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Activation of Host Wound Responses in Breast Cancer Microenvironment

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Abstract

Cancer progression is mediated by processes that are also important in wound repair. As a result, cancers have been conceptualized as overhealing wounds or wounds that do not heal, and gene expression signatures reflective of wound repair have shown value as predictors of breast cancer survival. Despite the widespread acknowledgment of commonalities between host responses to wounds and host responses to cancer, the gene expression responses of normal tissue adjacent to cancers have not been well characterized. Using RNA extracted from histologically normal breast tissue from 107 patients, including 60 reduction mammoplasty patients and 47 cancer patients, we measured whole genome expression profiles and identified a gene expression signature that is induced in response to breast cancer. This signature represents an *in vivo* wound response signature that is differentially expressed in the normal tissue of breast cancer patients compared to those without disease and is highly accurate (at least 92% sensitivity and 98% specificity) in distinguishing diseased and nondiseased. The *in vivo* wound response signature is highly prognostic of breast cancer survival and there is a strong association between the groups identified by this signature and those identified using serum-treated fibroblasts and other microenvironment-derived or -related signatures. The prevalence of the wound response signature in histologically normal tissue adjacent to breast cancer

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Statement of Translational Relevance

These data show that wound responses are activated in the gene expression profile of normal tissue adjacent to cancer and that this *in vivo* wound response signature predicts prognosis in independent tumor datasets. If normal tissue adjacent to breast cancer expresses genomic signatures with promoting characteristics, surgical interventions may need to evaluate wider margins consistent with the geographic range of these alterations. Furthermore, differential expression of wound response signatures across different tumors and tumor subtypes suggests that individualized targeting of microenvironment may be a viable treatment strategy in breast cancer. For example, significant heterogeneity in extracellular matrix (ECM) or angiogenesis gene expression may suggest differential efficacy of ECM- or angiogenesis-targeted drugs. These studies provide support for the notion that the normal microenvironment is an important variable in breast cancer progression and may be an important target for future clinical interventions.

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suggests that microenvironment response is an important variable in breast cancer progression and may be an important target for clinical interventions.

Keywords

breast cancer; microarray; microenvironment; normal breast; reduction mammoplasty; wound repair

INTRODUCTION

The concept of cancers as "wounds that do not heal" arose more than twenty years ago based on the observation of gross cellular and molecular similarities between wounds and cancer tissue (1,2). Wound response involves clotting and coagulation, tissue remodeling, cellular migration and proliferation, and angiogenesis. Many of these processes have well recognized roles in promoting cancer. Thus, the hypothesis that tumors induce wound responses in cancer microenvironments has been the subject of recent reviews (2), and has led to suggestions that targeting the microenvironment could lead to new cancer therapies (3,4) or chemoprevention opportunities (5). Other targets and drugs in the microenvironment have not been successful [reviewed in (6)], highlighting the need to better characterize of the microenvironment response to breast cancer.

The gene expression alterations present in the breast cancer microenvironment have been examined in a few previous studies. In a careful study by Allinen et al. (7), pure cell populations were isolated from two reduction mammoplasty, two ductal carcinoma in situ, and 10 invasive breast cancer patients. Serial analysis of gene expression (SAGE) of these purified cell populations documented widespread molecular changes in all cell types of breast cancer stroma (7). These molecular changes may reflect paracrine interactions among the diverse cell types that are present in the mammary gland during carcinogenesis: progression to carcinoma is associated with increases in myofibroblasts and immune cells, as well as increased vascularization (8–13). However, recent studies comparing stroma from reduction mammoplasty patients to stroma adjacent to breast cancers failed to identify any gene expression changes associated with disease status (14). Technical differences between these two studies may account for their distinct conclusions. Further research is needed to explain differences between these two studies. Since both studies relied on relatively small numbers of patients, little is known about interindividual variation in the microenvironment response. However, previous studies of breast cancers suggest that interindividual variation is an important feature of cancer-associated wound responses: based on the idea that fibroblasts *in vivo* are exposed to serum only during wounding, Chang et al. (15) used cultured fibroblasts from many different anatomical sites to derive a "wound response" signature, that is highly prognostic in breast cancer patients (15). Whether that signature is altered in the microenvironment adjacent to breast cancer has not been evaluated previously.

We hypothesized that with larger sample sizes, a reproducible gene expression signature of activated wound response would be detectable *in vivo* in the microenvironment of breast cancer. We used whole genome microarrays to investigate gene expression in 107 patients, including 60 reduction mammoplasty (RM) patients and 47 histologically normal samples from cancer patients (referred to as cancer-adjacent normal, or CN samples). Our results show that an *in vivo* wound response signature is identifiable in the histologically normal tissue adjacent to breast cancer and that this signature has strong predictive value in distinguishing normal tissue of cancer patients from that of disease free individuals. This signature is also prognostic, confirming the importance of wound response in breast cancer progression.

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METHODS

Patient samples

Patients were women undergoing elective reduction mammoplasty (RM) at Baystate Medical Center in Springfield, Massachusetts or mastectomy at University of North Carolina Hospitals in Chapel Hill, NC. All patients enrolled voluntarily under Institutional Review Board approved protocols. RM tissue was excluded if pathological assessment of patient-matched paraffin embedded tissues suggested any abnormal malignant or pre-malignant findings. For histologically normal tissue adjacent to breast cancer (CN), a pathologist from the Tissue Procurement Facility (TPF) at University of North Carolina at Chapel Hill confirmed histologically normal tissues. Only patients receiving no neoadjuvant therapy prior to surgery were included. All tissues were handled by snap freezing immediately after surgery and RNA was isolated using a single protocol as described in Hu et al. (16).

