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RESEARCH PAPER

Stromal biomarkers in breast cancer development and progression

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Abstract Breast cancer is a heterogeneous, multi-factorial disease of aberrant breast development whose etiology relies upon several microenvironmental changes within the tissue. Within the last decade, it has become widely accepted that tumor cells frequently rely on signals from an activated microenvironment in order to proliferate and survive within a tissue. This activated tissue microenvironment involves the appearance of αSMA + fibroblasts (referred to as "cancer associated fibroblasts"), the recruitment of various immune cells (macrophages, T cells, B cells, T regulatory cells), enhanced collagen I deposition, and epigenetic modifications of stromal cells. These stromal changes can predict patient survival and correlate with distinct breast tumor subtypes. Characterizing these stromal changes will facilitate their use as clinical biomarkers in breast cancer, and may facilitate their use as potential drug targets for adjuvant breast cancer therapy.

Keywords Breast · Stroma · Biomarkers · Cancer

Introduction

Over an entire lifespan, individual cells incur several detrimental genetic insults due to environmental exposures and physiologically induced reactive oxygen species. Every cell within the body is susceptible to these carcinogens, but each cell does not necessarily turn cancerous. Pioneering studies demonstrated tumor cells injected into the mouse blastocyst can give rise to normal, chimeric mice [1], while Rous sarcoma virus infection can readily transform fibroblasts in vitro, but fail to induce tumors in chick embryos despite widespread viral infection [2]. Moreover, heritable cancer syndromes (such as those driven by inherited mutant *BRCA1* or *Rb* alleles) only predispose to certain types of cancers, despite the fact that every cell within the body harbors the deleterious mutation in these essential genes. Based on these general observations, it is apparent that genetic alterations alone are not sufficient to drive cancer progression, and in some cases the tissue microenvironment may hold a dominant influence in tumor development.

Both mammary gland development and breast cancer development require stromal cues. However, breast cancer cells communicate with a drastically different stroma than do mammary epithelial cells within a disease free breast. It is well established that stroma associated with normal mammary gland development is strikingly different from that associated with breast carcinomas [3, 4]. When compared to normal tissues, the stroma accompanying breast tumors contains an increased number of fibroblasts, immune cell infiltrates, enhanced capillary density, increased collagen I and fibrin deposition [5, 6]. Compared to normal mammary gland stroma, breast tumor-associated stroma shows elevated expression of proteins such alpha smooth muscle actin (αSMA), type I collagen, fibroblast activated protein (FAP), cyclooxygenase-2 (Cox-2), tenascin, altered matrix deposition, and significant changes in gene expression. In this review, we discuss the differences in cellular composition, extracellular matrix (ECM) composition, and protein expression in breast tumor stroma and implicate these

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characteristics for possible use as clinical biomarkers in breast cancer.

Cancer associated fibroblast biomarkers

The majority of human breast cancers are associated with a strong desmoplastic stroma and inflammatory response that strikingly resembles the stromal response during chronic wound healing [3, 6, 7]. Both tumors and wounds elicit stromal reactions that are characterized by ECM remodeling, growth factor secretion, inflammation, cell migration, and angiogenesis. During normal wound healing, this stromal response is initiated by bone marrow-derived hematopoietic cells and is accompanied by a marked increase in vascular permeability; plasma extravasation, fibrin deposition, platelet activation and inflammatory cell infiltration, which together result in the release of numerous of cytokines and growth factors [6, 8]. This response leads to the generation of granulation tissue, which is characterized by angiogenesis, activation of fibroblasts into αSMA positive myofibroblasts, and matrix remodeling [9]. The αSMA positive myofibroblasts associated with the stroma of solid tumors are frequently referred to as "cancer associated fibroblasts" (CAFs) to distinguish them from myofibroblasts associated with wounded tissues, although functionally and molecularly, these cells may be indistinguishable. However, CAFs, unlike myofibroblasts associated with wounded tissues, co-evolve with tumor cells over time, causing dramatic alterations in their gene expression program [10–13]. This altered gene expression contributes to the tumor promoting functions of these cells in several different types of solid tumors, including breast tumors [14–18].

