant or quiescent state (5). Some of these cells may eventually become precursors of metastases that can arise many years after curative resection of the primary tumor (3). Current tumor staging procedures are unable to detect single disseminated tumor cells, and sensitive molecular procedures have, therefore, been developed to detect disseminated tumor cells in lymph nodes and distant organs, in particular, bone marrow.

Epithelial markers, such as cytokeratins, are widely used to detect disseminated tumor cells in mesenchymal organs, such as lymph nodes and bone marrow (3,6). The presence of micrometastatic tumor cells in histopathologically tumor-free lymph nodes is a strong prognostic factor for esophageal cancer (2). Twenty percent to 40% of patients with esophageal carcinoma harbor disseminated tumor cells in their bone marrow, even in the absence of lymph node metastasis (stage N0) or clinical signs of overt distant metastasis (stage M0) at the time of primary surgery, and such dissemination is also a predictor for the postoperative occurrence of overt metastasis at distant sites (2,7,8).

Chemokines are chemotactic factors that regulate the development and migration of various cell types. They are classified into four groups (CXC, CX3C, CC, and C) on the basis of the position of the first two highly conserved cysteines (C) in the amino acid sequence (where X is any amino acid residue). To mediate their chemical effects on target cells, chemokines use
G-protein-coupled receptors that are characterized structurally by seven transmembrane-spanning domains and are involved in the attraction of leukocytes to different organs. Chemokines play important roles in angiogenesis and tumor growth, and their role in metastasis has recently been explored (9–11). CXCR4 was initially described as the chemokine receptor that was involved in the homing of hematopoietic stem cells and lymphocytes to the bone marrow and to sites of inflammation (12,13). The chemokine ligand CXCL12, also known as stromal cell-derived factor (SDF)-1α, is expressed in lung, liver, and bone marrow and acts as a lymphocyte chemoattractant in these organs; it has been hypothesized that CXCR4 is involved in metastasis to these sites (14). Muller et al. (15) demonstrated a role for CXCR4 in breast cancer metastasis to lymph nodes and lungs. Other results (16–21) have identified CXCR4 expression in other tumor types such as melanoma; pancreatic, thyroid, renal, and small-cell lung cancers; and squamous cell carcinoma of the tongue. In some studies (21–23), the expression of CXCR4 was associated with decreased survival and increased lymph node metastasis. Blocking agents and antibodies against the CXCR4 receptor have been found to prevent metastasis in murine models of breast cancer and malignant melanoma (24–26).

Only one study (27), to our knowledge, has found an association between a chemokine receptor, namely CCR7, and lymph node metastasis in esophageal squamous cell carcinoma. The role of other chemokine receptors in the metastatic spread of esophageal cancer cells has yet to be determined.

The purpose of this study was to investigate CXCR4 expression in esophageal cancer specimens and, in particular, to determine whether CXCR4 might serve as a prognostic factor relevant to tumor cell homing to the regional lymph nodes or distant sites by use of bone marrow as an indicator organ.

Materials and Methods

Study Design and Patients

This study was approved by the ethics committee of the chamber of physicians at Hamburg, Germany. Written informed consent was obtained from all patients to use the resected samples and to perform bone marrow aspirates for research purposes. For this study, all 136 patients with esophageal cancer who underwent surgery in the Department of Surgery at University Medical Center Hamburg-Eppendorf between May 1, 1992, and September 30, 2003, were chosen retrospectively. Histopathologic examination of the surgical specimen found that the resected margins were tumor free. We found no evidence of distant metastasis for any patient. Tumor stage and grade were classified according to the tumor-node metastasis classification of the International Union Against Cancer (28).

