Macrophage Colony-Stimulating Factor-1 Receptor Expression Is Associated with Poor Outcome in Breast Cancer by Large Cohort Tissue Microarray Analysis

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ABSTRACT

Purpose: Macrophage colony-stimulating factor-1 receptor (CSF-1R) is a transmembrane tyrosine kinase receptor, which is abnormally expressed in invasive breast cancer. Small cohort studies have demonstrated that increased expression of CSF-1R is associated with ipsilateral breast cancer recurrence. Correlation with survival has not been reported. Our aim was to further evaluate the role of CSF-1R in breast cancer, by studying the expression of CSF-1R in a large cohort of clinical specimens.

Experimental Design: Tissue microarrays containing 301 node-negative and 280 node-positive cases were used. Immunohistochemical staining was performed and correlated with overall survival, nodal status, and other clinicopathological data.

Results: CSF-1R expression was strongly associated with nodal status. Of the node-negative cases, 114 (38.9%) stained positive for CSF-1R, whereas 189 (67.5%) of the node-positive cases expressed CSF-1R (P < 0.0001). CSF-1R expression is also associated with larger tumor size (P = 0.02). Positive staining was strongly associated with decreased survival (P = 0.0003). Among node-negative patients, CSF-1R expression was associated with decreased overall survival (P = 0.045), whereas among node-positive patients, it was not (P = 0.47). In multivariate analysis, CSF-1R was not independent of nodal status as a predictor of survival.

Conclusions: CSF-1R expression is a strong predictor of poor outcome in nonmetastatic breast cancer. It is significantly more frequently expressed in patients with nodal involvement. Among the node-negative patients, it has a stronger association with survival than among the node-positive patients. Our findings support other preclinical findings that CSF-1R may be involved in local invasion and metastasis. Thus, this receptor may be an effective target for therapeutic agents.

INTRODUCTION

The cfms proto-oncogene encodes the only known receptor for the macrophage colony-stimulating factor-1 (CSF-1). Colony-stimulating factor-1 receptor (CSF-1R) is a transmembrane tyrosine kinase receptor, and its ligand, CSF-1, has soluble, membrane bound, and cell matrix-associated isoforms (1–3). The CSF-1R/CSF-1 receptor/ligand pair has essential physiological functions in monocyte and macrophage differentiation (4, 5), embryonic implantation, placental development, and lachogenic differentiation of the human breast (6–8). Abnormally high CSF-1R expression has been associated with aggressive behavior in a variety of malignancies, including breast, prostate, ovarian, and endometrial cancer (9–16).

In previous studies (6), a spontaneously immortalized mammary epithelial cell line cultured from lactating mice was stably transfected with the CSF-1R oncogene. Coexpression of the endogenous ligand produced an autocrine system in all transfecants that express the receptor. Compared with the parental cells, transfecants expressing CSF-1R invaded 100-fold more efficiently through a barrier of reconstituted basement membrane (Matrigel), formed colonies in soft agar, and produced 10 times more lung metastases when injected i.v. in mice. These findings suggest a role for CSF-1R in tumor invasion and tumorigenesis of breast cancer cells. Other studies using mouse models (17) have demonstrated that CSF-1 and CSF-1R are important for tumor invasion and metastasis but not for growth of primary mammary tumors.

The role of CSF-1R in clinical specimens has been studied in relatively small patient cohorts. One study (13) demonstrated CSF-1R and CSF-1 expression in 41 of 48 invasive breast tumors but not in in situ carcinoma (n = 14). A second study (18) compared CSF-1R expression by immunohistochemical staining in 40 cases of stage I/II breast cancer that developed ipsilateral breast tumor recurrence as a primary site of relapse with 40 matched cases of stage I/II breast cancer that did not develop ipsilateral breast tumor recurrence. For patients who...
developed ipsilateral breast tumor recurrence, 28 cases (70%) demonstrated strong staining, whereas only 16 cases (40%) in the second group demonstrated high immunoreactivity \( P = 0.007 \). The CSF-1R staining showed a positive correlation for local relapse, but no correlation was found between CSF-1R expression and distant metastasis. These findings provide evidence for the role of CSF-1R in poor outcome for patients with early stage breast cancer.

