

The Macrophage Colony-Stimulating Factor 1 Response Signature in Breast Carcinoma

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Abstract Purpose: Macrophages play an important role in breast carcinogenesis. The pathways that mediate the macrophage contribution to breast cancer and the heterogeneity that exists within macrophages are incompletely understood. Macrophage colony-stimulating factor 1 (CSF1) is the primary regulator of tissue macrophages. The purpose of this study was to define a novel CSF1 response signature and to evaluate its clinical and biological significance in breast cancer.

Experimental Design: We defined the CSF1 response signature by identifying genes over-expressed in tenosynovial giant cell tumor and pigmented villonodular synovitis (tumors composed predominantly of macrophages recruited in response to the overexpression of CSF1) compared with desmoid-type fibromatosis and solitary fibrous tumor. To characterize the CSF1 response signature in breast cancer, we analyzed the expression of CSF1 response signature genes in eight published breast cancer gene expression data sets ($n = 982$) and did immunohistochemistry and *in situ* hybridization for CSF1 response genes on a breast cancer tissue microarray ($n = 283$).

Results: In both the gene microarray and tissue microarray analyses, a consistent subset (17-25%) of breast cancers shows the CSF1 response signature. The signature is associated with higher tumor grade, decreased expression of estrogen receptor, decreased expression of progesterone receptor, and increased *TP53* mutations ($P < 0.001$).

Conclusions: Our data show that the CSF1 response signature is consistently seen in a subset of breast carcinomas and correlates with biological features of the tumor. Our findings provide insight into macrophage biology and may facilitate the development of personalized therapy for patients most likely to benefit from CSF1-targeted treatments.

Clinical and experimental studies suggest that macrophages play an important role in breast carcinogenesis (1–3). Macrophage colony-stimulating factor 1 (CSF1) is the primary regulator of tissue macrophages. Studies on breast cancer have shown that the protein expression of CSF1 and CSF1 receptor (CSF1R) correlates with increased inflammation and poorer prognosis (4, 5). CSF1 has been shown to promote progression to malignancy in mouse mammary tumors (6) and blockade of CSF1 suppressed growth of human mammary tumor xenografts (7). Previous studies in

breast cancer have focused primarily on expression of CSF1 or CSF1R. Here, we show that an expanded list of CSF1-responsive genes can be used to identify subsets of breast carcinomas that may be uniquely sensitive to CSF1- and macrophage-targeted therapies.

Previously, we reported that the related soft tissue tumors tenosynovial giant cell tumor (TGCT) and pigmented villonodular synovitis (PVNS) are made up of a heterogeneous population of cells, in which a small subset of tumor cells contains a translocation involving *CSF1* resulting in the recruitment of CSF1R-expressing macrophages that constitute the majority of the tumor mass (8). We hypothesize that the distinct gene expression pattern of TGCT and PVNS can be seen as a surrogate for the macrophage response to CSF1.

In the current study, we first define a CSF1 response gene expression signature by identifying a set of genes specifically and highly expressed in TGCT and PVNS. We then evaluate the expression of these CSF1 response genes in multiple breast cancer gene expression data sets and a breast cancer tissue microarray (TMA) and find that a consistent subset of breast cancers expresses the CSF1 response signature. The characterization of this new pathway in breast cancer provides insight into the regulation of the breast cancer tumor microenvironment by CSF1. This finding may facilitate the development of personalized therapy for patients most likely to benefit from CSF1- and macrophage-targeted treatments.

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Translational Relevance

Breast cancer is the most common cancer among American women. Although it is known that the tumor microenvironment plays an important role in the pathogenesis of breast cancer, there are currently few available therapeutic agents to target the breast cancer microenvironment. Clinical and experimental studies suggest that macrophages play an important role in breast carcinogenesis. Macrophage colony-stimulating factor 1 (CSF1) is the primary regulator of tissue macrophages. In the current study, we have defined a novel CSF1 response signature. Using multiple breast cancer gene expression data sets ($n = 982$) and a tissue microarray ($n = 283$), we show that a reproducible subset of breast cancers (17-25%) is enriched with the CSF1 response signature. Breast cancers with the CSF1 response signature are significantly more likely to be higher grade, estrogen receptor negative, and progesterone receptor negative and contain *TP53* mutations (all $P < 0.001$). Therapies targeted at CSF1 and other mediators of macrophage behavior are currently being developed. We believe that the CSF1 response signature described in this article will facilitate the development of drugs to target previously unrecognized mediators of the CSF1 response in breast cancer and may provide a novel technique for identifying patients most likely to respond to CSF1- and macrophage-targeted therapies.

Materials and Methods

Determination of the CSF1 response gene signature. We have previously shown that in TGCT/PVNS, a translocation occurring in a small subset of tumor cells results in the recruitment of a macrophage-rich inflammatory infiltrate that composes the majority of the tumor mass (8). Therefore, we hypothesized that the expression profile of TGCT/PVNS primarily reflects the biology of the nonneoplastic macrophage-rich inflammatory infiltrate responding to secreted CSF1 by the minority of tumor cells with the CSF1 translocation. To define the CSF1 response signature, significance analysis of microarrays was done to identify genes that show significantly increased expression in PVNS ($n = 8$) and TGCT ($n = 7$) compared with desmoid-type fibromatosis ($n = 7$) and solitary fibrous tumor ($n = 6$), with a minimum fold change of 2.5 and false discovery rate of 0.02%, based on gene expression profiling previously done (8). This analysis resulted in the identification of 603 genes that constitute the CSF1 response signature (Supplementary Workbook).