Microarrays

All microarrays were performed at the University of North Carolina at Chapel Hill. Two-color Agilent human arrays were used. Cy3-labeled reference was produced from total RNA from Stratagene Universal Human Reference (spiked with 1:1000 with MCF-7 RNA and 1:1000 with ME16C RNA to increase expression of breast cancer genes) following amplification with Agilent's low RNA input amplification kit. The identical protocol was applied to total RNA from RM or CN tissue, but the patient samples were labeled with Cy5. Some samples from RM and CN groups were performed on 4X44k Agilent whole genome arrays and some of each type were performed on 244k Agilent custom arrays that included all of the probes from the 4by44K array. Only probes that were present on both formats were utilized in the final analysis and intraclass correlation coefficients for technical replicates assayed on the same platform (range: 0.73 – 0.97, median 0.89, n=8) did not show meaningful differences from those conducted on different platforms (range: 0.708 – 0.938, median 0.90, n=12). Principal component analysis (PCA) suggested no substantial difference between the two platforms. Duplicate microarrays corresponding to the same patient sample were combined by averaging. All data are publicly available through the Gene Expression Omnibus (GSEXXXX, in progress).

Data Normalization and Age-Adjustment

Data were lowess normalized and only genes with signal intensity >10 in both channels were included in analyses. Because the RM patients tended to be younger than the CN patients, we selectively adjusted for confounding by age using the following algorithm: First, we estimated the effect of tissue type (RM vs. CN) in a linear regression model that included tissue type as a main effect and age as a covariate (full model). We compared the main effect estimate from this full model to that of a reduced model that included only the main effect (no age parameter). We concluded that age was a confounder if inclusion of age in the model induced a change in the main effect parameter greater than 10% and that change-in-effect was in the top 80th percentile. To ensure that the change-in-effect estimate was stable, we performed 100 bootstrap samplings and confirmed that genes were similarly affected in the original analysis and in the bootstrap analysis. That is, in the majority of cases those genes that were unadjusted in the original data remained unadjusted in the bootstrap. We utilized the parameter estimates from the original analysis to select genes for age-adjustment, and as a result, we adjusted for age in 19% of genes. This is concordant with previous literature from lymphoblastoid cell lines suggesting that at least 10% of genes were significantly associated with donor age (17). Identical normalization parameters and age-adjustment parameters and algorithms applied to the training set were used to adjust for age in the test set.

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Prediction Analysis

The age-adjusted training dataset was analyzed using Distance Weighted Discrimination to identify genes that distinguished RM and CN samples (18). The top 200 DWD loadings were selected for the predictive signature based upon the observation that this was the smallest gene set that provided maximum predictive accuracy in the training set (by ten-fold cross validation). Average predictive accuracy in 10-fold cross validation was 95.4% (95% CI: 95.1%–96.3%) in the training data set, with 91.9% sensitivity (95%CI: 91.2%–94.1%) and 97.98% specificity (95%CI: 97.92%–97.92%) and the same set of genes was used to assign class membership in the test set.

To evaluate the Chang et al. (15) serum response as a predictor of CN vs. RM status, all patients in the training set were classified according to the Pearson correlation of its expression levels of the Core Serum Response (CSR) genes to the serum activated fibroblast centroid. Patients with correlation >-0.15 were classified as "activated" and patients with correlation ≤ -0.15 were classified as "quiescent", as described in the original paper.

Prediction of Breast Cancer Survival

To evaluate the prognostic value of our signature, we used published gene expression data for breast tumors from 295 patients, derived by researchers from Netherlands Cancer Institute (NKI) and Rosetta Inpharmatics-Merck using Agilent oligonucleotide microarrays (15,19, 20). To evaluate the prognostic value of our *in vivo* wound response signature, we mapped our 200 probes, representing 155 genes, to 112 genes on the NKI dataset. We then used PCA to define three groups of equal size. These groups were then evaluated in univariate Kaplan-Meier analyses using WinSTAT. We compared our prognostic signature to the wound response signature published for these tumors by Chang et al. (15). For that comparison, we used the group definitions from the original paper (two groups, Activated and Quiescent). In addition, we compared our signature to the Stroma-Derived Prognostic Predictor (SDPP) of Finak et al. (21) and the 66 gene desmoids-type fibromatosis (DTF) signature (22), both of which were used to define three groups of equal size by PCA.

Immunohistochemistry

A series of CN (n=7) and RM (n=8) samples that had been analyzed by microarray were also analyzed by immunohistochemistry. Five-micron sections cut from formalin-fixed paraffin-embedded tissue blocks were deparaffinized and rehydrated following standard protocol. Sections were incubated with antisera against COX-2 (Cayman Chemical) and CYR61 (H-78, Santa Cruz) following manufacturers' instructions. Antigen-antibody complexes were visualized using horseradish peroxidase labeled polymer kit following standard protocol (DAKO). Sections were counterstained in hematoxylin dehydrated through graded alcohols, cleared in xylene, and mounted in permount.

