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Variations in stromal signatures in breast and colorectal cancer metastases

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Abstract

The tumor microenvironment (TME) plays an important role in tumor survival and growth but little is known about the degree of preservation between different stromal response patterns found in primary tumors and their metastases. We have previously identified gene expression profiles for two distinct stromal signatures in breast carcinoma of fibroblast (aka DTF) and macrophage response (aka CSF1) and found them to be correlated with clinicopathologic features including outcome. In this study, we compare the DTF fibroblast and CSF1 macrophage stromal response patterns in primary breast and colorectal cancers to their matched lymph node metastases. In both breast and colorectal cancer, there was a significant, positive correlation between the CSF1 macrophage signature in the primary tumors and the matched lymph node metastases as assessed by immunohistochemical markers. No such correlation was observed for the DTF fibroblast signature. A similar result was seen in independent analysis of two published gene expression microarray datasets. The variations of these stromal reaction patterns from the primary to the metastasis shed light on the relationship between the neoplastic cells and the non-neoplastic cells in the TME. The preservation of the CSF1 macrophage response pattern in metastases lends support to targeting the CSF1 pathway in cancer.

Keywords

Breast cancer; metastasis; tumor microenvironment; gene expression

Introduction

The malignant cells in carcinomas are surrounded by varying amounts of stroma that is comprised of different elements that include fibroblasts, inflammatory cells, endothelial

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Statement of author contributions

JAW, AHB, MVDR, and RBW conceived the experiments.

JAW, AHB, MS, IE, KCJ, KDM, MVDR and RBW carried out experiments and analyzed data.

All authors were involved in writing the paper and had final approval of the submitted and published versions.

Supplemental Files

Supplemental files contain additional text for the Materials and methods and Results.

cells, adipocytes, and extracellular matrix.¹ In recent years it has been shown that this tumor microenvironment (TME) is more than a scaffold providing nutrition and mechanical support for the tumor cells. Studies have shown that the TME is involved in a wide range of interactive processes with the tumor cells that can influence tumor growth and may even play a role in the development of carcinoma.^{2–4}

We previously used gene expression profiles of soft tissue tumors including desmoid-type fibromatosis (DTF), solitary fibrous tumor (SFT), and tenosynovial giant cell tumor (TGCT), to identify stromal reaction patterns that are differentially expressed in breast cancer specimens. A core set of 66 DTF-associated genes (DTF fibroblast signature) was identified that was consistently and coordinately expressed in 25–35% of breast cancer cases in each of 5 published data sets. The cases with high level expression of the DTF core genes tended to be lower grade, express estrogen receptor, and show significantly increased survival.⁵ Analysis of TGCT produced a CSF1 macrophage response core gene set consisting of 112 genes that were consistently expressed as the CSF1 macrophage response signature in 17–25% of breast cancers. This CSF1 macrophage response signature was associated with higher tumor grade, decreased expression of estrogen and progesterone receptor, and increased TP53 mutations.⁶ The results of both of these gene expression profiling analyses were validated using tissue microarray technology, which demonstrated that proteins corresponding to the identified genes were expressed predominantly in the tumor stroma and showed the same correlation with prognostic markers and clinical outcomes as the gene signatures.^{5,6} These protein markers therefore can function as surrogate markers for the TME types discovered through gene expression profiling studies.

We have recently found that these stromal signatures are present in pre-invasive breast cancers and that they tend to be conserved in the associated invasive cancer.⁷ This finding suggests that these signatures may play a role in the induction and progression of oncogenesis. The current study seeks to expand our knowledge of these TME subtypes by investigating whether the DTF fibroblast and CSF1 macrophage response signatures as assessed at the protein and RNA levels are conserved between primary tumors and their lymph node metastases. Prior work on primary breast cancer and matched lymph node metastases focused primarily on analyzing gene expression with little attempt to distinguish between the epithelial and stromal compartments of the tumors.^{8–11}

Methods

Determination of the CSF1 macrophage response signature in primary breast cancer and matched lymph node metastases using tissue microarrays