Breast cancer data sets. We used five publicly available whole tumor breast cancer data sets [NKI (9), Perreard (10), GSE1379 (11), GSE1456 (12), and GSE3494 (13)] that contain gene expression data on a total of 856 cases with clinical follow-up, and we used three laser capture microdissection (LCM) breast cancer data sets [GSE5847 (14), GSE9014 (15), and GSE10797 (16)] that contain gene expression data obtained separately from the stroma and epithelium [GSE5847 (14) and GSE10797 (16)] or solely from the stroma [GSE9014 (15)] from 126 cases. Additional information on the data sets is provided in Supplementary Materials and Methods.

Gene expression data analysis. For all data sets, the expression data were downloaded and imported into the dChip 2006 software.⁴

Expression values were standardized gene wise by subtracting the mean and dividing by the SD of the expression values for each gene. Unsupervised hierarchical clustering was done in each data set with the Cluster 3.0 software⁵ using the uncentered Pearson correlation as the distance metric and average linkage clustering. The resulting heat map and dendrogram were visualized on Java TreeView.⁶

Determination of CSF1 response core gene and case clusters. We defined the CSF1 response core gene cluster as the largest cluster of genes in each whole tumor data set that showed >25% correlated expression. We defined the CSF1 response case cluster as the largest cluster of cases that showed high levels of expression of the CSF1 response core gene cluster and exhibited >10% correlated expression. After applying these rules, we defined the CSF1 response core gene set as being composed of genes that are present on the microarray platform in at least two whole tumor data sets and present in the CSF1 response core gene cluster in either all whole tumor data sets (if gene was present on platform in only two to three data sets) or absent in a single data set (if gene was present on platform in four to five data sets). Using these criteria, the original set of 603 CSF1 response genes identified through gene expression profiling on TGCT and PVNS was reduced to a "CSF1 response core gene set" of 112 genes (Supplementary Workbook) that were consistently and coordinately expressed in the five whole tumor breast cancer data sets.

Analysis of clinicopathologic variables. In the whole tumor data sets examined, the measured survival outcomes include disease-free survival (Perreard, GSE1379, and GSE1456; combined $n = 294$), disease-specific survival (GSE1456 and GSE3494; combined $n = 395$), and overall survival (NKI, Perreard, and GSE1456; combined $n = 529$). The Kaplan-Meier estimate was used to compute survival curves, and the log-rank P value was computed to assess statistical significance. For association tests, the Pearson χ^2 test was used. To compare ordinal or ratio variables in two independent groups, either the Mann-Whitney U test or Student's t test was done. Statistical computing was done using Statistical Package for the Social Sciences 15.0 for Windows.

Evaluation of CSF1 response protein localization in the breast cancer microenvironment. To determine the patterns of CSF1 response protein expression in the breast cancer microenvironment, we did immunohistochemistry and *in situ* hybridization on a breast cancer TMA (TA221), which contains samples from 283 breast carcinomas obtained from Stanford University Medical Center. We measured CSF1 and CSF1R RNA expression by *in situ* hybridization and the expression of four additional CSF1 response proteins (FCGR3a, FCGR2a, CTS1L1, and CD163) by immunohistochemistry. To select CSF1 response markers for evaluation by *in situ* hybridization or immunohistochemistry, we identified the 112 genes that were consistently present in the CSF1 response core cluster (the CSF1 response core gene set; Supplementary Workbook). Out of this list of 112 candidates, we identified FCGR3a, FCGR2a, CTS1L1, and CD163 as markers for which commercial available antibodies that did well on formalin-fixed, paraffin-embedded tissue were available. The primary antibodies used were FCGR3a (CD16; MCA1816, mouse monoclonal; AbD Serotec), CTS1L1 (MCA2374, mouse monoclonal; AbD Serotec), FCGR2a (CD32; AB45143, rabbit monoclonal; Abcam), and CD163 (NCL-CD163, mouse monoclonal; Novocastra). The immunohistochemical reactions were visualized using mouse and rabbit versions of the EnVision+ system (DAKO) using diaminobenzidine. CD163 staining was done with the Ventana Benchmark Autostainer. *In situ* hybridization of TMA sections for CSF1 and CSF1R was done based on a protocol published previously (8, 17–19). The immunohistochemical and *in situ* hybridization studies were interpreted by histopathologic evaluation by a surgical pathologist (I.E.). A case was determined to show the CSF1 response signature if it showed coordinate expression (score ≥ 1) of at least four of the five markers. The digital images, collected using

⁴ <http://biosun1.harvard.edu/complab/dchip/>

⁵ <http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm#ctv>

⁶ <http://jtreeview.sourceforge.net/>

computerized microscopes [BLISS (Bacus Labs) and Ariol (Applied Imaging, Inc.)], are available for all stained cores through the accompanying Web site.⁷ Additional detailed information on the antibodies used, staining procedure, and scoring technique can be found in Supplementary Materials and Methods. Institutional review board approval was obtained for these studies.

Functional gene set analysis. To determine the functional significance of gene sets, we used the DAVID: Database for Annotation, Visualization, and Integrated Discovery (20). To generate protein-protein interaction (PPI) networks, gene sets were uploaded into STRING 7.0 (21), and the following active prediction methods were used (neighborhood, coexpression, gene fusion, experiments, cooccurrence, database, and text mining) with a medium confidence score (0.400). The Cytoscape software platform (22) was used to visualize the PPI networks, and the Cytoscape plug-in Network Analyzer 2.52 was used to evaluate the topological characteristics of the networks (23).

Results

A reproducible core subset of CSF1 response genes shows coordinate expression in breast carcinoma. We did two-way unsupervised hierarchical clustering on the five whole tumor breast cancer gene expression data sets with the 603 CSF1 response genes. In each data set, we observed a core cluster of CSF1 response genes that showed high levels of coordinated expression among a cluster of breast cancer cases. We defined the CSF1 response core gene set as being composed of the genes that were present in the CSF1 response core gene cluster most consistently across the five whole tumor data sets (see Materials and Methods). The resulting CSF1 response core gene set consists of 112 CSF1 response genes (Supplementary Workbook).