RESULTS

Activated Wound Responses in Normal Tissue Adjacent to Breast Cancer

Using data from 48 reduction mammoplasty (RM) samples and 34 histologically normal samples adjacent to cancer (CN), we performed Distance Weighted Discrimination to select the top 200 probes that were differentially expressed between RM and CN patients. In ten fold cross validation, these 200 probes, representing 155 genes had 98% accuracy in predicting RM vs. CN status. Figure 1 shows a hierarchical cluster of these 82 patients using this gene list (complete gene list and values for all 200 probes shown in Supplemental Table 1).

The CN signature appears to be enriched for many genes previously shown to have an important role in wound healing and inflammation (Figure 1, Supplemental Table 2). For example,

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extracellular matrix (ECM) deposition is an important process in repairing of wounds and ECM genes are differentially expressed between RM and CN samples. Figure 1C shows a group of genes more highly expressed in RM tissue than in CN tissue that includes genes in the Gene Ontology categories for extracellular matrix such as EGFL6, BMPER, ELA2, and TFPI2. TFPI2 is the negative regulator of F3, and consistent with lower expression of its inhibitor, the transcript levels of F3 are higher in CN samples (Figure 1D), along with a number of other genes involved in extracellular matrix formation such as ADAMTS4, OGN, PDGFRL, FBLN1, LUM, EFEMP1. Thus, clusters 1C and 1D show dysregulation of ECM in the microenvironment of cancer. Functional annotation clustering with DAVID (http://david.abcc.ncifcrf.gov/) shows ECM genes are significantly overrepresented in our *in vivo* signature for response to cancer (Supplemental Table 2). Three functional annotation clusters including extracellular matrix genes were identified with GO terms such as extracellular region (Benjamini p=1.0e-11), extracellular region part (2.7e-8) and extracellular matrix (3.7e-4) statistically overrepresented. Supplemental Table 2 shows DAVID functional annotation clustering results.

Angiogenesis is an important process in wound healing. Consistent with a role for angiogenesis in the cancer microenvironment, Figure 1E includes important immediate early (IE) angiogenesis genes such as PTGS2/COX-2, CYR61 and CTGF (23–26). Furthermore, well-documented binding partners for CYR61 and CTGF (including THBS1 and BMPER) are upregulated in CNs. Thus, these pathways and the CCN family of genes may be important in regulating the host response to cancer. Both PTGS2 and CTGF are immediate early genes that are in the GO category “response to wounding”, which was enriched in our data (p=1.4e-5), and the role of CYR61 in wound response is well established in recent literature (27). PTGS2 has been previously shown to be elevated in the microenvironment of breast cancer (28). Figure 1E therefore represents an important wound response angiogenic signature that is activated in the breast cancer microenvironment.

Genes from the GO “cellular adhesion” category were represented in Figure 1F, including LAMA3 and LAMC2. The cluster in Figure 1F also included a number of chemotaxis genes such as IL1B, CXCL1, and CXCL14, the latter of which has previously been shown to be upregulated in the histologically normal tissue of breast cancer patients (7). Figure 1G includes a wide range of genes involved in re-epithelialization after wounding, including keratins 6A, 6B, 6C and 14, 14p, 16 (29–31). Re-epithelialization is one of the major processes necessary for successful wound repair (32). Keratins are overrepresented in our functional clusters (p=5.2e-5), in a cluster that includes significant enrichment for GO categories epidermis development (p=2.8e-4), ectoderm development (p=2.8e-4) and cell communication (p=5.5e-6). Figure 1G shows overrepresentation of cellular adhesion genes, including TP63 (33), DST, COL17A1, DSC3. In sum, Figures 1F and 1G show remodeling clusters that are dependent upon paracrine signaling and heterotypic interactions between cell types. Taken as a whole, Figure 1 demonstrates that the wound healing processes that have been well documented in skin (32) are strongly induced in the microenvironment of normal breast tissue adjacent to breast cancers.

The original work hypothesizing that tumors resemble chronic wounds was referring to the tumor stroma rather than the adjacent normal tissue (1). While we defined a wound response signature in adjacent normal tissue, we were interested to examine the relative expression of this signature in the tumors themselves. Supplemental Figure 1 depicts the expression of the 200 probes across the 82 patients, including the paired tumor tissue for each of the cancer patients, with the groups of genes labeled according to the corresponding clusters from Figure 1. This figure shows that for some clusters (e.g. Figure 1C) where CN has lower expression than RM, the decreased expression is even more pronounced in the tumors. Likewise, many of the genes in Figure 1F that were upregulated in CN are more strongly upregulated in tumors.

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However, there are groups of genes from Figure 1D–F that are homogeneously expressed in CN tissue, and heterogeneous expression in tumors, and there are genes (eg. Figure 1G), that are upregulated in CN and expressed at lower levels in tumors. In the case of the cluster in Figure 1G, many of these genes are immune response/chemotaxis genes and therefore downregulation in the immunosuppressive tumor microenvironment, but not the adjacent normal, may be expected. In sum, our gene list has a unique expression profile in each of the tissue types (CN vs. RM vs. tumor) consistent with some of the known biological features of each tissue type, and our signature reliably segregates normal breast samples from patients with disease from those without disease (Figure 1B and dendrogram in Supplemental Figure 1).