Immunohistochemical Staining for CXCR4 and Evaluation of Intensity

Tumor tissue samples that had been snap-frozen in liquid nitrogen were embedded in Optimal Cutting Temperature Compound (Tissue-Tek) and sectioned at 5 mm. Sections were placed on slides coated with 3-triethoxysilylpropylamine (Merck, Darmstadt, Germany), air-dried, and then fixed for 5 minutes in acetone at room temperature. After rehydration in Tris-buffered saline (TBS = 0.05 M Tris-HCl at pH 7.6 and 0.15 M NaCl), sections were incubated with human AB serum (Biotest Diagnostics, Dreieich, Germany) diluted 1:10 in TBS for 20 minutes to block unspecific binding. Sections were stained with anti-human CXCR4 monoclonal antibody (immunoglobulin G subclass 2a [IgG2a], clone 12G5; R&D Systems, Minneapolis, MN) at a dilution of 1:100 overnight at 4 °C. An irrelevant murine IgG1 monoclonal antibody (MOPC21; Sigma, Deisenhofen, Germany) was used as a negative control. At least two representative tissue sections from each patient with esophageal cancer were examined. Colon mucosa from healthy control individuals served as positive staining control for CXCR4 (29). The antibody reaction was developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique including secondary rabbit anti-mouse polyclonal antibody (clone Z0259; Dako, Hamburg, Germany) and APAAP staining complex (Dako) combined with a new fuchsin stain (Sena, Heidelberg, Germany) for visualization (2). Sections were counterstained with Mayer’s hematoxylin solution (Merck). Specimens were considered immunopositive for CXCR4 when more than 20% of all tumor cells within one section were clearly immunostained. Results did not vary when another nearby cutoff (e.g., 5% positive tumor cells as a cutoff) was tested. The tumor samples were then classified as having either absent to low staining (CXCR4 negative) if 20% or fewer tumor cells expressed CXCR4 or moderate to strong staining (CXCR4 positive) if more than 20% of tumor cells expressed CXCR4. Immunohistochemical analysis and scoring were performed by two independent investigators who were unaware of patient outcome or other clinical findings (J. T. Kaiﬁ and D. Obonyo). For 95% of the slides, the observers’ evaluations were identical; the remaining slides were reevaluated, and consensus decisions were made.

Detection of Tumor Cells in Bone Marrow

Bone marrow aspirates of 4–8 mL were obtained from the iliac crest of patients on the day of surgery and processed as described previously (2). Briefly, the aspirates were collected in heparin, and mononuclear cells were isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400 × g for 30 minutes. Cells were then subjected to cytocentrifugation at 150 × g for 3 minutes at room temperature, to apply cells to a glass slide. To detect tumor cells in bone marrow, we used monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany) that detects an epitope on various cytokeratins, including cytokeratins 8, 18, and 19. Unspecific IgG1 was used as a control antibody. Visualization was performed by the APAAP technique described above. Counterstaining was done with Mayer’s hematoxylin. We used the Automated Cellular Imaging System (ChromaVision, Medical Systems Inc., CA) to screen immunostained bone marrow slides for disseminated tumor cells (30).

Immunohistochemical Detection of Lymph Node Microinvolvement

Lymph nodes were systematically sampled during lymphadenectomy as described previously (2). Each lymph node that was removed at the time of surgery was divided into two parts. One part was embedded in paraffin for routine histopathologic staging and stained with hematoxylin and eosin; the other part was snap-frozen in liquid nitrogen. Lymph nodes from patients without evidence of nodal metastasis on routine histopathologic examination were screened for the presence of epithelial tumor cells with...
the Ber-EP4 monoclonal antibody (IgG1; Dako), as described previously (2). This antibody is directed against the epithelial cell adhesion molecule on the surface and in the cytoplasm of nearly all epithelial cells, and it does not react with mesenchymal tissue, including lymphoid tissue (31). Two cryostat sections (5–6 μm thick) were cut at three different levels in each lymph node. Sections were stained by the APAAP technique. Normal esophageal mucosa served as the positive staining control. The slides stained with hematoxylin and eosin or the immunostained sections were evaluated in a blinded fashion by observers working independently. Minimal tumor cell involvement in a lymph node that was considered to be tumor free by routine histopathologic staining was defined as the presence of from one to 10 Ber-EP4–positive cells in the body of the lymph node.

Clinicopathologic Data

All data including sex, histology, depth of tumor invasion, lymph node metastasis, tumor type, and disease stage were obtained from the clinical and pathologic records. Patients whose death was clearly documented as attributable to esophageal cancer were considered to have died of that disease; other deaths were not considered to have been caused by esophageal cancer. Clinical follow-up data were obtained by reviewing the hospital records, by direct communication with the attending physicians, and from the Cancer Registry of Hamburg. Overall survival was calculated from the date of surgical excision of the tumor to the date of death or last follow-up. When disease-specific survival was calculated, data for patients who died from other causes were censored at their time of death.