The purpose of this work was to study the associations among CSF-1R expression, nodal status, and other clinicopathological variables and outcome in a large retrospective series with long-term follow-up.

**MATERIALS AND METHODS**

**Tissue Microarray Construction.** Tissue microarrays were constructed as described previously (19, 20) and as reviewed recently (21). Cores measuring 0.6 mm were spaced 0.8 mm apart. Expression was evaluated on two arrays, one containing node-negative cases, and the other containing node-positive cases. Both cohorts were constructed from paraffin-embedded formalin-fixed blocks obtained from the Yale University Department of Pathology archives. The specimens were resected between 1962 and 1980, with a follow-up range of breast cancer history between 4 months and 53 years, with a mean follow-up time of 12.6 years. Complete treatment information was not available for the entire cohort; however, most patients were treated with local irradiation. None of the node-negative patients were given adjuvant systemic therapy. Among the node-positive patients, approximately 15% were given chemotherapy, and approximately 27% were given tamoxifen (post-1978). Time between tumor resection and fixation was not available for this cohort. Slides from all blocks were reviewed by a pathologist to select representative areas of invasive tumor to be cored and placed on the tissue microarray using a Tissue Micorarray (Beecher Instruments, Silver Spring, MD). The tissue microarrays were then cut to 5-µm sections and placed on glass slides using an adhesive tape-transfer system (Instumedics, Inc., Hackensack, NJ) and UV cross-linking.

**Immunohistochemistry.** The tissue microarray slides were deparafinized by rinsing with xylene and transferred through two changes of 100% ethanol. Endogenous peroxidase activity was blocked using the peroxidase block from the DAKO EnVision TM System (Dako, Carpinteria, CA), incubating for 30 min at room temperature. The slides were then boiled in a pressure cooker containing a sodium citrate buffer (pH 6.0) for antigen retrieval. After washing the slides in water, they were incubated at room temperature for 30 min in 0.3% Tris-buffered saline to reduce nonspecific background staining. After incubation in 0.3%BSA/1 Tris-buffered saline, the primary antibody (rabbit polyclonal anti-CSF-1R, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added at a dilution of 1:500, and the slides were incubated overnight at 4°C in a wet chamber. The following day, the slides were rinsed three times in 1 Tris-buffered saline/0.05% Tween 20. Bound antibody was detected by applying antirabbit horseradish peroxidase-labeled polymer secondary antibody from the DAKO EnVision kit. The slides were washed in Tris-buffered saline-Tween 20 as above, and incubated for 10 min in 3,3’-diaminobenzidine in buffered substrate (Dako). Counterstaining was then performed with hematoxylin, and slides were mounted with Immunomount (Shandon, Pittsburgh, PA).

**Evaluation of the Immunohistochemical Staining.** The regions of most intense staining were scored by eye for each spot. Only cytoplasmic/membranous staining of the malignant epithelial cells was scored. Very few spots had strong nuclear staining; therefore, nuclear staining was not scored. Because of...
the small size of the histospot (0.6 mm in diameter) and the uniform staining seen in nearly all of the spots, no area variable was included in the scoring. The staining was graded using the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Specimens with no infiltrating carcinoma or specimens that were not interpretable were excluded from the analysis. The tissue microarrays were scored separately by two independent observers (H. M. Kluger and M. Dolled-Filhart), with a very high correlation between scorers ($P < 0.0001$). A consensus score was determined for spots with discrepant scoring between the two observers.

Statistical Analysis. The Statview 5.0.1 (SAS Institute Inc., Cary, NC) software was used for all analyses. The correlation between scores of both scorers and the relationship of CSF-1R expression and clinicopathological parameters was done using the $\chi^2$ test. The prognostic significance of the parameters was assessed for predictive value using the Cox proportional hazards model with overall survival as an end point. Survival curves were calculated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox log-rank test.