Localization of PTGS2/COX-2 and CYR61 by Immunohistochemistry

To confirm the changes that we observed by microarray and to identify the cell types responsible for altered expression, we performed immunohistochemistry for two markers from the Angiogenesis/IE cluster in Figure 1E. Representative images are shown in Supplemental Figure 2. COX-2 was more highly expressed in the luminal epithelium of CN patients than RM patients (two-tailed t-test with unequal variance, p=0.0005), consistent with previous observations (28). Less than half of the RM samples (5 of 13) were positive for COX-2 staining, with average score of 0.7 (range from 1 to 2). However, 16 of 18 (89%) of cancer-adjacent normal samples were positive for COX-2, with an average score of 2.1 (range from 1 to 3). CYR61 was moderately or strongly expressed in the luminal epithelium of both CN and RM patients, and we also observed CYR61 expression in fibroblasts, endothelial cells, plasma cells, and lymphocytes. Thus, the 2–4 fold change in CYR61 RNA levels in CN relative to RM may reflect changes in expression levels of specific cellular populations or may reflect changes in the distribution of cell types present in the tissue.

Predictive Accuracy of the Wound Response Signature

Our *in vivo* wound response signature distinguished CN samples from RM samples with 98% accuracy in the training set, with 94% sensitivity and 100% specificity. This accuracy and the striking expression differences between CN and RM shown in Figure 1 suggest that this signature can be used to predict the disease status of histologically normal samples in an independent test set. To our knowledge, there are no comparable, publicly available dataset of grossly dissected human breast tissue, and therefore we collected a separate test set of 25 additional arrays (12 RM and 13 CN). In this independent test set, we correctly predicted CN vs. RM status with 96% accuracy (92% sensitivity and 100% specificity), with only one of 13 CN samples incorrectly classified as an RM.

The population undergoing RM surgery may be systematically different from general population. While some women undergo unilateral RM for cosmetic reasons, others undergo bilateral RM to address ergonomic problems. This latter group may have more fatty tissue and larger breasts, and therefore it is possible that some of these patients have biology that differs from normal breast biology in the general population. To evaluate generalizability of our study, we obtained a second test set comprised of ten additional samples of normal breast tissue, none of which were from reduction mammoplasty patients: three normal breast samples from women with benign diagnoses (1 benign cyst and 2 fibroadenoma) and seven normal breast tissue samples adjacent to ductal carcinoma in situ (DCISN for DCIS-adjacent normal). In this independent test set, we applied the same normalization, data preprocessing algorithms and parameters as were applied to the training set. Of the 200 probes that comprised our classifier in the training set, 149 were present with >80% good data in this second independent test set. All 3 of the normal tissues adjacent to benign lesions were classified as RM-like and 4 of 7 DCISN samples were identified as cancer-adjacent normal. These results suggest that the normal breast signature that we observed in RM is not peculiar to women who elect reduction

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surgery, and furthermore, documents that the CN-like signature is also present adjacent to DCIS in some patients, even before the disease becomes invasive.

Because Chang et al. had previously published a serum treatment/wound response signature (15), we were interested in whether their *in vitro* signature, which had little overlap in terms of exact gene content, but substantial overlap in terms of the processes represented, would also predict CN vs. RM status. We classified each of our samples as 'Activated' or 'Quiescent' using their signature and classification parameters, expecting that perhaps 'Activated' would be more common in the CN samples. However, approximately 37% percent of RM samples were classified as 'Activated' and 35% of CN samples were classified as 'Activated', demonstrating that the *in vitro* serum response signature does not offer predictive value in distinguishing RM and CN samples (Chi square test, 1 df, p=0.8). This suggests that while many of the processes involved in wound response may be common to different experimental and observational systems, the specific signatures of wound response may be tissue/ and or cell-type dependent. Our wound response signature, being breast tissue derived, appears to offer advantages over the *in vitro* serum-derived signatures in identifying abnormal breast tissues.

In Vivo Wound Response and Patient Survival

Because the aforementioned signature derived from serum-treated fibroblasts, commonly referred to as the "wound response" signature, was highly prognostic for breast cancer patient outcomes, we hypothesized that our *in vivo*, 200-probe, breast tissue-derived wound response signature might also have prognostic value. Thus we evaluated the prognostic value of our signature on a test set of 295 patients from the Netherlands Cancer Institute (NKI) (15,34). These 295 patients were divided into three groups based on the rank order of the first principal component of our *in vivo* wound response signature across their tumor expression profiles (gene expression shown in Figure 2) and we conducted Kaplan-Meier analysis to evaluate the association between these groups and overall and relapse free survival (Figure 3).

In Figure 2, the poor prognosis group (Group I) had low expression of immediate early genes CTGF and CYR61 and low expression of GREM1 and GREM2. GREM1 is an important developmental signaling molecule that is part of an FGF/GREM1 inhibitory loop that halts tissue outgrowth during development and regeneration (35). These tumors also have low expression of angiogenesis inhibitor THBSP1 (36). In contrast, the good prognosis group III tumors had high expression of these same genes. The Group I poor prognosis tumors had high expression of a number of chemotaxis and immunoregulatory genes including IL-1β, TLR2, CCL2, and indoleamine 2,3-dioxygenase, a targetable enzyme that modulates the response of T-cells to tumors (37). These tumors have high expression of Keratin 6 and 14, the former of which contains an IL-1 responsive DNA element (38). Keratin 6 is expressed in the skin in hyperproliferative disorders and in response to stressful stimuli such as wounding (29). Keratin 14 (K14) is a basal keratinocyte marker that is differentially expressed in wound repair (30). We observed that basal-like tumors were more likely to have the Group I wound response signature, while luminal tumors were more likely to be Group III (Figure 2). The correlations between subtype and some of the gene expression changes were consistent with those in previous studies. For example, PTGS2 has been shown to be highly expressed in basal-like tumors (39) previously, and we also observed this here.