Statistical Analysis

We used SPSS for Windows (version 11.5.1; SPSS Inc., Chicago, IL) for statistical analysis. Survival was analyzed by the Kaplan–Meier method, and the log-rank test was used for univariate analysis. To assess the independent association of CXCR4 expression and esophageal carcinoma outcome simultaneously with covariates that were statistically significantly associated with disease-specific and overall survival in univariate analysis, such as lymph node metastasis, histologic grading, lymph node microinvolvement, and bone marrow micrometastasis, we performed Cox regression analysis for multivariable analysis. Relationships between the immunostaining results of CXCR4 and clinical factors were calculated with cross-tables, and statistical significance of these associations was determined with Fisher’s test. Statistical significance was determined as P values from two-sided tests of less than .05. All statistical tests were two-sided.

RESULTS

Patient Characteristics

We included 136 patients with esophageal cancer in the study. Characteristics of the patients are listed in Table 1. Briefly, the median age of the study population was 59.5 years; 107 (79%) of the 136 patients were male, and 29 (21%) were female. Adenocarcinoma was the histologic subtype in 71 (52%) of the 136 patients, and squamous cell carcinoma was in 65 (48%) patients. The invasion depth was classified as pT1 in 36 (26%) of the 136 patients, pT2 in 49 (36%), pT3 in 49 (36%), and pT4 in 2 (2%) patients; 63 (46%) of the 136 patients were staged as pN0, and 73 (54%) had lymph node metastasis by histopathologic evaluation. The median follow-up of all patients was 28 months (range = 2–199 months). The 5-year overall survival rate for all patients included was 39% (95% confidence interval [CI] = 30% to 48%). The median survival was 49 months (range = 2–199 months). In 60 (44%) of the 136 patients, disseminated tumor cells were detected in the bone marrow aspirate. Among the 136 patients, 58 (43%) had tumor cells in histopathologically tumor-free lymph nodes.

Immunohistochemical Analysis of CXCR4 in Esophageal Cancer Tissues

CXCR4 expression of 136 esophageal cancer specimens was determined by immunohistochemistry. Fig. 1 shows representative staining patterns for CXCR4 in esophageal adenocarcinoma and squamous cell carcinoma specimens. Staining was not detected in normal esophageal epithelium (data not shown). Lack of staining or weak staining of CXCR4 (i.e., ≤20% of tumor cells expressed CXCR4) was classified as CXCR4-negative expression, and moderate to strong staining (i.e., >20% of tumor cells expressed CXCR4) was classified as CXCR4-positive expression. A total of 75 (55%) of the 136 tumors were CXCR4 positive and 61 (45%) samples were CXCR4 negative (Table 2). There were no statistically significant differences in CXCR4 expression results with respect to sex.

Overall and Disease-Specific Survival and the Expression of CXCR4

We next examined the relationship between CXCR4 expression and survival of patients with esophageal carcinoma. Survival was analyzed by the Kaplan–Meier method, and the log-rank test was used for univariate analysis. Overall survival and disease-specific survival rates among patients with CXCR4-negative tumors compared with patients with CXCR4-positive tumors are shown in Fig. 2. CXCR4-positive expression in the primary
tumor was statistically significantly associated with poorer overall and disease-specific survival than CXCR4-negative expression (P<.001 by log-rank test). The median overall survival associated with CXCR4-positive tumors was 20 months, and the median overall survival associated with CXCR4-negative tumors was 76 months (difference = 56 months, 95% CI = 4 to 108 months; P<.001). The median disease-specific survival associated with CXCR4-positive tumors was 25 months, and the median disease-specific survival associated with CXCR4-negative patients was 97 months (difference = 72 months, 95% CI = 34 to 110 months; P<.001).