Results

Immunohistochemical Staining of Breast Cancer Tissue Microarrays. Of the 672 breast cancer tumors on the tissue microarrays, 581 (86%) were interpretable for cytoplasmic CSF-1R staining. These specimens included 301 node-negative and 280 node-positive cases. Spots that were deemed uninterpretable had insufficient tumor cells in the spot, loss of tissue in the spot, or an abundance of necrotic tissue. A total of 515 tumor cores (76.7%) also had associated survival information. Examples of scores of 3+ and 0 are shown in Fig. 1. The distribution of the scores is shown in Fig. 2. Of the 515 cases, 250 were node-positive, and 265 were node-negative. Table 1 shows the distribution of CSF-1R staining in these two patient populations. There were significantly more CSF-1R-positive tumors among the node-positive patients compared with the node-negative patients ($P < 0.0001$).

Survival Analysis. The cytoplasmic CSF-1R expression was correlated with overall survival of the patients using 20 year follow-up. Kaplan-Meier survival curves generated for CSF-1R were split by ordinal score, as shown in Fig. 3A. These curves show that increased expression is correlated with worse outcome (log-rank $P = 0.0018$). Although these data are only semiquantitative, the curves suggest a splitting of the data to define scores of 0 and 1 as “low” or “negative” expression and 2 and 3 as “high” or “positive.” These designations are used for the remainder of the analyses. This result is shown in a Kaplan-Meier plot in Fig. 3B. In breast cancer, it is often informative to examine patient cohorts split by nodal status. The curves generated for the node-negative subset and node-positive subset of patients are shown in Fig. 4, A and B. Significant survival

<table>
<thead>
<tr>
<th>Nodal status</th>
<th>Score of 0</th>
<th>Score of 1</th>
<th>Score of 2</th>
<th>Score of 3</th>
<th>Total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node-negative</td>
<td>73</td>
<td>114</td>
<td>93</td>
<td>21</td>
<td>301</td>
</tr>
<tr>
<td>Node-positive</td>
<td>8</td>
<td>83</td>
<td>99</td>
<td>90</td>
<td>280</td>
</tr>
<tr>
<td>Total patients</td>
<td>81</td>
<td>197</td>
<td>192</td>
<td>111</td>
<td>581</td>
</tr>
</tbody>
</table>

* CSF-1R, colony stimulating factor-1 receptor.
differences were seen for CSF-1R staining on all patients ($P = 0.0003$ by Mantel-Cox test) and for the node-negative patients ($P = 0.0445$) with the high CSF-1R expressor patients having shorter overall survival. The node positive subset does not show a difference in outcome ($P = 0.465$). Univariate analysis was performed using the Mantel-Cox test on all patients as well as on the nodal subsets, and the data are shown in Table 2.

**Clinicopathological Correlations and Multivariate Analyses.** Using the Cox proportional hazards model, we performed multivariate analyses to assess the independent predictive value of positive CSF-1R cytoplasmic staining. The prognostic variables used to perform these analyses included tumor size, nodal status, estrogen receptor staining, progesterone receptor staining, human epidermal growth factor receptor 2 staining, nuclear grade, and patient age at diagnosis. CSF-1R was not an independent predictor of survival, and the only three independent predictors of survival were nodal status, nuclear grade, and tumor size.

Correlation between clinicopathological variables and CSF-1R staining was further examined, and the results are shown in Table 3. CSF-1R staining was most strongly associated with nodal involvement ($P < 0.0001$) and had weaker associations with age and tumor size ($P = 0.033$ and $P = 0.02$, respectively).

High CSF-1R expression is associated with nodal involvement in tumors $<2$ mm in size ($\chi^2$ test, $P = 0.0002$) as well as in tumors $2$ mm in size or larger ($\chi^2$ test, $P < 0.0001$). In addition, positive CSF-1R staining is associated with poor outcomes in both the smaller ($P = 0.02$) and larger ($P = 0.021$) tumors.

**DISCUSSION**

Receptor tyrosine kinases are a group of growth factor receptors that are being widely studied, because of the recent development of specific and relatively nontoxic agents that target them. Many of these receptors, CSF-1R among them, are mechanistically important in malignant transformation.

Our results reveal a very strong association between CSF-1R expression and nodal status ($P < 0.0001$). There is a weaker association with tumor size ($P = 0.02$) and with advanced age ($P = 0.033$). In our cohort, CSF-1R staining is associated with nodal involvement in both small and large tumors; therefore, the strong association between CSF-1R staining and nodal involvement is independent of tumor size. Within the node-negative subset of patients, CSF-1R expression is associated with poor survival. Within the node-positive subset, there are more CSF-1R expressors, but there does not appear to be a survival difference between CSF-1R high expressors and low expressors.

