Prognostic Significance of Colony-stimulating Factor Receptor Expression in Ipsilateral Breast Cancer Recurrence

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ABSTRACT

The macrophage colony-stimulating factor receptor (CSF-1R), the product of the c-fms proto-oncogene, regulates normal proliferation and differentiation of macrophages and monocytes. Recent research found abnormal expression of CSF-1R in human carcinomas of the breast, endometrium, and ovary. Furthermore, activation of CSF-1R by its ligand has been shown to regulate invasiveness and anchorage-independent growth in breast carcinoma cells. To study the significance of CSF-1R expression in breast cancer, we designed a case-controlled immunohistochemical study. We chose 80 patients from a database of 1200 early stage I or II breast cancer patients treated with conservative surgery and radiation therapy. Expression of CSF-1R in the tumors of 40 patients who experienced an ipsilateral breast tumor recurrence (IBTR) as a primary site of relapse were compared with 40 patients who had not experienced an IBTR. The index and control patients were matched by age, clinical stage, nodal status, and follow-up. Paraffin-embedded sections were immunostained with antibodies directed toward CSF-1R. For the CSF-1R antibody, a total of 28 index cases (70%) demonstrated strong staining, whereas only 16 control cases (40%) demonstrated high immunoreactivity (P = 0.007). The CSF-1R antibody showed a positive correlation for local relapse, but no correlation was found between CSF-1R expression and distant metastasis. In summary, our findings provide evidence for the poor prognostic role of CSF-1R in IBTR.

INTRODUCTION

The conservative management of breast cancer with lumpectomy followed by radiation therapy to the intact breast is now a widely instituted and acceptable standard of care for patients with early stage I or II breast cancer (1, 2). Although long-term survival and disease-free survival in conservatively treated patients are equivalent to those in mastectomy-treated patients, long-term IBTR rates have been reported consistently at 10–20% (3, 4). Identification of clinical and pathological prognostic factors for IBTR remains an active area of clinical investigation. Although there have been conflicting reports, many prognostic variables have been shown to be potential risk factors for IBTR in patients who have been treated with conservative therapy (5–7). Factors now reported to be of prognostic significance are the patient’s age, presence of tumor at resected margin, histology, extent of intraductal component, lymphohypocellular reaction within the tumor stroma, nodal status, radiation dose, and timing of systemic therapy (8, 9).

A number of other biological features and new molecular approaches have also been explored to delineate additional prognostic factors for ipsilateral breast cancer recurrence including various biochemical markers of cell proliferation, growth factors, and oncogene amplification (10, 11). Recent studies done by our laboratories, as well as others, have reported correlations between the expression of oncogenes, including p53 and HER-2/Neu, and growth factor receptors, such as insulin-like growth factor-1 receptor, and the propensity to experience an IBTR (10, 12–15).

The macrophage CSF, CSF-1 (or M-CSF), is a hematopoietic growth factor that stimulates the proliferation and differentiation of monocytes, macrophages, and their committed bone marrow progenitors (16). CSF-1 is also synthesized by a variety of mesenchymal cells, including fibroblasts and endothelium, and exerts its functions by binding to a single class of high-affinity cell surface receptors expressed primarily on mature mononuclear phagocytes (17). There is also evidence for the expression of this receptor by tumors of the female reproductive tract and in breast cancer (18–21).

CSF-1R is encoded by the c-fms proto-oncogene and is a member of a family of growth factor receptors with intrinsic tyrosine kinase activity, similar to the platelet-derived growth factor and the c-kit receptors (22, 23). Binding of CSF-1 to its receptor (CSF-1R) activates the receptor kinase activity and leads to autophosphorylation of the receptor on specific tyrosine sites, internalization and degradation of receptor-ligand complexes, and phosphorylation of a series of specific cellular proteins, some of which are important in transducing growth-promoting signals from the plasma membrane to the cell nucleus (17). Furthermore, recent research from our laboratory suggests that autocrine activation of the wild-type CSF-1R in a normal mammary epithelial cell line stimulates invasion via a uroki-

1 The abbreviations used are: IBTR, ipsilateral breast tumor recurrence; CSF, colony-stimulating factor; CSF-1R, CSF-1 receptor; CS, conservative surgery; RT, radiotherapy; ER, estrogen receptor; PR, progesterone receptor.