Two tissue microarrays were used in this study. The tumors were collected and studied using HIPAA compliant Stanford University Medical Center institutional review board approval. TA221 contains samples from 283 primary breast carcinomas obtained from Stanford University Medical Center.^{5,6} The second tissue microarray (TA248) contains samples of lymph node metastases of breast carcinoma from 49 whose primary tumors were included in TA221 and had available metastasis. We selected 4 proteins (FCGR3a, FCGR2a, CTS1, and CD163) that were highly ranked in the 112 CSF1 response gene list identified in a prior study⁶ that also had commercially available antibodies that performed well in immunohistochemistry on formalin-fixed, paraffin-embedded tissue (also see Supplemental Methods).⁷ The primary antibodies used were FCGR3A (CD16, AbD Serotec, MCA1816, 1:40 dilution), CTS1 (AbD Serotec, MCA2374, 1:25 dilution), FCGR2A (CD32; Abcam, AB45143, 1:200 dilution), and CD163 (Novocastra, NCL-CD163, 1:200 dilution). CD163 was detected with the Ventana Benchmark autostainer while the remaining 3 stains were manually applied and were visualized using mouse and rabbit versions of the EnVision+ system (DAKO) using diaminobenzidine. The immunohistochemical studies were

interpreted by histopathologic evaluation by J.W., I.E. and R.W. based on prior scoring criteria.⁶

Determination of the DTF fibroblast stromal response signature in primary breast cancer and matched lymph node metastases

The same tissue microarrays (TA221 and TA248) were used to evaluate the DTF fibroblast stromal response signature in breast cancer. The primary antibodies were chosen in the same manner as above and included SPARC (Zymed, 1:1000 dilution), VCAN (CSPG2, Santa Cruz Biotechnology incorporated, 1:150 dilution), CDH11 (Invitrogen, Cat# 32-1700, 1:10 dilution), SDC1 (CD138, Serotec, cat# MCA681H, 1:400 dilution) and MMP11 (Calbiochem, Cat# IM86, 1:200 dilution). CD138 was detected with the Ventana Benchmark autostainer, the remaining 4 stains were manually applied and were visualized using mouse and rabbit versions of the EnVision+ system (DAKO) using diaminobenzidine. The immunohistochemical studies were reviewed together by histopathologic evaluation by J.W., I.E. and R.W. based on prior scoring criteria^{5,7} and challenging cases were discussed to achieve consensus. The distinction between normal lymph node tissue and metastatic tissue was made based on morphology.

Analysis of the DTF fibroblast and CSF1 macrophage response signatures in primary breast cancer and lymph node metastases

For each marker, the stain was scored as negative (−2), weak positive (1), or strong positive (2). For a given patient we summarized the expression of markers from a signature by taking the sum of the scores of the evaluable stains and dividing by the number of evaluable stains. If fewer than 3 of the 4 (CSF1 signature) or fewer than 4 of the 5 markers (DTF signature) were evaluable, the signature score was not included in the final analysis. The scores for multiple lymph node metastases within a single case were averaged to produce a single lymph node score for each case.

Evaluation of the DTF fibroblast and CSF1 macrophage response signatures from published breast cancer gene expression profiling data

We used a previously published gene microarray dataset comparing 15 primary breast cancers and matched lymph node metastases¹² and a previously published gene microarray dataset comparing 8 primary breast cancers and matched distant metastases.⁸ Genes from the DTF signature and CSF1 signature were mapped to these platforms and used in the analysis.^{5,6}

Determination of the DTF fibroblast and CSF1 macrophage response signatures in primary colorectal cancer and matched metastases using tissue microarrays

A tissue microarray (TA151) was constructed with samples of primary colon cancer from 37 patients from Stanford University Medical Center. Of these 37 cases, 17 had lymph node metastases at the time of resection and two had liver metastases without lymph node metastases. Duplicate cores were used for all primary and metastatic tumors in the colon samples and the most positive of the two scores for each marker was used in computing the average CSF1 and DTF stromal signature scores.

Statistics—For association analysis, Spearman's rho was calculated in R (version 2.9.0), and a two-tailed P-value was calculated to assess significance. To compare ordinal variables in two groups, the student's t-test was performed.