Among the 49 patients without detectable micrometastasis in bone marrow and lymph nodes (14 with CXCR4-positive and 35 with CXCR4-negative tumors), CXCR4-positive expression (median overall survival = 34 months) was associated with reduced survival compared with CXCR4-negative expression (median overall survival = 97 months) (difference = 63 months, 95% CI = 18 to 108 months; P= .005). The group with best survival had CXCR4-negative tumors, independent of the presence or absence of lymph node or bone marrow micrometastasis, whereas the group with worst survival had CXCR4-positive tumors and both lymph node and bone marrow micrometastasis (n = 24; median overall survival 15 months; 95% CI = 7 to 22 months). The survival data for the other groups were as follows. Among patients with CXCR4-positive tumors, with bone marrow micrometastasis, and without lymph node micrometastasis (n = 19), the median overall survival was 16 months (95% CI = 12 to 20 months). Among patients with CXCR4-positive tumors, without bone marrow micrometastasis, and with lymph node micrometastasis (n = 18), the median overall survival was 26 months (95% CI = 12 to 41 months). Among patients with CXCR4-negative tumors, with bone marrow micrometastasis, and with lymph node micrometastasis (n = 7), the median overall survival was 25 months (95% CI = 15 to 36 months). Among patients with CXCR4-negative tumors, without bone marrow micrometastasis, and with lymph node micrometastasis (n = 9), median overall survival was 31 months (95% CI = 23 to 38 months).

**CXCR4 Expression, Tumor Depth, and Lymph Node Metastasis**

To evaluate the association between CXCR4 expression and clinicopathologic findings, we investigated the association between the expression of CXCR4 and several clinicopathologic factors (Table 2). We determined associations between clinicopathologic factors and sex, histologic tumor type and grade, tumor depth (pT), and lymph node metastasis (pN). Microinvolvement of the lymph nodes in histopathologically tumor-free lymph nodes, compared with its noninvolvement, was associated with statistically significantly poorer overall and disease-specific survival (P = .001). The median overall survival of patients with lymph node microinvolvement was 24 months and without lymph node microinvolvement was 48 months (difference = 24 months, 95% CI = 1 to 47 months; P = .001). Median disease-specific survival of patients with lymph node microinvolvement was 25 months and without was 75 months (difference = 50 months, 95% CI = 15 to 85 months; P = .001). The presence of micrometastatic tumor cells in bone marrow, compared with its absence, was also statistically significantly associated with poorer overall and disease-specific survival (P<.001). Median overall survival of patients with bone marrow micrometastasis was 18 months and without was 64 months (difference = 46 months, 95% CI = 14 to 68 months; P<.001). Median disease-specific survival of patients with bone marrow

<table>
<thead>
<tr>
<th>Variable</th>
<th>CXCR4 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Total, No. (%)</td>
<td>61 (45)</td>
<td>75 (55)</td>
</tr>
<tr>
<td>Mean age, y ± SD</td>
<td>58.81 ± 12.57</td>
<td>58.72 ± 12.56</td>
</tr>
<tr>
<td>Sex, No. (%)</td>
<td>Male: 44 (41)</td>
<td>63 (59)</td>
</tr>
<tr>
<td></td>
<td>Female: 17 (59)</td>
<td>12 (41)</td>
</tr>
<tr>
<td>.140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type, No. (%)</td>
<td>Squamous cell carcinoma: 25 (38)</td>
<td>40 (62)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma: 36 (51)</td>
<td>35 (49)</td>
</tr>
<tr>
<td>Tumor histology</td>
<td>G1/G2: 40 (48)</td>
<td>44 (52)</td>
</tr>
<tr>
<td></td>
<td>G3: 21 (44)</td>
<td>31 (56)</td>
</tr>
<tr>
<td>Tumor depth (pT), No. (%)</td>
<td>pT1/pT2: 50 (59)</td>
<td>35 (41)</td>
</tr>
<tr>
<td></td>
<td>pT3/pT4: 11 (22)</td>
<td>40 (78)</td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td>pN0: 36 (57)</td>
<td>27 (43)</td>
</tr>
<tr>
<td></td>
<td>pN1: 25 (34)</td>
<td>48 (66)</td>
</tr>
<tr>
<td>LNMM, No. (%)</td>
<td>Negative: 45 (58)</td>
<td>33 (42)</td>
</tr>
<tr>
<td></td>
<td>Positive: 16 (28)</td>
<td>42 (72)</td>
</tr>
<tr>
<td>BMNNM, No. (%)</td>
<td>Negative: 44 (47)</td>
<td>32 (53)</td>
</tr>
<tr>
<td></td>
<td>Positive: 17 (28)</td>
<td>43 (72)</td>
</tr>
</tbody>
</table>