Based on the three groups of equal size identified in Figure 2, we then evaluated associations with survival. The three groups had different relapse free (Figure 3A) and overall survival (Figure 3B) and the predictive value of these signatures compare favorably with other prognostic signatures that have used microenvironment biology to predict outcome. For example, our signatures show prognostic value similar to that provided by the Chang et al.

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wound response signature in predicting relapse free (Figure 3C) and overall (Figure 3D) survival.

To further evaluate concordance of our *in vivo* wound response with previous wound response or microenvironment-related signatures we applied methods described in Fan et al. (34). Concordance between predictors was evaluated by classifying each patient and then conducting a Chi-square test to identify associations between classifications made by each predictor. Table 1 shows that there was high concordance between those classified as "activated" by Chang et al, and our Group I and II tumors. Likewise, our predictor showed significant concordance with the three-class SDPP predictor (21) and DTF signatures (22), both of which were more likely to classify group III tumors as good prognosis and group I tumors as poor prognosis.

While our signature represents many of the processes observed to occur in healing skin (32), we sought to strengthen the claim that our signature reflects wound repair using results from previous gene expression studies of healing skin and wound response in breast. Using the same concordance evaluation methods (34) that we applied for SDPP and DTF signatures, we found a significant association between our signature and a previously published gene expression signature for the early response of skin to injury (40). This signature was measured only 30 or 60 minutes after skin injury, so does not reflect chronic injury and it is also derived from skin not breast. Thus, we also sought comparisons with breast tissue injury. A breast injury signature has not been reported, but mammary gland involution has established features of wound repair (41). Therefore we examined concordance with the mammary gland involution signature and found a strong and significant correlation. The results are presented in Table 1. Taken together, these findings show reproducible concordance between our signature and wound repair- and microenvironment-based signatures.

DISCUSSION

It has been argued that solid tumors are composed of two distinct but interdependent compartments: the malignant parenchyma and its supporting microenvironment (42). In this paper, we have studied how the normal microenvironment responds to the presence of the tumor. Our results show that a wound response signature is activated in the histologically normal tissue of cancer patients. Wound healing includes numerous overlapping processes such as blood clotting, inflammation, extracellular matrix alterations, angiogenesis, and tissue remodeling [reviewed in (2,32)]. Many of these processes are expressed differentially in normal tissue adjacent to breast cancer and in tumors, but not in the normal tissue of women undergoing reduction mammoplasty.

Previous microarray studies have examined molecular similarities between wound repair and cancer using *in vitro* models. First, Iyer et al. studied the response of fibroblasts to serum (43), based on the idea that serum has a very specific meaning in vivo: cells encounter serum only in the event of local injury. In response, tissues mount a rapid, concerted multicellular response to preserve tissue integrity. Serum promotes growth and survival of normal and cancer cells in culture, and in parallel, serum causes rapid cell proliferation in tissue-level wound repair responses. Given these parallels, Iyer et al. were able to document a serum response of foreskin fibroblasts that included genes involved in clotting and coagulation during remodeling, including tissue factor 3 (TF3), genes promoting migration and proliferation of fibroblasts (e.g. CTGF), and genes involved in angiogenesis (e.g. COX2 and IL1β). Many of these same genes were modified in our in vivo wound response signature. To extend the observations of Iyer et al., Chang et al. hypothesized that a canonical gene expression signature might be identified for serum response of fibroblasts from different anatomic sites (15). Using gene expression profiles of 50 samples from ten sites, Chang et al. recapitulated a wound response signature originally reported by Iyer et al. and documented that this Core Serum

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Response (CSR) was evident in a subset of breast tumors. Breast tumors that expressed the serum response signature had a poorer prognosis than tumors with a 'quiescent' serum response phenotype. While the in vitro wound response signatures of Iyer et al. and Chang et al. did not have predictive accuracy in distinguishing RM and CN tissues in our dataset, we did observe that the same biological processes are activated in association with breast cancer *in vivo,* outside the boundaries of the tumor itself.

The association between microenvironment characteristics, survival and breast cancer subtype has been reported in previous papers. For example, in a tissue microarray study of 479 invasive breast carcinomas using 28 different markers, basal-like breast cancers were more likely to highly express genes (such as laminin) that are involved in ECM remodeling (44). In a separate study, the ECM composition of 28 primary breast carcinomas were examined using microarrays and a gene expression profile of 278 ECM-related genes were used to divide the tumors into four main groups that correlated with outcome and basal-like subtype (45). The current results confirm that many ECM genes are enriched in basal-like cancers and that these genes have prognostic value, but suggest that the enrichment may be part of a broader, chronic response of the host as it attempts to repair the tissue injury caused by cancer.

Strengths of our investigation include the large number of samples relative to previous studies of histologically normal tissue, the application of methods to control for confounding by age, and the systematic comparison with previous microenvironment-derived signatures. Additionally, we documented the generalizability of our findings to women with premalignant or benign breast disease and showed the validity of our predictor in two independent test sets. Our results demonstrate that use of whole tissue, which includes epithelial cells as well as stromal cells, does not hamper our ability to detect important microenvironment signatures. Many of the genes we detected as differentially expressed are reported to be expressed by fibroblasts (e.g. CTGF) and neutrophils (e.g. IL1B) for example, while others are epithelial contributors (keratins 6 and 14). This reiterates the findings of Perou et al. (46) showing that signatures from distinct cellular compartments can be identified in microarray data from mixed cell populations.