Results

The CSF1 macrophage response signature in breast and colon cancer is conserved in metastases

We have previously found that the CSF1 macrophage response signature significantly varies between both invasive and in situ breast carcinomas.^{6,7} To determine whether this signature is conserved between primary tumors and their metastases, we first examined the CSF1 macrophage response signature between matched breast cancer primary and regional (axillary) lymph node metastases by immunohistochemistry using 4 previously established markers (FCGR3a, FCGR2a, CTSL1, and CD163) on 49 cases represented on a tissue microarray.⁶ These markers were scored based on their expression in cells in the stromal compartment, which typically represented mononuclear inflammatory cells, including macrophages (Figure 1). Figure 2 shows the plot of the average score (2 to -2) for each primary and the metastatic case. The CSF1 macrophage response stromal signature score, as measured by the reactivity of these 4 markers in the stroma within the tumor, showed a moderate, statistically significant positive correlation ($\rho=0.4$, $p=0.003$, Spearman's rank correlation coefficient) for the 49 primary breast tumors and matched lymph node metastases. In an individual marker analysis, only CD16 demonstrated significant correlation (see Supplement Results).

To validate our immunohistochemistry findings, we examined the CSF1 macrophage response signature in gene expression profiling data using published gene expression microarray data from a study comparing 15 primary breast tumors and their matched regional lymph node metastases.¹² We have previously identified the core set of genes representing the CSF1 macrophage response through the analysis of 5 independent breast cancer microarray datasets⁶. We determined the CSF1 macrophage response signature score for the microarray dataset with matched primary and regional lymph node metastatic breast cancers by summing the mean centered ratios for all CSF1 macrophage response genes represented in the dataset. The Spearman's rank correlation coefficient based on these gene sums demonstrated a significant, positive correlation ($\rho=0.66$, $p=0.009$) for the pairs of primary tumors and metastases (Figure 3A). We also examined a small gene expression profile dataset of 8 matched primary and distant metastatic breast cancers.⁸ This dataset included one metastasis to the lung, one to the skin of the arm, one to a distant (supraclavicular) lymph node, and four to the ovary. Though the CSF1 macrophage response signature score was not significant by Spearman's rank correlation coefficient (Figure 3B), 2 of the 4 cases with a CSF1 macrophage response signature sum of gene ratios greater than 10 in the primary cancer also had a positive CSF1 macrophage response signature score in the distant metastasis.

To assess whether the correlation between the CSF1 macrophage response signature in primary tumor and metastasis was specific to breast cancer, we examined another common cancer type, colorectal carcinoma, for the conservation of the CSF1 macrophage response signature using the four immunohistochemical markers. The Spearman's rank correlation coefficient demonstrated a significant, positive correlation ($\rho=0.609$, $p=0.035$) for the primary colon tumors and their matched metastases (Figure 4).

Stromal expression of the DTF fibroblast stromal signature in primary tumors and matched metastases

The DTF fibroblast stromal signature was studied by the stromal expression of five markers (SPARC, VCAN, CDH11, SDC1 and MMP11), derived from the core gene set of the DTF fibroblast stromal signature (Figure 1).^{5,7} We examined primary breast and matched regional lymph node metastases using immunohistochemistry on the same set of breast

cancers studied for the CSF1 macrophage response signature. Based on positivity of the five DTF fibroblast stromal signature markers in the stromal compartment only for both the primary and metastatic breast cancers, the Spearman's rank correlation coefficient demonstrated no significant evidence of correlation (Spearman's $\rho=0.22$, $p=.13$) for the pairs of primary tumors and metastases (Figure 5). CD138 and Cadherin11 demonstrated weak correlation in individual marker analysis (see Supplemental Results).

The immunohistochemical markers that we used had been selected because they react predominately with the cells in the TME and do not react with the malignant cells. However, part of the gene expression profile signature (that is derived from whole tumor samples) may not be confined to the stromal compartment and may be expressed in the epithelial (neoplastic) compartment as well. To ascertain whether the epithelial compartment still has remnants of the DTF fibroblast stromal signature in the metastatic environment even when the stroma does not, we examined the DTF fibroblast stromal signature with the published gene microarray data of 15 primary breast tumors and their matched regional lymph node metastases in a manner similar to that used for the macrophage stromal signature.¹² We determined the DTF fibroblast stromal signature score for the microarray dataset with matched primary and metastatic breast cancers by summing the mean centered ratios for all DTF fibroblast genes represented in the dataset (Figure 6A). In contrast to the immunohistochemistry findings obtained on tissue microarrays, the Spearman's rank correlation coefficient of the sums demonstrated a significant positive correlation ($\rho=0.72$, $p=0.003$) when analyzing the pairs of primary tumors and metastases in the published dataset of 15 breast tumors for the fibroblast stromal signature.¹³ These findings suggest that at least part of the DTF fibroblast stromal signature is maintained in metastasis. Further studies are necessary to determine to what extent the DTF fibroblast signature in metastasis is expressed in the stroma or the epithelial component of the tumor.