The most prominent upregulated genes in CAFs include components of the ECM and matrix metalloproteases (MMPs), responsible for stromal remodeling as well as secreted and cell surface proteins, such as stromal derived factor-1 alpha (SDF1α) [14, 19] and interleukin-6 (IL-6) [20, 21]. While CAFs may have a distinct secretome compared to disease-free fibroblasts [15], markers of these cells have been difficult to pinpoint; this is largely due to in vitro, 2D culture techniques used to characterize these cells. It is known that fibroblasts isolated from human tissues and cultured on 2D plastic will acquire a myofibroblast, activated phenotype, characterized by αSMA expression and stress fiber formation. Because of this, in vitro techniques used to elucidate protein expression differences in CAFs versus their disease free fibroblast counterparts often mask true discrepancies in gene and protein expression [21, 22]. Moreover, given the complex heterogeneity within human breast cancers, tumor associated stromal markers in vivo may even differ among patients and different breast tumor subtypes. Despite these hindrances, some of the markers commonly used to identify CAFs in human breast tumors (in addition to αSMA) are the following:

FAP (Fibroblast activation protein alpha)

Fibroblast activation protein alpha (FAPα, Seprase, FAP) is a type II integral membrane serine protease with dipeptidyl peptidase, gelatinase, and collagenase activity [23, 24]. Interestingly, while FAP is generally not expressed in disease-free stroma, it is significantly upregulated in reactive stroma of solid tumors and wounded tissues [25–27]. Because of this restricted expression pattern, and reports demonstrating overexpression of FAP increases tumor growth and metastasis [28], it has been an attractive target for therapeutic targeting of tumor stroma [25, 29–31]. Vaccinating mice with an oral DNA vaccine targeting FAP significantly reduces mammary tumor development through cytotoxic T cell mediated elimination of CAFs [30]. Interestingly, these tumor promoting functions of FAP appear to be independent of its proteolytic activity, suggesting that FAP may act as a membrane bound signaling modulator for intracellular gene expression, possibly in conjunction with integrins [28].

Cav1 (Caveolin-1 (Cav1))

Caveolin-1 (Cav1) is an integral membrane protein localized to caveoli in fibroblasts, adipocytes, and myoepithelial cells in the mammary gland [32]. Downregulation of stromal Cav1 expression in invasive ductal carcinoma correlates with tumor recurrence, estrogen receptor (ERα) negative status [33], advanced tumor stage and lymph node metastases [34]. However, the prevalence of stromal Cav1 downregulation in human breast tumors remains unclear. While some reports suggest reduced Cav1 expression is a hallmark of CAFs [35, 36], other reports demonstrate that upregulation of Cav1 is essential for fibroblast contraction and matrix remodeling, with CAFs having significantly higher levels of Cav1 as compared to disease free breast tissue [37]. We failed to identify differences in Cav1 expression in cultured CAFs from various breast tumor patient samples of varying hormone status, HER2 status and grade, as compared to cultured disease-free breast fibroblasts [21]. These contradictory results are likely a reflection of fibroblast response to in vitro culture conditions, as well as the inherent heterogeneity of both cancerous and disease-free breast tissues. Thus, it remains unclear whether Cav1 will serve as useful stromal biomarker in human breast cancers.

TN-C (Tenascin-C)

TN-C is an alternatively spliced ECM glycoprotein predominantly expressed during embryogenesis, wound

healing and tumorigenesis [38]. Because the expression of TN-C is highly upregulated in the desmoplastic stroma of human breast tumors compared to levels in disease-free breast tissue [39], it was originally postulated that TN-C is a marker solely for CAFs. However, further investigation has shown that TN-C is expressed in some breast cancer cell lines, both cultured and primary human mammary epithelial cells, and breast tumor cells in vivo [40–43]. While TN-C expression is not restricted to tumor associated stroma, increased stromal expression of TN-C predicts recurrence [44, 45], increased risk of death in patients with node-negative breast cancer [46], and distant metastasis [47]. Recent findings demonstrate TN-C secretion by stromal fibroblasts [17] as well as breast tumor cells [48] may support metastatic colonization of tumor cells but not significantly impact primary tumor growth. These results are consistent with TN-C listed as a component of the "lung metastasis gene-expression signature," which identifies genes mediating experimental breast cancer metastasis selectively to the lung and associate with increased risk of lung metastases in human breast cancer patients [49, 50].