Our results suggest some important questions for future investigation related to the spatial and temporal parameters of the wound response to cancer. While our tissue procurement protocols ensured that the histologically normal tissue was collected from regions outside of tumor margins, the distance to the margin was not measured precisely so we are unable to document the geographic zones of gene expression alterations. Methylation changes have been reported as far as 4 cm from the site of the primary tumor (47), however the geographic zones for gene expression alterations have not been characterized. Based on the notion that wound response is a local phenomenon, it might be expected that the wound response would not be present in contralateral breast, or that it may dissipate as the distance from the primary tumor increases, but this has not been investigated. The geographic limits of the wound response to cancer may have translational implications. For example, most tumor recurrences occur at the site of previous resection even when margins are clear (48). If gene expression alterations with promoting characteristics have a defined range of action outside the primary tumor margins, future guidelines for surgical intervention may need to consider wider margins consistent with the geographic range of microenvironment alterations. It is also not known how early in development of breast cancer gene expression alterations are activated in adjacent normal tissue. Leukocyte infiltration occurs early in carcinogenesis (49), suggesting that aberrations in gene expression may already be present during DCIS, but this has not been investigated. What is clear from our results is that wound response signatures are strongly activated in histologically normal tissue adjacent to invasive tumors.

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A role for wound response in breast carcinogenesis is increasingly well documented. Differential expression of wound response signatures in different tumors suggests that individualized targeting of microenvironment may be a viable treatment strategy in breast cancer. For example, there is significant heterogeneity in extracellular matrix across tumors (44,45) suggesting differential efficacy of ECM targeting drugs. The strong angiogenesis signature observed in Group I tumors, which are enriched for basal-like breast cancers, supports the application of chemotherapy combinations that target VEGF and angiogenesis in triple negative breast cancers (50). By better characterizing microenvironment heterogeneity, improved strategies for application of novel and existing therapies may be possible. The similarities between cancer and wound response are compelling and continued investigation of host responses to cancer may lead to new biological insights and translational opportunities.

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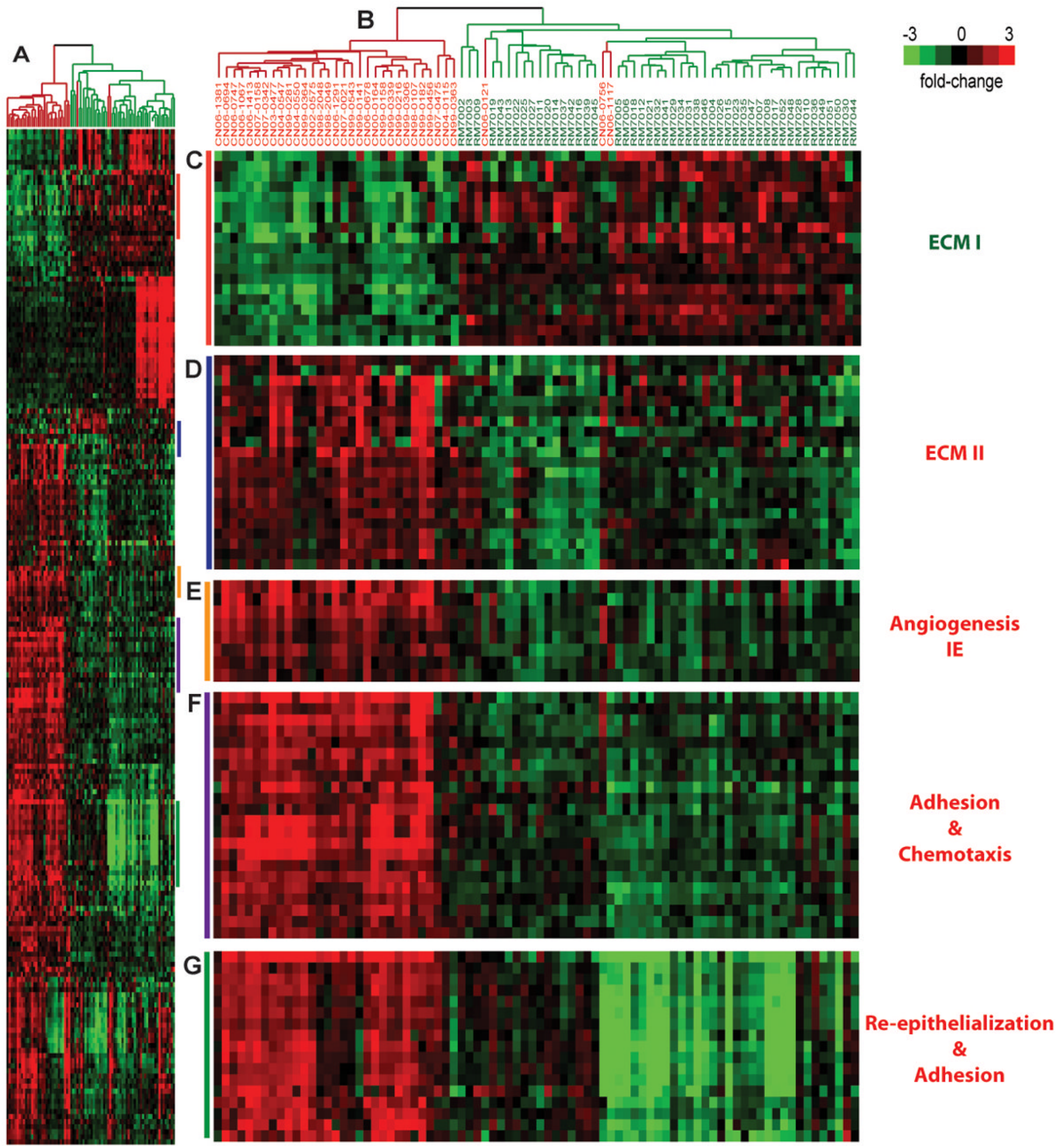