We examined the gene expression microarray dataset of 8 matched primary and distant metastatic breast cancers for the DTF fibroblast stromal signature (Figure 6B).⁸ Unlike the CSF1 macrophage response signature results, none of the 3 cases with a DTF fibroblast response signature sum of gene ratios greater than 10 in the primary cancer score retained the DTF fibroblast response signature in the distant metastasis.

Using the same colonic adenocarcinoma samples studied for the analysis of the macrophage response signature, we determined the DTF fibroblast stromal signature score by immunohistochemistry for matched primary colonic adenocarcinomas and lymph node metastases with evaluable data for at least four of the five DTF response proteins. The Spearman's rank correlation coefficient of the averaged scores demonstrated no evidence of correlation ($\rho=-0.23$, $p=0.47$) for the pairs of primary tumors and metastases (data not shown).

Discussion

Growth, survival and progression in carcinoma depends in part on a complex crosstalk between the epithelial cells within the tumor and the stroma that surrounds them.¹ By using gene expression profiles of soft tissue tumors as surrogates for stromal reaction patterns, we have previously described gene signatures that can be used to identify different stromal response patterns to breast carcinoma.^{5,6,13} While these studies have focused on the stromal signatures in primary tumors, the tumor microenvironment is clearly important in metastasis as well. There are competing theories regarding the choice of the metastatic site that address "tissue tropism" versus a more passive role of the microenvironment but it is clear that for a successful tumor growth at the site of metastasis there must be a productive interaction with the stroma. In the current study we asked whether stromal signatures established at the site

of metastasis are similar to those established in the primary organ or whether novel interactions with the stroma are created. This would help determine whether the tumor brings with it the stromal expression signature that it established as it developed at the primary site and imposes this signature on the metastatic site or whether the tumor must develop an entirely novel gene expression pattern to interact with the metastatic stroma at the new site.

Analysis of the CSF1 macrophage stromal response signature demonstrated conservation of the signature between primary tumors and regional lymph node metastases in both breast and colon carcinoma types by two different assays, immunohistochemistry for protein expression and gene expression profiling for RNA expression. Although the correlations are not as tight for the immunohistochemistry study, they are still statistically significant and the difference is likely due to the limited number of immunohistochemistry markers. Taken together, these findings suggest that in both breast and colorectal cancer the CSF1 macrophage stromal response is determined by the intrinsic biology of the tumor and that it is independent of the TME that the tumor resides in. That we do not find a significant correlation in the distant metastases could be due to the small number of cases available for analysis. Despite this, 2 of the 4 cases with a positive CSF1 macrophage response signature in the primary also had a positive signature in the metastasis. It is possible that with greater numbers this would be significant. However, it is also possible that the differences reflect an important difference between metastases which would become apparent in a larger dataset.

Analysis of the DTF fibroblast stromal response signature demonstrated an absence of significant correlation between primary tumors and lymph node metastases by immunohistochemical analysis of the stromal compartment. This lack of correlation was observed in both breast cancer ($\rho=0.22$, $p=0.13$) and colorectal cancer ($\rho=-0.23$, $p=0.47$). These findings could suggest that the DTF fibroblast stromal response is independent of the intrinsic biology of the tumor and may vary with the tissue in which the response is observed. However, the gene microarray data demonstrated conservation of the DTF fibroblast gene signature between primary tumors and regional lymph node metastases ($\rho=0.72$, $p=0.003$) in breast cancer. It is important to note that the gene microarray studies analyzed mRNA expression levels in homogenized tumors where epithelial tumor cells are intermixed with stroma, while the tissue microarray studies only evaluated the tumor stroma. This might indicate that the observed difference in the consistency of the DTF fibroblast stromal response for primary breast cancers and matched regional lymph node metastases may be a consequence of the measurement of mRNA levels within the epithelial elements of the tumor in the gene microarray analysis as compared to the evaluation of stromal elements in the tissue microarray analysis. It is possible that the DTF fibroblast stromal response in the primary tumor is initially generated from the stroma surrounding the tumor and not the tumor. In this scenario, the expression of the DTF fibroblast stromal response is mostly contained within the stroma but also involves cross-talk with the cancer cells. When the tumor metastasizes, it does not bring with it the stroma and so that gene signature is lost.