*Data are presented in cross-tables. P values were determined by using two-sided Fisher’s tests. LNMM = lymph node micrometastasis; BMNNM = bone marrow micrometastasis; SD = standard deviation.
CXCR4 Expression and Lymph Node Microinvolvement

Lymph nodes were examined immediately after surgical removal. Histopathologically tumor-free lymph nodes were sectioned, and sections were stained for disseminated tumor cells with Ber-EP4 monoclonal antibody against epithelial cell adhesion molecule; 58 (43%) of 136 patients had disseminated tumor cells in their lymph nodes. As mentioned above, lymph node micrometastasis was strongly and negatively associated with overall and disease-specific survival in a Kaplan–Meier analysis. We used Fisher’s test to compare bone marrow micrometastasis with and without CXCR4 expression in the primary tumor and found a statistically significant positive association between CXCR4-positive expression of esophageal cancer cells in the primary tumor and the presence of esophageal tumor cells in the bone marrow ($P<.001$); 43 (72%) of 60 patients with micrometastatic tumor cells in their bone marrow expressed CXCR4 in their primary tumor. Among patients without micrometastatic tumor cells in the bone marrow, 32 (53%) of 76 patients had CXCR4-positive tumors.

CXCR4 Expression and Bone Marrow Micrometastasis

Mononuclear cells, isolated from bone marrow aspirates were obtained from the iliac crest of patients on the day of surgery, were analyzed for the presence of tumor cells with the pan-cytokeratin A45-B/B3 monoclonal antibody. Tumor cells were found in the bone marrow from 60 (44%) of 136 patients. As mentioned above, bone marrow micrometastasis was strongly and negatively associated with overall and disease-specific survival in a Kaplan–Meier analysis. We used Fisher’s test to compare bone marrow micrometastasis with and without CXCR4 expression in the primary tumor and found a statistically significant positive association between CXCR4-positive expression of esophageal cancer cells in the primary tumor and found a statistically significant positive association between CXCR4-positive expression of esophageal cancer cells in the primary tumor and the presence of esophageal tumor cells in the bone marrow ($P<.001$); 43 (72%) of 60 patients with micrometastatic tumor cells in their bone marrow expressed CXCR4 in their primary tumor. Among patients without micrometastatic tumor cells in the bone marrow, 32 (53%) of 76 patients had CXCR4-positive tumors.

CXCR4 Expression as an Independent Prognostic Factor

We used a Cox regression model for multivariable analysis to examine whether various factors were associated with reduced overall and disease-specific survival (Table 3). The following covariates that were statistically significantly associated with worse survival by univariate analysis by the log-rank test were included...
in the model as potential risk factors: lymph node status (pN0 or pN1), grading (G1–G3), bone marrow and lymph node micrometastasis, and CXCR4 expression. We found that survival was independently associated with CXCR4 expression, lymph node metastasis status, and histologic grade. Lymph node micrometastasis and bone marrow micrometastasis were associated with decreased overall survival (for lymph node micrometastasis, relative risk [RR] of death = 1.49, 95% CI = 0.94 to 2.36; for bone marrow micrometastasis, RR of death = 1.55, 95% CI = 0.97 to 2.47), although not statistically significantly so by multivariable analysis. Disease-specific survival was independently associated with bone marrow micrometastasis, compared with the absence of bone marrow micrometastasis (RR of death = 1.69, 95% CI = 1.03 to 2.78), but not with lymph node micrometastasis, compared with the absence of lymph node micrometastasis (RR of death = 1.58, 95% CI = 0.97 to 2.57). Furthermore, CXCR4-positive expression, compared with CXCR4-negative expression (for overall survival, RR of death = 2.18, 95% CI = 1.33 to 3.59; for disease-specific survival, RR of death = 2.03, 95% CI = 1.20 to 3.41) was more strongly associated with worse outcome than was the presence of lymph node metastasis (i.e., comparing pN1 with pN0) (for overall survival, RR of death = 1.97, 95% CI = 1.18 to 3.29; for disease-specific survival, RR of death = 1.88, 95% CI = 1.08 to 3.27) and grade (i.e., comparing G3 with G1/G2) (for overall survival, RR of death = 1.65, 95% CI = 1.03 to 2.63; and for disease-specific survival, RR of death = 2.07, 95% CI = 1.26 to 3.40) (Table 3).