Figure 1. Wound response signatures are differentially expressed between reduction mammoplasty patients (RM) and histologically normal tissue of cancer patients (cancer normal, or CN)

Figure A shows the complete dendrogram for 200 probes that distinguish RM from CN tissues with 98% accuracy in predictive analyses. The dendrogram in B shows CN samples (red) and RM samples (green) form distinct branches, with only a few CN tissues clustering with RM samples. The clusters show that various biological processes involved in wound repair are differentially expressed between the two groups, including extracellular matrix alterations (C & D), immediate early (IE) genes involved in angiogenesis (E), Re-epithelialization and celluar adhesion (F), and cellular adhesion and chemotaxis (G). Supplemental Table 1 lists $\log_2$(R/G) values for each sample by probe ID and Supplemental Table 2 provides DAVID analysis results for the 200 probe gene set.

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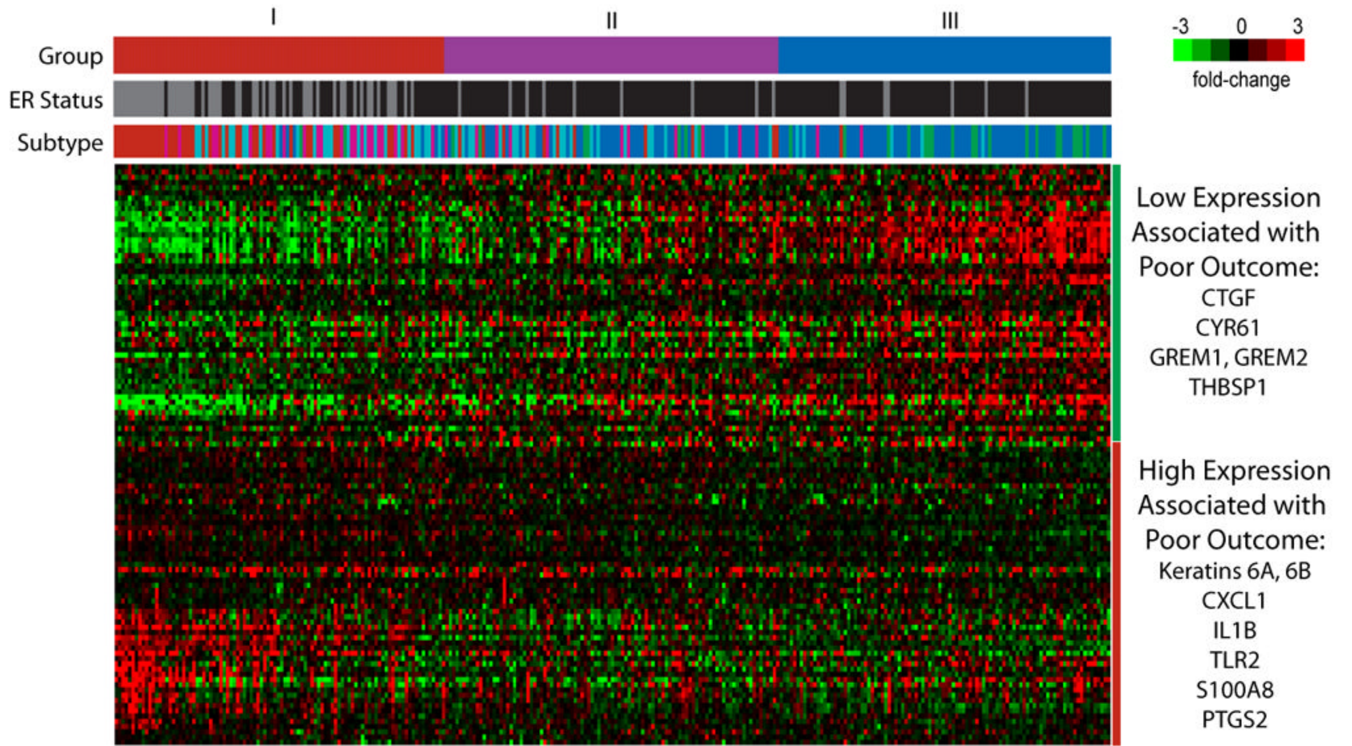

Figure 2. Differential expression of wound response signatures in breast cancer

Expression of our *in vivo* wound response signature (Figure 1) was examined in 295 tumors from the Netherlands Cancer Institute (15). Principal Component Analysis was used to divide the samples into three groups of equal size (Groups I, II, and III) and the samples are ordered according to their assigned Group. ER status is indicated (gray = ER negative, black= ER positive), along with breast cancer subtype (red = basal-like, magenta=HER2-enriched, light blue = luminal B, dark blue = luminal A, green = normal-like). Two gene clusters were identified: one where low expression predicted poor outcome and one where high expression predicted poor outcome.