Functional gene set analysis shows that the core CSF1 response gene set is most highly associated with annotation terms relating to cellular defense and immune response [response to biotic stimulus, defense response, immune response, and response to stimulus; all Fisher's exact $P \leq 1.5e-15$ computed using the DAVID: Database for Annotation, Visualization, and Integrated Discovery (20); see Supplementary Workbook for full annotation results]. The core CSF1 response genes associated with these annotation terms include *CSF1R*, *FCGR3a*, *FCGR2a*, and *CD163*. The core gene set contains several members of the cathepsin class of lysosomal cysteine proteases (*CTSL1*, *CTSS*, and *CTSC*), which are expressed by macrophages and thought to be important for regulating antigen presentation (24).

We did PPI network analysis on both the core CSF1 response genes and the noncore CSF1 response genes. This analysis shows that the PPI network created by the core CSF1 response genes has a higher average clustering coefficient, average number of connections to other proteins, average neighborhood connectivity, and average closeness centrality (all $P \leq 0.002$) compared with the network created with the noncore CSF1 response genes (Supplementary Fig. S1 and Supplementary Table S1). These findings suggest that the PPI network created by the CSF1 response core gene set is more centralized and tightly connected than the PPI network created by the CSF1 response noncore proteins.

These functional gene set and PPI network analyses suggest that by filtering the 603 CSF1 response gene set to the 112 core

CSF1 response genes, we have selected for the core genes that are likely to operate in a common CSF1-induced immune response module in the breast carcinoma microenvironment.

A reproducible subset of breast carcinomas shows the CSF1 response signature. In each of the five whole tumor breast carcinoma data sets, a subset of similar size (17-25%) of breast carcinomas shows the CSF1 response gene signature (Fig. 1). Cases of breast cancer with the CSF1 response signature were more likely to be estrogen receptor (ER) negative, progesterone receptor (PR) negative, higher grade, and larger (Supplementary Table S2).

The GSE3494 data set contains information pertaining to the *TP53* mutation status of 251 cases of breast carcinoma. From this data set, Miller et al. (13) defined a p53 expression signature and used diagonal linear discriminant analysis to classify breast cancer cases according to the signature. In our analysis of this data set, we find that breast cancers with the CSF1 response signature are significantly more likely to harbor a *TP53* mutation and to be enriched with the *TP53* mutation gene expression signature (Supplementary Table S2).

The NKI and GSE1456 data sets have previously been stratified by others into molecular subcategories based on initial gene expression studies by Perou and colleagues and Sorlie and colleagues (basal, ERBB2⁺, luminal A, luminal B, and normal like; refs. 25, 26). Breast cancers with the CSF1 response signature were significantly more likely to be basal or ERBB2 and less likely to be normal like, with no significant association with the luminal A and luminal B molecular subtypes (Supplementary Table S2).

The CSF1 response signature shows a variable association with survival. We evaluated the relationship of the CSF1 signature with patient survival in each of the five whole tumor data sets. In the NKI data set, which is limited to patients younger than 53 years old with stage I or II disease, the CSF1 response signature showed an association with decreased overall survival (10-year survival = 64% in tumors with CSF1 response signature versus 73%; log-rank $P = 0.044$; Fig. 2A). In all other data sets, we were unable to identify a statistically significant association with survival (all $P > 0.15$; Fig. 2A). When survival data are pooled from the five data sets, we find a trend for an association of the CSF1 response signature with decreased survival but are unable to identify a statistically significant association ($P \geq 0.099$; Fig. 2B; Supplementary Table S2).

The lack of a consistent association of the CSF1 response signature with poor survival was unanticipated, given the fact that the signature is highly and consistently correlated with features known to predict poor outcome (higher grade, ER negativity, PR negativity, *TP53* mutations, and basal and ERBB2 molecular subtypes). To further evaluate the complex relationship of the CSF1 response signature with survival, we did several subset analyses. When the survival analysis was limited to ER-negative tumors, the CSF1 response signature showed a trend for an association with improved overall survival (10-year survival of 54% in tumors with CSF1 response signature versus 36%; log-rank $P = 0.103$; Fig. 3A) and improved disease-specific survival (10-year survival of 90% in tumors with CSF1 response signature versus 65%; log-rank $P = 0.099$). Among cases that are grade 1 or 2, the CSF1 response signature is associated with decreased survival (10-year survival of 75% in tumors with CSF1 response signature versus 84%; log-rank $P = 0.006$; Fig. 3B). Among cases enriched with the *TP53* mutation

⁷ http://tma.stanford.edu/tma_portal/CSF1_breast

signature, the CSF1 response signature showed an association with improved disease-specific survival (10-year survival of 72% in tumors with CSF1 response signature versus 49%; log-rank $P = 0.047$; Fig. 3C). These subset analyses suggest that the CSF1 response signature shows a complex relationship with survival, in which the signature is associated with poor prognosis among low-grade tumors and shows a trend for an association with improved prognosis among ER-negative tumors and among tumors with a *TP53* mutation gene expression signature. When the data are not substratified by

these factors, the CSF1 response signature does not show a statistically significant relationship with prognosis.