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Given the underlying biology of CSF-1R and additional research linking its expression to poor prognosis in epithelial ovarian carcinoma (18), we decided to investigate what role CSF-1R might play in IBTR after CS and RT for early stage breast cancer. We designed a case-control study to test our hypothesis that tumors that express high levels of CSF-1R would have increases in local recurrence after conservative therapy of lumpectomy and RT. All of the patients in the study were chosen from an overall database of 1200 stage I or II invasive ductal carcinoma patients all treated with CS + RT. The index cases had known IBTR, while their matched control cases did not experience a local recurrence. Our data demonstrate a statistically significant correlation between high levels of expression of CSF-1R and local recurrence in this cohort of patients.

MATERIALS AND METHODS

Patient Characteristics. The patient population base for this study included 1200 patients with early stage I or II breast cancer treated with CS + RT to the intact breast at Yale-New Haven Hospital between 1970 and 1992. The overall patient characteristics, treatment, and outcome of these patients have been reported previously (25). As of September 1996, the database was reviewed, and patients experiencing an IBTR as the first site of disease were chosen for the study. IBTR was defined as histologically confirmed recurrent invasive breast carcinoma occurring in the ipsilateral breast within the previously irradiated field. The index cases were selected from a population of 93 patients with IBTR, who also fulfilled the following criteria. All patients had a date of diagnosis prior to December 1992 to optimize the posttreatment follow-up period. All index cases carried a diagnosis of infiltrating ductal carcinoma, were treated with lumpectomy followed by RT to the intact breast (CS + RT), and had an available paraffin-embedded block for immunostaining. The index cases were pulled chronologically from the overall population without selection bias until we retrieved a target number of index cases. It was determined that 35–40 index case/control pairs would be required to show a meaningful statistically significant difference in protein expression between the cases and controls. Therefore, an initial target number of 45–50 cases was selected, because it was anticipated that inadequate tissue in some patients would reduce the number of evaluable samples to a final target sample size of 35–40 case/control pairs, which corresponds to just less than 50% of the total number of 93 invasive ductal carcinoma relapses in our database.

Each index case was then matched with a control case. The control cases were pulled from our overall database of 1200 breast cancer patients, and an effort was made to match each index case to a control case with the following criteria: age, clinical stage, nodal status, CS + RT, and follow-up. Because margins were not routinely assessed in the time span of this study, we did not attempt to match the groups by margin status. Analysis of margin status, however, showed no differences between the case and control group as shown below. By definition, each control case was also treated with lumpectomy followed by RT and had available a paraffin-embedded block but did not develop a local breast cancer recurrence in the ipsilateral breast.

Thus, the patient population base for this study consisted of 80 patients treated by lumpectomy with or without axillary lymph node dissection followed by a full course of RT to the intact breast. The index cases sustained a local relapse, whereas the matched control patients did not sustain a local recurrence. Distant disease was present in both groups, and although we did not intentionally match for distant disease, the groups were evenly balanced with respect to this end point. A protocol for the study was approved by the Human Investigations Committee at the Yale University School of Medicine (HIC protocol 08419).

Immunohistochemical Studies. For tumor histology verification, the paraffin-embedded specimens of each of the index cases and matched controls were sectioned at 5 μm and mounted on poly-L-lysine-coated slides for H&E staining. The subsequent section was used for immunohistochemical evaluation. The presence of breast cancer in the specimens was determined in a blind manner by a pathologist (D.C.), who also assigned a nuclear and histological grade to each specimen. Estrogen and progesterone receptor status of the archival paraffin-embedded blocks was determined by immunohistochemistry (ERICA method) using monoclonal antibodies to nuclear antigens (Abbott Laboratories, Chicago, IL; Ref. 26) as described previously. After processing of all of the tissue blocks, 40 index and 40 control pairs were evaluated for this study.