Intriguingly, TN-C knockout mice have reduced inflammatory responses [51]. Chronic inflammation associated with arthritis induces TN-C expression, which activates toll-like receptor 4 (TLR-4) signaling in fibroblasts and myeloid cells resulting in enhanced cytokine secretion, creating a positive feedback loop for upregulation of TN-C [52]. Given these findings, TN-C upregulation in the stromal microenvironment may enhance tumor associated inflammation and fibroblast secretion of tumor promoting, pro-inflammatory cytokines.

Inflammatory biomarkers

Given the striking parallel between tumor progression and wound healing, it is not surprising that chronically inflamed/wounded tissues are more likely to instigate the development of carcinomas, as demonstrated by *H. pylori* induced gastritis [53], inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [54], and obesity-induced hepatosteatosis [55]. Interestingly, adaptive immunity may not only initiate the formation of carcinomas [56–58], but can also drive their progression to more invasive, poorly differentiated phenotypes. In fact, recent studies have demonstrated that inflammation can drive the progression of breast tumors despite any known breast specific inflammatory conditions that predispose this tissue to tumorigenesis. These inflammatory tumor promoting mechanisms driving breast cancer progression result from the stromal recruitment of $CD4^+$ cells [59], regulatory T cells [60], $Gr1^+CD11b^+$ myeloid cells [61], and type II macrophages [62]; together, these cells cultivate an activated microenvironment that supports breast cancer cell proliferation, growth, survival and even metastatic spread. The percentage of these inflammatory cells within human breast tissues can have significant prognostic value [63–65].

Leukocytes

CD45+ cells (leukocytes) are present in disease-free breast tissue, but breast cancer tissue contains a significantly higher percentage of these cells [66]. Specifically, activated T lymphocytes predominate in tumor tissue, while myeloid cells are more prevalent within disease-free breast tissue. These compositions reportedly shift in response to chemotherapy [67]. High lymphocytic infiltration is associated with increased survival in patients less than 40 years old with ductal and lobular invasive carcinoma; however this is not true for patients over 40 [68]. Moreover, the correlation between the degree of lymphocytic infiltration and patient prognosis may depend not only on the age of the patient, but also the hormone receptor status of the tumor. ERα+ tumors with a high degree of lymphocyte infiltration associate with shorter survival, whereas ERα− tumors with a high degree of lymphocyte infiltration associate with longer survival [69].

TAMs (Tumor associated macrophages)

Tumor associated macrophages (TAMs, $CD68^+$ cells) have been shown to represent up to 80 % of the total number of leukocytes present within late stage mouse mammary tumors [59]. In human breast tumors, infiltrating TAMs correlate with poor prognostic features [67, 70], higher tumor grade [71], and decreased disease-free survival [72, 73]. This may be, in part, due to increased angiogenesis, as shown in mouse models. For example, mice expressing polyoma middle T antigen (PyMT) under control of the mouse mammary tumor virus (MMTV) promoter develop aggressive mammary adenocarcinomas and lung metastases in a macrophage dependent manner, and this is in part due to macrophage mediated induction of the angiogenic switch [74]. Stromal derived molecules such as monocyte chemoattractant protein 1 (MCP-1) recruit TAMs, and MCP-1 blocking antibodies can significantly decrease macrophage infiltration, angiogenesis and mammary tumor growth in mice [75]. Moreover, studies have found the percentage of $CD68^+$ in human breast tumors correlates with increased microvessel density and vascular endothelial growth factor (VEGF) expression in the primary tumor [76, 77]. While CD68 is an accepted marker for human macrophages, recent reports suggest CD68 alone cannot accurately determine the number of macrophages present in human breast tissue [66] because it's expression is not solely limited to macrophages, but other stromal cell types

that do not necessarily express CD45, such as CAFs. Certain CD68 antibodies recognizing specific glycosylation sites within the protein may be more reliable when specifically analyzing macrophage populations [78], and may support the use of CD68 as a stromal biomarker and prognosis indicator in human breast cancers.