The CSF1 response signature is present in LCM breast cancer data sets. To further assess the pattern of CSF1 response gene expression in the epithelium and stroma of breast carcinomas, we evaluated the expression of CSF1 response genes in three publicly available LCM breast carcinoma data sets. Two of the data sets (GSE5847 and GSE9014) contain samples from both breast cancer epithelium and stroma. Unsupervised hierarchical clustering of both of these data sets shows that a subset of cases

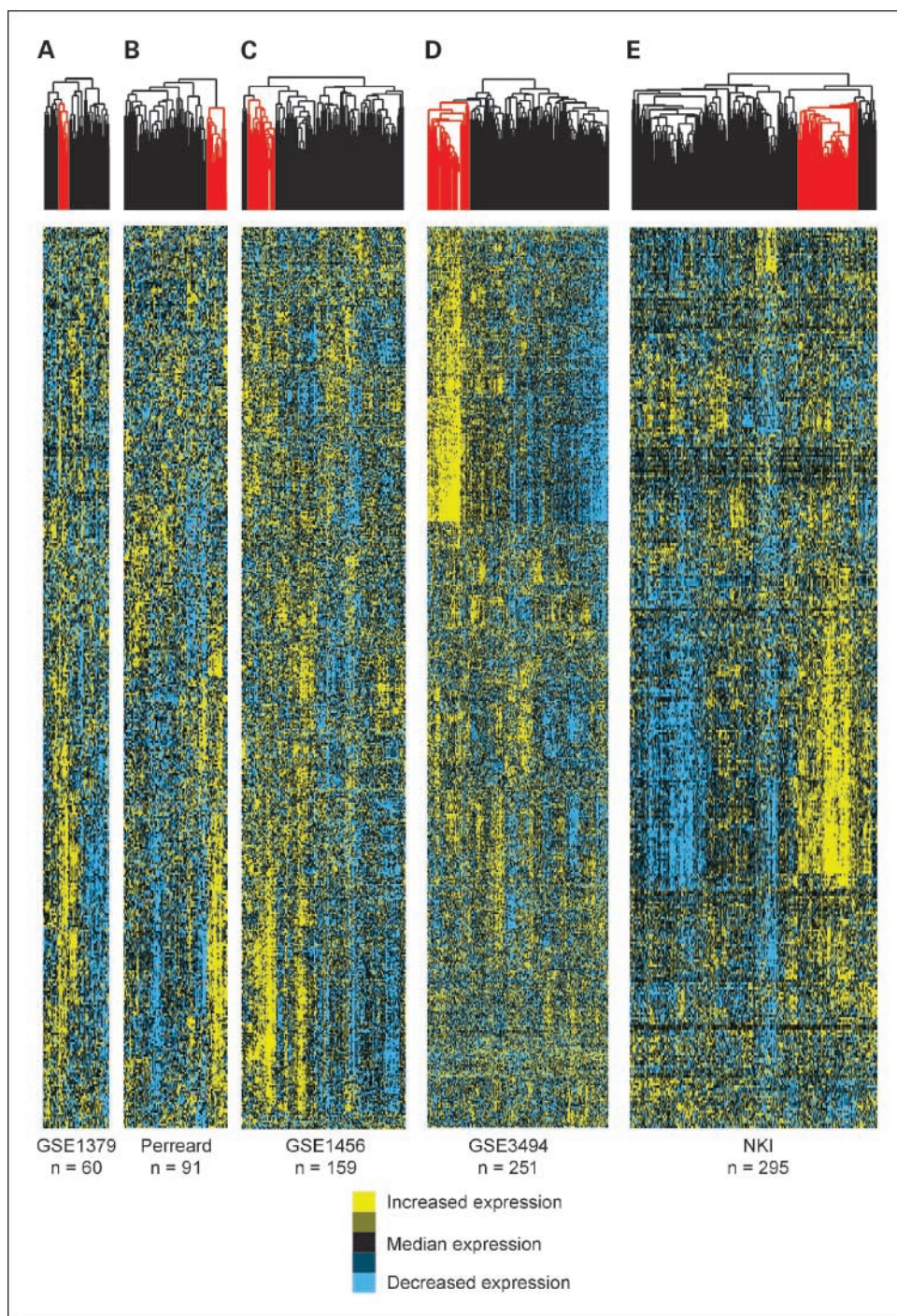


Fig. 1. Unsupervised hierarchical clustering of breast carcinomas with CSF1 response genes in five data sets. *A.* GSE1379 ($n = 60$). *B.* Perreard ($n = 91$). *C.* GSE1456 ($n = 159$). *D.* GSE3494 ($n = 251$). *E.* NKI ($n = 295$). Within the heat map, yellow represents high expression, black represents median expression, and blue represents low expression. The red highlighted region of the dendrograms above the heat maps indicates the cluster of breast cancer cases with the CSF1 response signature in each data set.