Immunoperoxidase-based histochemistry was performed using anti-CSF-1R (UBI; 4069B) at a 1:200 dilution. All antibodies were diluted in PBS plus 1% BSA. A total of 80 archival surgical breast specimens was used in this study. They had been previously fixed in 10% neutral buffered formalin and paraffin-embedded by the standard procedure. Five-μm sections were deparaffinized and rehydrated through graded alcohols. Endogenous peroxidase activity was quenched using 2% hydrogen peroxide in methanol for 30 min at room temperature. The tissue sections were rehydrated through graded alcohols, washed with PBS for 10 min, incubated with diluted goat serum (1:200) for 20 min at room temperature, and followed by incubation with primary antibody overnight at 4°C in a humidified chamber. Slides were then rinsed with 1% Triton-PBS, washed with PBS for 10 min, incubated with biotin-conjugated goat anti-rabbit IgG (1:250) for 30 min at room temperature, and rinsed again in PBS for 10 min. Immunoperoxidase staining was carried out using the Vectastain ABC Elite and 3,3'-diaminobenzidine peroxidase substrate kits (Vector) following the manufacturer's protocol. Slides were counterstained with Harris hematoxylin solution (Sigma Chemical Corp.).

Microscopy and Histological Grading. The pathologists, who were blinded to the clinical information, evaluated the slides under light microscopy. The staining on each slide was rated on a 4-point scale: A score of zero corresponded to no tumor staining (Fig. 1d). Slight staining, either focal or diffuse, was scored as 1; staining, either focal or diffuse, was considered 1+(Fig. 1c); more intense staining, focal or diffuse, was considered 2+ (Fig. 1b); and intense staining, if focal, 3+ (Fig. 1a), or if diffuse, 4+ (not shown). For the purposes of this study, it was determined that a score of ≥2 would be considered significant staining for CSF-1R, and a score of 0, 1, or 1+ would be considered negative. Grading of the nuclear staining for estrogen and progesterone receptors used the H-score method, defined as the product of intensity times distribution. A score of ≤75 was used for the cut for ER and PR positivity. Once all of the slides were evaluated, the data were unblinded for statistical analysis.
performed by the Pearson $\chi^2$ test as a nonparametric test of association, with $P \leq 0.05$ considered significant. Survival curves were calculated by the life table method, with differences between curves tested by the Mantel Haensel $\chi^2$ statistic.

**RESULTS**

We have attempted to evaluate the prognostic significance of expression of CSF-1R in the primary breast tumors of patients who have experienced an IBTR as a primary site of relapse. Of an overall database of 1200 stage I or II breast cancer patients, 80 patients’ tumor blocks were used for this case-control study. We used immunohistochemistry to detect the expression of CSF-1R antigen in the primary tumors of 40 index cases who had experienced an IBTR and 40 matched control patients who had not. After immunohistochemical staining for CSF-1R, ER, and PR of the archival paraffin-embedded tumors, we found the following results.

**Patient Population.** Table 1 summarizes the overall characteristics of both the index and control patient populations. By design of the study, patients in the index group and control group were matched with respect to age, clinical stage, nodal status, systemic adjuvant therapy, and follow-up. For each case-control pair, 35 of the 40 pairs had infiltrating ductal carcinoma, and 30 cases had intraductal components in tissue sections. There were four invasive lobular carcinomas and one medullary carcinoma.

Index cases and control cases were not originally matched for margin status. However, when the tissue specimens were analyzed for the presence or absence of tumor at the margins, the numbers of cases with tumor margins indicated to be either not assessed, positive, or negative was equal. Therefore, there was no significant difference between cases and controls with respect to margin status of the primary tumor (Table 1).