**DISCUSSION**

We report, to our knowledge for the first time, that CXCR4 is expressed in esophageal cancer and that its expression is associated with survival data and with tumor cell dissemination into lymph nodes as an indicator for local disease and bone marrow as an indicator for systemic spread. In this retrospective study, we found that CXCR4 expression in primary tumor tissue was associated with worse disease-specific and overall survival in patients with esophageal cancer. A multivariable analysis that examined the association between death from esophageal cancer and other prognostic factors by univariate analysis, including lymph node status (pN0 versus pN1) and histologic grade, revealed that CXCR4 expression in the tumor was the factor most strongly associated with outcome for esophageal cancer patients.

It is noteworthy that CXCR4 expression was not detected in normal esophageal epithelium but was detected in both types of esophageal cancer tumors—adenocarcinoma and squamous cell carcinoma. Thus, our data provide evidence that expression of CXCR4 by primary tumor cells is associated with malignant transformation in esophageal cancer. These results are consistent with those from a breast cancer study (15). Although the mechanism of CXCR4 activity is not known, CXCR4 may induce or support carcinogenesis through the interaction of CXCR4 with its ligand SDF-1, which mediates the activation of phosphatidylinositol 3-kinase and Akt, resulting in cell proliferation (32,33). In our study, we used immunohistochemical analysis to detect CXCR4 protein expression in tumor cells directly. To avoid cutpoint optimization as discussed by Altman et al. (34), we chose a cutpoint of 20% CXCR4-positive tumor cells to separate immunostained tumors into a group that expressed CXCR4 and a group that did not express it. As a control that the results were not cutpoint sensitive, the results did not vary when other nearby cutpoints were tested.

The homing mechanism that guides tumor cells to the bone marrow is not well understood. Because the migration of hematopoietic stem cells to the bone marrow is mediated through an interaction between the CXCR4 receptor and its ligand SDF-1, which is secreted by bone marrow stromal cells (35), we hypothesized that the CXCR4 receptor participates in the bone-marrow homing mechanism used by tumor cells in general and by esophageal cancer cells in particular. Furthermore, it has been shown that the CXCR4 receptor plays roles in the bone metastasis of prostate carcinoma, as shown with a murine model (36), and in the bone marrow metastasis of myeloma and neuroblastoma cells (12,37). CXCR4 expression is increased by hypoxia-inducible factor (HIF)-1α (19), and a recent study revealed higher expression of HIF-1α in breast carcinomas of patients with bone marrow micrometastasis, but these data were generated with a cDNA microarray that did not include CXCR4 (38). Consequently, no other study has directly examined the role of CXCR4 in bone marrow tumor cell dissemination in cancer patients.

In esophageal cancer, the presence of micrometastatic tumor cells in lymph nodes and bone marrow has been associated with poorer survival (2,39,40). Although the presence of micrometastatic cells in regional lymph nodes was not surprising, we found esophageal cancer cells in the bone marrow more frequently than expected, given the rather infrequent rate of overt skeletal metastases of esophageal cancer cells generally observed. Thus, disseminated tumor cells in the bone marrow may enter circulation again and migrate to other organs, such as the liver or lung, in which better growth conditions for esophageal cancer cells might exist and esophageal cancer metastasis usually occurs (41,42). Recent work by Pierga et al. (30) suggested that circulating tumor cells in peripheral blood that can find their way to the bone marrow and survive there appear to have an increased ability to develop into overt metastases. Our results suggest a potential role of CXCR4 in the preferential formation of micrometastases at these sites in esophageal carcinoma.

Because the interaction between esophageal cancer–expressed CXCR4 and SDF-1, which is produced elsewhere, may be a major event in directing esophageal cancer cells to lymph nodes.