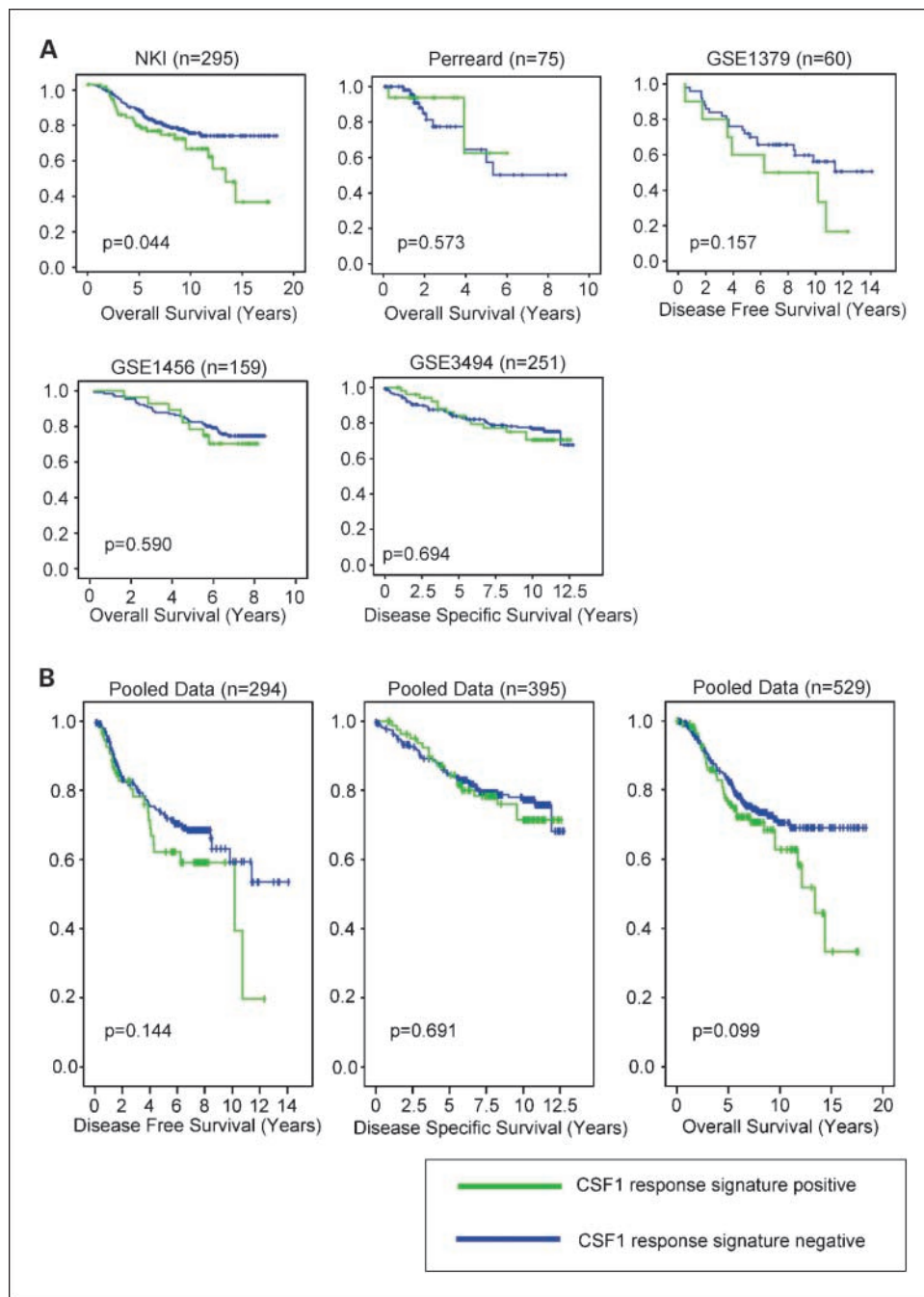


Fig. 2. Kaplan-Meier survival curves for breast cancer cases stratified by CSF1 response signature. *A*, the survival curves are displayed individually for each of the five data sets. *B*, the survival curves display data pooled from the five data sets for disease-free survival, disease-specific survival, and overall survival.

(18 of 74, 24%) shows the CSF1 response signature (Supplementary Fig. S2A and B). Most cases (13 of 18) with the CSF1 response signature express the signature coordinately in both the epithelium and stroma, whereas 4 of 18 cases express the signature in only the epithelial sample and 1 of 18 in only the stromal sample. It should be noted that inflammatory cells may infiltrate the breast cancer epithelium, and consequently, their gene expression may be included in either the "epithelial" or the "stromal" compartment of LCM data sets. The third LCM data set (GSE10797) contains gene expression data from stromal samples of 52 breast cancers. Unsupervised hierarchical clustering done on this data set shows a cluster of 13 of 52 (25%) stromal samples with the CSF1 response signature

(Supplementary Fig. S2C). The GSE10797 data set contains information on tumor grade and recurrence-free survival for all samples. Analysis of these data shows that the CSF1 response signature identified in stromal samples is associated with higher tumor grade (10 of 13 CSF1 response stromal samples from grade 3 carcinomas versus 16 of 39; $P = 0.025$), which agrees with the association seen in the whole tumor gene expression profiling.

The expression of CSF1 is correlated with stromal expression of CSF1 response proteins in breast carcinoma. To confirm the existence of a CSF1 response signature in breast cancer by a different technique and to determine the cellular localization and patterns of expression of CSF1 and CSF1 response proteins