By study design, the two groups were evenly matched with respect to age and follow-up. The median age of the index group was $52.7 \pm 14.8$ years, and the median age of the control group was $53.9 \pm 13.9$ years. As of September 1997, the median follow-up time of the index population was $10.5 \pm 5.2$ years, whereas the median follow-up of the control group was $9.4 \pm 4.3$ years.

**CSF-1R Receptor Staining.** Fig. 1 illustrates the immunohistochemical staining with the commercial CSF-1R antibody 4069B. The CSF-1R staining was homogeneous and predominately cytoplasmic with some membranous staining, which is consistent with prior reports on this antibody (19, 28). In this figure, $a$ is an example of $3+$ staining, $b$ is $2+$ staining, $c$ is $1+$ staining, and $d$ is faint or no staining. As stated previously, the $\geq 2$ cutoff value designated positive staining and the signif-

![Fig. 1 Immunohistochemical staining with a commercial CSF-1R antibody. The above CSF-1R staining is homogeneous, cytoplasmic, and membranous. $a$, example of $3+$ staining; $b$, $2+$ staining; $c$, $1+$ staining; $d$, faint or no staining.](image-url)
significant presence of CSF-1R in the cell. Values of 0 or 1 were considered negative.

The use of this rating system generated the following results (Fig. 2). Twenty-eight of the 40 index cases (70%) exhibited significant positive expression of CSF-1R, whereas only 16 of the 40 control cases (40%) exhibited high immunoreactivity ($P = 0.007$). The degree of staining also correlated incrementally with the percentage of local recurrence. In Fig. 3, we show that an increase in staining of the 1+ degree is associated with a 43% increase in local recurrence, whereas an increase of 2+ or greater staining is associated with a 63% increase in local recurrence ($P = 0.004$). Expression of CSF-1R in primary breast cancers did not, however, show a statistically significant decrease in long-term survival by the Kaplan-Meier life table analysis ($P$, not significant; Fig. 4).

**ER and PR Staining.** The estrogen and progesterone status of the paraffin-embedded tumors was determined by immunohistochemistry, and the histology (nuclear staining) was evaluated using the above-mentioned H-score rating system. The following results were obtained. Fourteen of the 40 index cases (35%) had a positive H-score for the ER, whereas 15 of 40 control cases (37.5%) had a positive H-score. There was no difference between the cases and controls in this study with respect to ER status ($P = 0.485$). Additionally, for the PR status, 9 of the 40 index cases (22.5%) had a positive H-score for the PR, whereas 16 of 40 control cases (40%) had a positive H-score. Therefore, there was a statistically significant difference between the cases and controls in this study with respect to the PR status ($P = 0.0334$).

**DISCUSSION**

Patients whose breast cancer has been conservatively treated by lumpectomy with RT have a long-term risk for relapse in the breast of 10–20% (29, 30). After local recurrence, salvage mastectomy can be performed, offering 59–84% survival at 5 years (8, 25, 31, 32). As demonstrated in numerous randomized clinical trials, overall survival and disease-free survival in conservatively treated patients is similar to their counterparts treated by mastectomy (1). Identification of risk factors for local recurrence in the conservatively treated breast cancer patient, however, remains an active area of clinical investigation.

In the current case-control study, we selected for analysis only those patients who were treated prior to 1992 to achieve a long, adequate follow-up period, given the known long natural history of local recurrences in the conservatively treated breast.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of cases and controls</th>
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<tbody>
<tr>
<td></td>
<td>Cases (n = 40)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>52.7 ± 14.8</td>
</tr>
<tr>
<td>Follow-up years</td>
<td>10.5 ± 5.2</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>29 (72.5%)</td>
</tr>
<tr>
<td>II</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td>Nodal status</td>
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<tr>
<td>Unknown</td>
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<td>Negative</td>
<td>19 (47.5%)</td>
</tr>
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<td>4 (10.0%)</td>
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<td>Surgical margins</td>
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<td>Not assessed</td>
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<tr>
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<td>ER status</td>
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<td>26 (65.0%)</td>
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<tr>
<td>Positive</td>
<td>14 (35.0%)</td>
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<tr>
<td>PR status</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>Negative</td>
<td>31 (77.5%)</td>
</tr>
<tr>
<td>Positive</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>Adjuvant hormone</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7 (17.5%)</td>
</tr>
</tbody>
</table>