The full cohort of patients analyzed in this study was very large, and the $P$ value for prediction of survival based on CSF-1R staining in this large cohort was highly significant ($P = 0.0003$). When the patients were divided according to nodal status, there was still a significant decrease in survival with CSF-1R staining in the node-negative cohort, albeit less significant ($P = 0.0445$). This is because of (a) the decrease in the size of the cohort when analyzing the node-negative subset alone and (b) the fact that many more of the node-positive patients were CSF-1R positive than the node-negative group, with the node-positive patients having shorter survival, regardless of the CSF-1R status of their tumors.

We conclude that CSF-1R expression might be mechanistically important in the ability of a primary tumor to metastasize to lymph nodes or elsewhere. Once tumors have already metastasized to the nodes, regardless of the molecular elements involved in the process, expression of CSF-1R does not affect survival.

In summary, CSF-1R expression is a strong predictor of survival via its association with nodal involvement. Our data

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Prediction of overall survival</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node-positive patients</td>
<td>$P = 0.47$</td>
<td>1.135 (0.81–1.6)</td>
<td>0.53</td>
</tr>
<tr>
<td>Node-negative patients</td>
<td>$P = 0.045$</td>
<td>1.49 (1.01–2.21)</td>
<td>3.98</td>
</tr>
<tr>
<td>All nonmetastatic patients</td>
<td>$P = 0.0003$</td>
<td>1.58 (1.24–2.04)</td>
<td>20.46</td>
</tr>
</tbody>
</table>

$^a$ CSF-1R, colony stimulating factor-1 receptor.
suggest that this marker may be clinically useful for assessing prognosis, particularly among node-negative patients. It might also be clinically beneficial in predicting nodal involvement, such as in the setting of administering neoadjuvant chemotherapy or when lymph node dissection cannot be performed. Moreover, CSF-1R may have a role in promoting invasion and metastatic capability. With the recent development of new drugs that inhibit receptor tyrosine kinases and monoclonal antibodies to these receptors, CSF-1R may represent a future valuable therapeutic target in breast cancer therapy.

**ACKNOWLEDGMENTS**

We thank Aaron Berger for assistance in preparing the figures.

**REFERENCES**


**Table 3** Association between CSF-1R* staining and clinical/pathological variables$^a$

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>High CSF-1R expression ($n = 303$)</th>
<th>Low CSF-1R expression ($n = 278$)</th>
<th>Total patients ($n = 581$)</th>
<th>$\chi^2$ test, $P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High nuclear grade (3/3)</td>
<td>86 (28)</td>
<td>66 (23)</td>
<td>152 (26)</td>
<td>0.1</td>
</tr>
<tr>
<td>Estrogen receptor negative</td>
<td>127 (42)</td>
<td>125 (45)</td>
<td>252 (43)</td>
<td>0.197</td>
</tr>
<tr>
<td>Progesterone receptor negative</td>
<td>135 (45)</td>
<td>120 (43)</td>
<td>255 (44)</td>
<td>0.76</td>
</tr>
<tr>
<td>Her-2/neu positive (2–3/3)β</td>
<td>36 (12)</td>
<td>42 (15)</td>
<td>78 (13)</td>
<td>0.36</td>
</tr>
<tr>
<td>Age &lt;50 yr</td>
<td>78 (26)</td>
<td>94 (34)</td>
<td>172 (30)</td>
<td>0.033</td>
</tr>
<tr>
<td>Size &gt; 2 cm</td>
<td>196 (65)</td>
<td>150 (54)</td>
<td>346 (60)</td>
<td>0.02</td>
</tr>
<tr>
<td>Node-positive</td>
<td>189 (62)</td>
<td>91 (33)</td>
<td>280 (48)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*a* CSF-1R, colony stimulating factor-1 receptor.

The percentage of cases in each group (high CSF-1R expression, low CSF-1R expression, and total patients) that were associated with a particular clinicopathological variable is shown in parenthesis.

β Scored on a scale of 0–3, with 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.
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