**Table 3. Multivariable Cox regression analysis for overall and disease-specific survival for various factors***

<table>
<thead>
<tr>
<th>Variable</th>
<th>RR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0 vs. pN1</td>
<td>1.97 (1.18 to 3.29)</td>
<td>.010</td>
</tr>
<tr>
<td>G1 vs. G3</td>
<td>1.65 (1.03 to 2.63)</td>
<td>.036</td>
</tr>
<tr>
<td>CXCR4+ vs. CXCR4−</td>
<td>2.18 (1.33 to 3.59)</td>
<td>.002</td>
</tr>
<tr>
<td>LNMM</td>
<td>1.49 (0.94 to 2.36)</td>
<td>.091</td>
</tr>
<tr>
<td>BMMM</td>
<td>1.55 (0.97 to 2.47)</td>
<td>.066</td>
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<tr>
<td>Disease-specific survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0 vs. pN1</td>
<td>1.88 (1.08 to 3.27)</td>
<td>.025</td>
</tr>
<tr>
<td>G1 vs. G3</td>
<td>2.07 (1.26 to 3.40)</td>
<td>.004</td>
</tr>
<tr>
<td>CXCR4+ vs. CXCR4−</td>
<td>2.03 (1.20 to 3.41)</td>
<td>.008</td>
</tr>
<tr>
<td>LNMM</td>
<td>1.58 (0.97 to 2.57)</td>
<td>.067</td>
</tr>
<tr>
<td>BMMM</td>
<td>1.69 (1.03 to 2.78)</td>
<td>.038</td>
</tr>
</tbody>
</table>

*RR = risk ratio; CI = confidence interval; pN = lymph node metastasis; G = histologic grade; LNMM = lymph node micrometastasis; BMMM = bone marrow micrometastasis; + = positive expression; − = negative expression. Statistics were done by multivariable Cox regression analysis. All statistical tests were two-sided. RRs of death presented are for overall and disease-specific survival.
and bone marrow, this interaction may also account for metastasis from other tumors. Muller et al. (15) demonstrated that CXCR4 is involved in breast cancer metastasis to sites with high expression of SDF-1. The interaction between CXCR4 and SDF-1 appears to be involved in the homing to the bone marrow of multiple myeloma cells and neuroblastoma cells (12,37) and of prostate cancer cells, as shown in a murine model of prostate carcinoma (36). Increased expression of HIF-1α has been detected in patients with breast cancer who also have bone marrow micrometastasis (38). Whether HIF-1α has a role in the metastasis of esophageal cancer cells is yet to be determined. We found associations between CXCR4 receptor expression and lymph node micrometastases and lymph node macrometastasis. CXCR4 has been shown in a mouse model to be involved in colon carcinoma micrometastasis to the lungs (43), and a positive association between CXCR4 expression and lymph node micrometastasis has been found in other tumor types, including squamous cell carcinoma of the tongue or in breast carcinoma (21,22). Our results indicate a possible role for CXCR4 in the dissemination and outgrowth of esophageal cancer cells in the lymph nodes.

The current study has several limitations. One is that the patients were selected retrospectively; consequently, these results need to be confirmed in larger prospective trials. In addition, a role of CXCR4 in homing of esophageal cancer cells to lymph nodes and bone marrow is probable; however, a correlation does not prove a direct role of CXCR4 in this process. Double staining of micrometastatic tumor cells should be performed in future experiments to show coexpression of CXCR4 and epithelial markers in single disseminated tumor cells. Finally, a causal relationship needs to be examined by functional experiments in vitro or in vivo models.

A previous investigation (44) of the expression of CXCR4 in esophageal cancer that used reverse transcriptase–polymerase chain reaction did not detect higher expression in tumor tissue than in noncancerous tissue. The fact that CXCR4 is also expressed by lymphocytes and endothelial cells may explain these false-positive results and discordant findings in noncancerous tissue when a nonquantitative reverse transcriptase–polymerase chain reaction was used. In contrast, we detected CXCR4 by immunohistochemical staining directly on the tumor cell surface and excluded staining of nonepithelial cells. These results, however, must be validated in an independent prospective trial. Various blocking agents against CXCR4 have been developed (24,26), and these agents should be used to validate this chemokine receptor as a molecular target for the treatment of esophageal cancer in patients who have not received toxic systemic treatment.

REFERENCES


NOTES

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