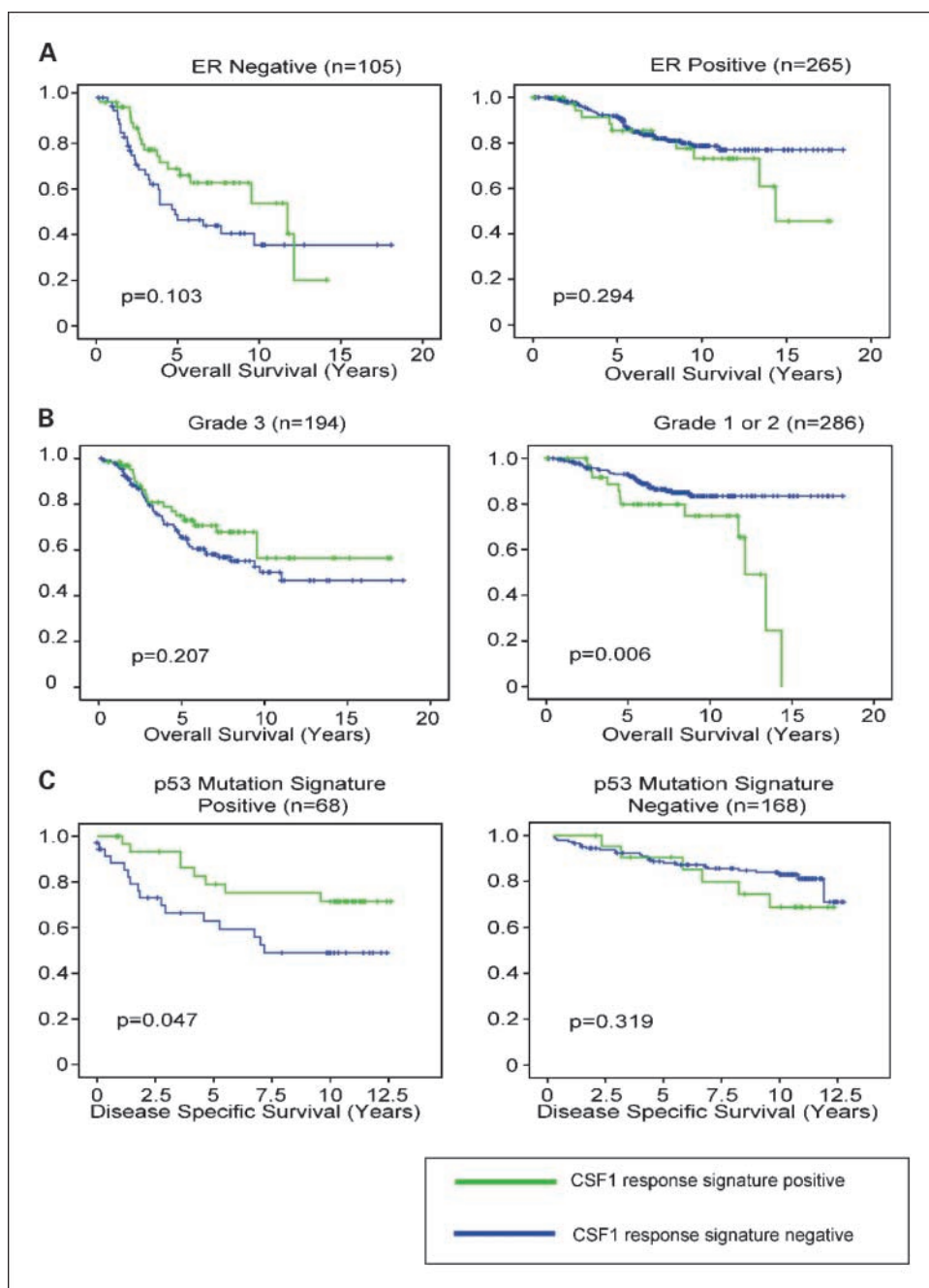
in breast carcinoma, we examined the expression of CSF1 and five CSF1 response markers by *in situ* hybridization and immunohistochemistry on a breast cancer TMA containing samples from a total of 283 patients.

Of the 206 cases with evaluable data for CSF1 and at least four of the five CSF1 response proteins, CSF1 was expressed in the stroma in 72 cases (35%) and in the malignant epithelium in 107 cases (52%). The expression of CSF1 in either the epithelium or stroma was associated with stromal expression of CSF1 response proteins: 41 of 107 (38%) of cases with epithelial CSF1 expression showed coordinate stromal expression of at least four CSF1 response proteins versus 17 of 99 (17%) for cases with no epithelial CSF1 ($P = 0.001$), and 37 of

72 (51%) of cases with stromal CSF1 expression showed coordinate stromal expression of at least four CSF1 response proteins versus 21 of 134 (16%) of cases with no stromal CSF1 expression ($P = 5.49e-008$). These data show that either the epithelial or the stromal expression of CSF1 is significantly correlated with the stromal expression of CSF1 response genes and further suggest that the expression of the CSF1 gene plays a major role in coordinating the CSF1 response signature.

Unsupervised hierarchical clustering of stromal and epithelial CSF1 with the CSF1 response proteins shows that CSF1R and stromal CSF1 expression show the strongest pairwise correlation (Fig. 4). Of the CSF1 response proteins, CD163 (macrophage-associated antigen) stains the highest percentage

Fig. 3. Kaplan-Meier survival curves for CSF1 response signature subset analyses. Kaplan-Meier survival curves are displayed for breast cancer cases stratified by CSF1 response signature for subsets determined by ER status (A), grade (B), and p53 mutation signature (C).



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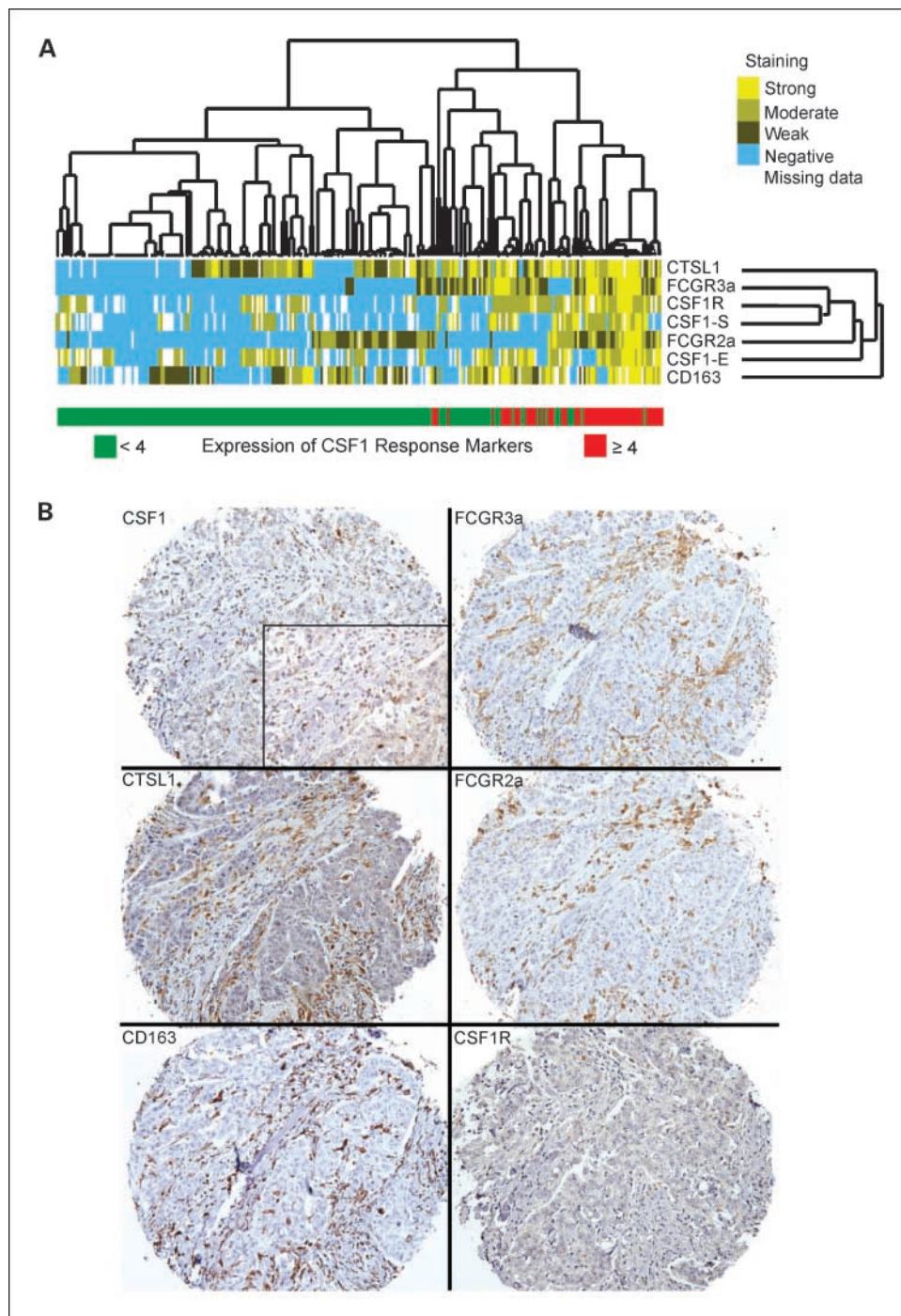