Others have found that the 70-gene and molecular subtype signatures are retained from the primary to the metastasis.¹² However, these signatures are intrinsic to the cancer cells themselves and do not raise a conflict with our findings that are focused on the tumor stroma. In fact, our findings correlate with prior studies comparing primary and metastasis expression where it was noted that it is only some ECM genes that vary between primary and metastasis.¹²

It is important to note that while we use the term “fibroblast” and “macrophage” to describe these signatures, these designations are not meant to suggest that the genes in these signatures are exclusively expressed in these cell types. However, the bulk of the genes from

the 2 signatures appear to be involved in either fibroblast or macrophage function and this has been borne out by prior gene pathway analysis^{5,6} and morphologic analysis in this study and prior studies.^{5–7} Our findings do not clarify the inducing event for the signatures and from what cell or cell interactions this induction originates. Others have found that there is both CSF1 and CSF1R expression in breast cancer cells.¹⁴ It may be that the stromal CSF1 signature is associated primarily by the secretion of CSF1 by tumor cells.

The finding of a conserved CSF1 macrophage stromal response in 2 different types of metastatic cancer suggests that targeting therapeutics to this pathway may represent an effective therapeutic strategy for patients with metastatic disease. The CSF1 macrophage stromal response has opposite prognostic significance in multiple cancer types^{15–24}. For example it is associated with poor outcome in breast cancer but with improved outcome in colon cancer. The DTF fibroblast stromal response appears to be more complicated and is not entirely conserved between primary tumors and their metastases. Future studies are needed to determine which factors within these different tissues influence the fibroblast stromal response. The DTF fibroblast stromal response has been shown to be associated with a better prognosis in breast cancer, while no conclusions have been drawn about its prognostic significance in colorectal cancer.⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative images of the immunohistochemistry for selective markers. A) CTSL in a primary invasive ductal carcinoma of the breast. B) CTSL in a breast metastasis. C) CSPG2 in a primary invasive ductal carcinoma of the breast. D) CSPG2 in a breast metastasis. E) CD163 in a primary colonic adenocarcinoma. F) CD163 in a colonic adenocarcinoma metastasis. G) CD163 in a breast metastasis. H) SPARC in a primary colonic adenocarcinoma. I) SPARC in a colonic adenocarcinoma metastasis. J) SPARC in a breast metastasis.

Figure 2.

Analysis of CSF1 macrophage response signature in 49 matched primary and regional lymph node metastatic breast cancer as measured by 4 immunohistochemical markers and displayed as a scatter plot of average marker score for each primary and regional lymph node metastatic breast cancer pair.

Figure 3.

A) Gene expression profiling of 15 matched primary and regional lymph node metastatic breast cancers with the CSF1 macrophage response signature core gene set. The bar graph shows the sums of CSF1 macrophage response signature core for each matched pair, blue represents the primary and red represents the metastasis. B) Gene expression profiling of 8 matched primary and distant metastatic breast cancers. The bar graph shows the sums of CSF1 macrophage response signature core for each matched pair.

Figure 4. Results from the of CSF1 macrophage response signature in matched primary and metastatic colorectal cancer as a scatter plot of average marker score for each primary and regional lymph node metastatic breast cancer pair.

Figure 5.

Analysis of DTF fibroblast stromal signature in matched primary and regional lymph node metastatic breast cancer for 5 DTF fibroblast response signature markers for a set of 49 primary and regional lymph node metastatic breast cancer pairings as a scatter plot of average marker score for each primary and regional lymph node metastatic breast cancer pair.

Figure 6.

A) Gene expression profiling of 15 matched primary and regional lymph node metastatic breast cancers with the DTF fibroblast response signature core gene set. The bar graph shows the sums of DTF fibroblast response signature core for each matched pair, blue represents the primary and red represents the metastasis. B) Gene expression profiling of 8 matched primary and distant metastatic breast cancers. The bar graph shows the sums of DTF fibroblast response signature core for each matched pair.