Fig. 2 CSF-1R expression (4069B) correlates with local recurrence. This histogram shows expression of CSF-1R in the index cases and in the control cases. In this case-control study, 28 of the 40 index cases (70%) exhibited significant positive expression of CSF-1R, whereas only 16 of the 40 control cases (40%) exhibited high immunoreactivity for CSF-1R. ■, cases (n = 40); □, controls (n = 40).

Fig. 3 Increasing CSF-1R expression (4069B) correlates with increasing local recurrences. This line graph shows the degree of intensity of staining with CSF-1R in the tumor sections. An increase in staining of the 1+ degree was associated with a 43% increase in local recurrence, whereas an increase of 2+ or greater staining is associated with a 63% increase in local recurrence.
The design of the study was to perform a matched case-control series where each control patient was evenly matched to the index cases with respect to primary histology, clinical, pathological stage, patient age, and follow-up.

Many studies have attempted to identify risk factors associated with local recurrence, both at the clinical and molecular levels (5, 15, 33, 34). It has also been found that the biology and the natural history of local recurrence in the conservatively treated breast is different from the processes underlying distant metastases (4, 32). Certain clinical factors, such as tumor size and axillary lymph node status, have been shown to have some prognostic significance with respect to distant metastasis, but they have not been as helpful with respect to isolating those patients at greatest risk of local tumor recurrence in the conservatively treated breast. Similarly, the hormone receptors ER and PR have been shown to be significant prognostic indicators of distant metastasis and overall survival, but they have failed to predict long-term incidence of IBTR (35). In this communication, our results agree with the observation reported previously that ER failed to have prognostic significance in local recurrence. The association between PR and local relapse noted in the present study warrants further investigation. Other factors have had prognostic value in determining those at risk for local recurrence, including absence of RT and systemic chemotherapy, lymphocytic/inflammatory infiltrate/stromal reaction (33), peritumoral lymphatic invasion, extensive intraductal component, presence of tumor necrosis, and young patient age. These factors have all been demonstrated in various studies to be of some prognostic value with respect to IBTR.

The present study was undertaken in an effort to evaluate the prognostic significance of expression of CSF-1R, the c-fms proto-oncogene, with respect to IBTRs. In this cohort of conservatively treated breast cancer patients, CSF-1R has been shown to correlate with local recurrence. Chambers et al. (18) reported recently that CSF-1R expression on the surface of ovarian epithelial carcinoma proved to be a strong independent poor prognostic factor for outcome in stage III invasive ovarian cancers. Our findings also show a prognostic value for CSF-1R in local recurrence of invasive ductal carcinoma of the breast, and, therefore, agree with prior reports in the literature that CSF-1R may be used as a poor prognostic factor. The results presented here, demonstrating a statistically significant association of expression of CSF-1R with locally recurrent breast cancer, require confirmation from other series and larger patient numbers prior to using these factors for clinical decision making. Although the prognostic data were highly significant and the index cases and controls were well matched, other confounding factors, changes in treatment strategies, such as detailed attention to surgical margins and the use of adjuvant systemic therapy, may alter the prognostic value of this marker. It is important, however, to investigate CSF-1R and other molecular markers as possible prognostic factors in IBTR. If expression of these markers consistently predicts for local relapse, it may be possible, through drug and/or gene therapy, to alter such expression and ultimately to improve local control in the conservatively treated breast cancer patient.

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