Fig. 4. Coordinate expression of CSF1 and CSF1 response proteins in breast cancer. **A**, unsupervised hierarchical clustering of 272 breast carcinomas based on TMA staining for CSF1 and five CSF1 response markers (CSF1R, CD163, FCGR3a, FCGR2a, and CTSL1). The breast cancer cases are arranged along the X axis above the heat map, and CSF1 and the CSF1 response markers are arranged along the Y axis to the right of the heat map. The CSF1-E row represents CSF1 epithelial expression and the CSF1-S represents CSF1 stromal expression. The color bar underlying the heatmap summarizes the expression levels for all five CSF1 response markers and shows red for cases expressing at least four CSF1 response markers and green for cases expressing less than four CSF1 response markers. **B**, example of a breast cancer case with stromal expression of CSF1 (*in situ* hybridization) and five CSF1 response proteins: CSF1R (*in situ* hybridization) and CD163, FCGR3a, FCGR2a, and CTSL1 (immunohistochemistry). Digital images from all TMA cores can be viewed at http://tma.stanford.edu/tma_portal/CSF1_breast.

of cases (149 of 226, 66%) and shows the least coordinated expression with the other markers (Fig. 4). CD163 is a member of the scavenger receptor cysteine-rich superfamily and is expressed on most subpopulations of mature tissue macrophages (27). These findings suggest that CD163 labels the majority of macrophages, and the CSF1 response signature captures a distinct and novel subset of CD163-expressing macrophages.

The coordinate expression of CSF1 response proteins in the breast cancer microenvironment correlates with the clinicopathologic features observed in the gene expression profiling

analysis. There were at least four of five evaluable CSF1 response markers for 252 cases on the breast cancer TMA. Of these 252 cases, 64 (25%) showed coordinate expression of at least four of the CSF1 response proteins. These cases were significantly more likely to be ER negative, PR negative, higher grade, epidermal growth factor receptor (EGFR) positive, and ductal differentiation and show significantly higher numbers of proliferating malignant epithelial cells as measured by Ki67% (Supplementary Table S3). The expression of CSF1 response proteins showed no association with the presence of lymph node metastasis at time of diagnosis, tumor size, or

expression of HER2 (all $P > 0.48$; Supplementary Table S3). The proportion of cases coordinately expressing CSF1 response proteins in the TMA data (25%) is similar to the proportion of cases with the CSF1 response signature seen in the whole tumor gene expression data (17-25%) and the LCM gene expression data (25%). The clinicopathologic associations seen in the TMA data correlate with the findings of the gene expression data.

Discussion

Clinical and experimental studies have shown that tumor-associated macrophages play an important role in breast carcinogenesis (1–3, 6, 7). Macrophages represent a heterogeneous cell type, but the clinical and biological significance of this heterogeneity is incompletely understood (28). CSF1 is a growth factor that acts through the cell surface receptor CSF1R. Activation of CSF1R by CSF1 stimulates the proliferation, differentiation, and survival of macrophages and influences macrophage chemotaxis, phagocytosis, and synthesis and secretion of proteolytic enzymes and cytokines (3). Lin and colleagues (6) have used a mouse breast cancer model to show that CSF1 promotes malignant progression in mammary tumors. The specific cellular pathways that mediate CSF1 behavior in breast cancer are largely undefined.

In the current study, we define a CSF1 response signature obtained by gene expression profiling of TGCT and PVNS, two related soft tissue tumors composed predominantly of macrophages that express CSF1R and are recruited in response to the expression of CSF1 by neoplastic tumor cells (8). The profiling of these tumors captures a CSF1 response because the CSF1R-expressing macrophages far outnumber the neoplastic cells, which typically compose ~10% of the tumor cells; thus, the RNA measured in the profiling experiments is predominately derived from macrophages responding to CSF1. We hypothesized that this CSF1 response occurs in a subset of breast carcinomas, in which carcinoma cells or stromal cells secrete CSF1, resulting in the recruitment of a macrophage-rich inflammatory infiltrate, and we posit that evidence of this process will be observable in gene expression profiling data sets.