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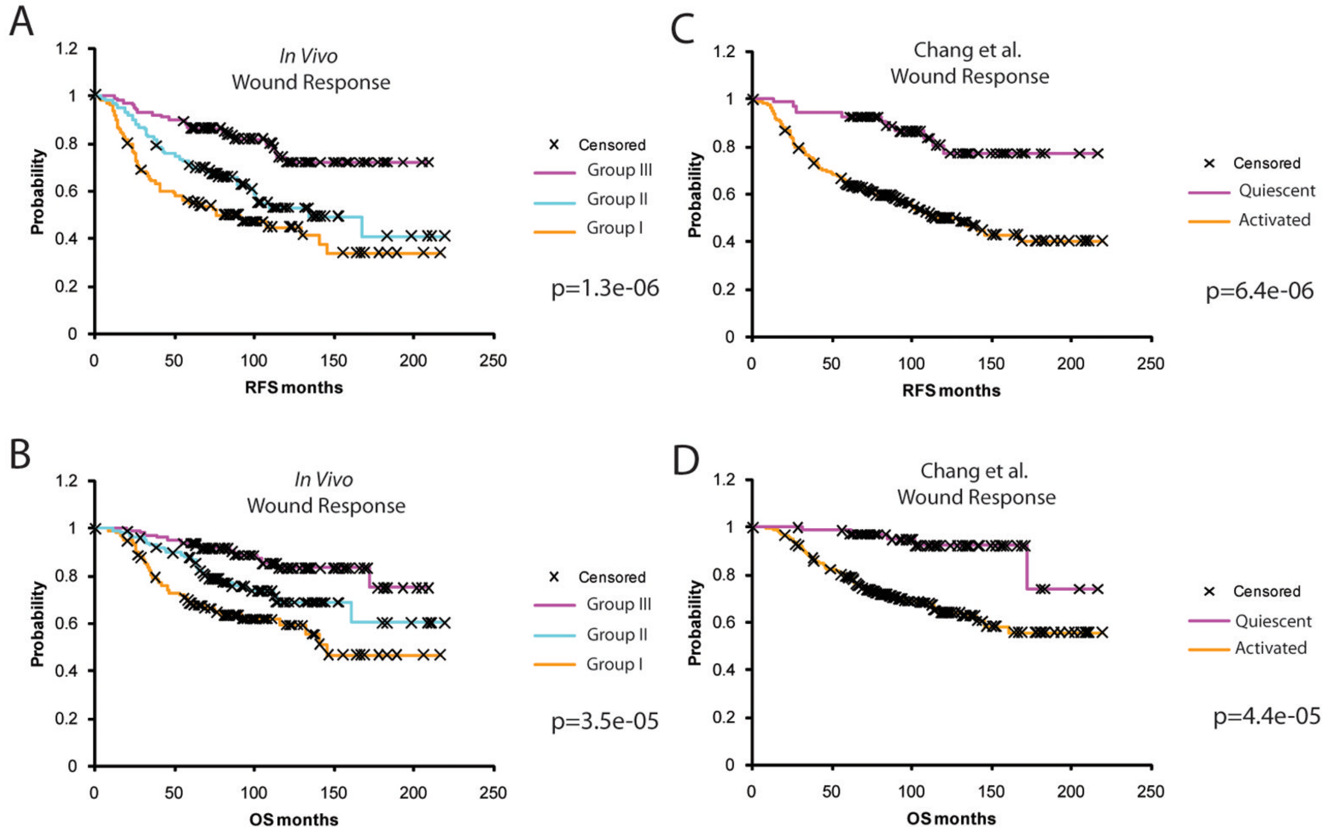

Figure 3. *In vivo* wound response signature and previously published serum response signature show similar prognostic value

Kaplan-Meier analyses were conducted using the PCA-assigned Group I, II, or III (A and B) and using the Chang et al. core serum response assignments (C and D). Both classification schemes are associated with overall and relapse free survival.

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Table 1

Concordance in classifications between the In Vivo Wound Response signature and previous serum response or microenvironment-related prognostic signatures[a].

In Vivo Wound Response		Core Serum Response (15)		SDPP (19)		DTF signature (20)		Skin Injury (40)		Involution (41)	
class	no. of patients	class	no. of patients	class	no. of patients	class	no. of patients	class	no. of patients	class	no. of patients
Group III	98	Quiescent	50	good	67	good	50	good	56	good	70
		Activated	48	mixed	25	mixed	19	mixed	36	mixed	23
				poor	6	poor	29	poor	6	poor	5
Group II	99	Activated	88	mixed	51	mixed	30	mixed	39	mixed	43
		Quiescent	11	good	28	good	39	good	34	good	23
				poor	20	poor	30	poor	26	poor	33
Group I	98	Activated	92	poor	72	poor	40	poor	66	poor	60
		Quiescent	6	mixed	23	mixed	49	mixed	24	mixed	33
				good	3	good	9	good	8	good	5
		χ^2=67.7, 2 df, p<0.001		χ^2=152.9, 4 df p<0.001		χ^2=44.0, 4 df, p<0.0001		χ^2=96.6, 4 df, p<0.0001		χ^2=121.7, 4 df, p<0.0001	

[a]The rows are arranged so that the number of the Group I, II, or III samples that are represented in the analogous category from each of the other predictors is visible by looking across the gray bar.