To evaluate this hypothesis, we first examined the expression of CSF1 response genes in five breast cancer data sets. We defined the CSF1 response gene set by identifying the 603 genes that showed the highest levels of expression in TGCT/PVNS compared with desmoid-type fibromatosis and solitary fibrous tumor. We then evaluated the expression of these genes in five breast carcinoma data sets and filtered the original list of 603 genes down to the 112 core CSF1 response genes that showed the highest level of coordinated expression across the five breast cancer data sets.

This core group of CSF1 response genes is highly enriched for annotation terms relating to immune defense (Supplementary Workbook). The core gene set includes multiple genes known to be expressed by macrophages and involved in macrophage function, including *CSF1R*, *FCGRI*, *FCGR3a*, *FCGR2a*, *CD163*, and *CCL5*, which is a chemoattractant for macrophages and has recently been shown to be secreted by mesenchymal stem cells to enhance the motility, invasion, and metastasis of breast cancer cells (29). The core gene set includes several cathepsins (*CTSL1*, *CTSS*, and *CTSC*), which are lysosomal proteases expressed by macrophages and important for antigen presen-

tation (24). The macrophage lineage encompasses a heterogeneous group of macrophage subpopulations, and the biological and functional significance of these subpopulations is only beginning to be elucidated (28). A study by Grage-Griebenow and colleagues (30) identified a novel dendritic cell-like subtype of monocyte that expresses FCGRI (CD64) and FCGR3a (CD16) and is characterized by high accessory capacity for activated lymphocytes and high expression of HLA-DR and CD86. All four of these markers (FCGRI, FCGR3a, HLA-DR, and CD86) were identified in our study as members of the core CSF1 response gene set. In addition to these selected genes, we have identified numerous other core CSF1 response genes whose behavior in macrophages or in relation to CSF1 has not previously been characterized (Supplementary Workbook).

In both our gene microarray and TMA analyses, we find that the CSF1 response signature is associated with a characteristic clinicopathologic phenotype in breast cancer (higher histologic grade, increased frequency of *TP53* mutations, decreased expression of ER and PR, increased expression of EGFR, and increased Ki67 proliferation index). The association of tumor expression of EGFR with expression of CSF1 response proteins in the tumor microenvironment is particularly interesting, as the expression of CSF1 by breast cancer cells has been shown to promote the expression of EGF by macrophages, which in turn promotes the expression of CSF1 by breast carcinoma cells leading to the adoption of a more invasive phenotype in a positive feedback loop (31, 32). It is known that EGFR is expressed by basal subtype breast cancers (33), and in the current study, we find an association of basal molecular subtype with the CSF1 response signature (Supplementary Table 2).

Despite the fact that breast cancers enriched with the CSF1 response signature harbor several molecular (p53 mutations and basal and ERBB2 molecular subtypes) and histopathologic (larger tumor size and higher histologic grade) features conferring poor prognosis, we find an association with poor prognosis in only one of the five breast cancer data sets and no significant association is identified when pooling data from all five data sets (Fig. 2; Supplementary Table S2).

To better define the relationship of the CSF1 response signature with prognosis, we did several subset analyses. Interestingly, we find that among grade 1 and 2 tumors, the CSF1 response signature is associated with decreased survival (Fig. 3B). In contrast, among tumors harboring a *TP53* mutation signature, the CSF1 response signature shows an association with improved prognosis (Fig. 3C). This finding is compatible with work by Lee and colleagues (34) showing that CSF1 activates p53-independent pathways to induce growth arrest of human breast cancer cells. This finding suggests that in cases with a *TP53* mutation, the CSF1 response pathway could potentially stimulate *TP53*-independent growth arrest. In an additional subset analysis, we find that among ER-negative cases, there is a trend for increased survival in cases enriched with the CSF1 response signature (Fig. 3A). It has been shown that tumor-associated macrophages may promote malignant progression by secreting estrogens (35, 36), and therefore, this mechanism of tumor pathogenesis might be prevented in ER-negative breast cancers. It has recently been shown by Teschendorff and colleagues (37) that increased expression of immune response genes correlates with improved prognosis in ER-negative breast cancer. These

findings suggest that several immune response pathways (including the CSF1 response pathway) may play a protective role in ER-negative breast cancers.

These data show that the influence of CSF1 and the CSF1 response in breast cancer depends not only on the behavior of stromal and inflammatory cells but also on the particular genotypic and phenotypic characteristics of the carcinoma cells. A recent study by Tamimi and colleagues (38) underscores the complex relationship between CSF1 expression and host characteristics and shows that increased serum levels of CSF1 are associated with decreased risk of breast cancer in premenopausal women and increased risk of breast cancer in postmenopausal women.

In the current study, we have defined a novel CSF1 response signature seen in breast carcinoma. We believe that our findings will not only afford a more comprehensive understanding of the mediators and pathways involved in the macrophage CSF1 response in breast cancer but will also provide a valuable resource for the development of additional therapeutic agents to target heretofore unrecognized mediators in the CSF1 response in breast cancer. Furthermore, the measurement of genes from the CSF1 response core gene set in clinical samples

from breast cancer patients may provide a novel technique for identifying patients most likely to respond to CSF1- and macrophage-targeted therapies. It is well documented that unrecognized molecular heterogeneity within a clinical cancer trial may lead to underestimation of therapeutic benefits if potential responders and nonresponders are not identified before treatment (39). Potent therapies directed against gene targets, such as ER and HER2 in breast cancer, only work well in tumors that highly express those gene products. Recent studies have shown the ability to use genomic signatures to guide the use of chemotherapeutics (40, 41), and the technique has been validated in clinical trials in ovarian (42) and breast cancer (43). Therapies targeted at CSF1 and other mediators of macrophage behavior are currently being developed (7, 44, 45). We believe that the CSF1 response signature could potentially serve as a resource for drug development and as a clinically useful tool for identifying patients most likely to respond to CSF1- and macrophage-targeted therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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