Cox-2 (Cyclooxygenase-2)

Cox-2, is an inducible enzyme upregulated during tissue inflammation and tumorigenesis [79]. Both Cox-2 and it's isoform, Cox-1, catalyze the synthesis of the pro-inflammatory hormone prostaglandin E2 (PGE2) via arachidonic acid, although Cox-1 is thought to be constitutively expressed in most tissues and likely responsible for producing the levels of prostaglandins required for normal tissue function [80]. Cox-2 has been implicated as a biomarker and prognostic factor in breast cancer. Cox-2 expression in human breast cancer correlates with overall decreased disease-free survival [81], Her-2 positive status [82, 83], higher grade [83], and hormone receptor negative status [82, 83]. It is also listed as part of the "lung metastasis gene-expression signature" mentioned previously [50].

In particular, a large amount of evidence implicates epithelial Cox-2 expression as a biomarker for the progression of early stage breast cancer to a more invasive, later stage disease [82, 84]. Recently, epithelial Cox-2 expression has been associated with the transition of ductal carcinoma in situ (DCIS) to invasive carcinoma in a xenograft mouse model of breast cancer. The upregulation of Cox-2 in hyperplastic cells increases expression of MMP14 and VEGF, contributing to the invasive phenotype [85]. These results correlate with previous reports regarding the correlation between tumor cell expression of Cox-2 and angiogenesis in both mouse models [86] and human breast cancer [87].

The studies in mice correlate well with the immunohistochemical analysis of human breast tumor tissue. In a recent study evaluating the use of biomarkers for DCIS patients treated with lumpectomy alone, DCIS lesions that were positive for Cox-2, p16, and Ki67 were statistically significantly associated with subsequent invasive cancer [88]. With the exception of p16, both Cox-2 and Ki67 overexpression may predict the risk of atypical hyperplasia, a precursor stage to DCIS, to development of breast cancer in the first 10 years after diagnosis [89].

While the aforementioned studies implicate epithelial Cox-2 expression in breast cancer progression, some evidence suggests that stromal Cox-2 expression may also contribute to progression of disease. Yang et al. [90] demonstrated that stromal Cox-2 expression specifically was differentially expressed in individuals with high mammographic density, a well-established and large risk factor for development of breast cancer. It is known that stromal fibroblasts express Cox-2 and this contributes to their tumor promoting capabilities [21, 91, 92]. In addition, we recently reported that fibroblast tumor promoting ability could be attributed to the production and secretion of PGE2 [21]. These findings could have important clinical applications for breast cancer patients whose desmoplastic stroma is strongly immunoreactive for Cox-2 expression. Such patients may benefit greatly from an adjuvant therapy targeting both the tumor cells as well as tumor promoting fibroblasts. Although the use of Cox-2 inhibitors as anticancer agents and remedies for inflammatory based diseases reportedly have had deleterious side effects in patients [79, 93], new generations of non-steroidal anti-inflammatory drugs (NSAIDs) may have promising results in the treatment of breast cancer.

Matrix associated biomarkers

Cells within tissues are continuously exposed to physical forces including hydrostatic pressure, shear stress, compression forces and tension forces. The nature of these forces change dramatically during cancer progression [94]. Breast epithelial cells are exposed to isometric force or tension generated by cell–cell or cell–ECM interactions, both of which modulate cell behavior and tissue organization [95]. It is known that ECM attachment regulates growth, differentiation and the tumorigenic capacity of breast epithelial cells, likely by modulating biochemical and biomechanical signaling events; interruption with specific ECM attachments significantly impairs mammary tumor growth. Pioneering studies by Weaver et al. [96] demonstrated transformed human mammary epithelial cells treated with a $\beta 1$ integrin (receptor for ECM) blocking antibody form significantly smaller tumors in vivo and re-assemble normal mammary gland architecture in a 3D culture model. These studies highlight the importance of ECM composition and signaling in mediating the malignant phenotype.

COL1

The desmoplastic stroma associated with breast tumors is significantly stiffer than that of the disease-free breast [97], which is likely why most breast tumors are detected as a stiff palpable mass [98]. This stiffening is, in part, due to altered type I collagen deposition, cross-linking and cleavage. Crossing Col1a1$^{tm/ae}$ transgenic mice (which carry mutations near the highly conserved MMP cleave site for type I collagen, resulting in a significant imbalance in collagen homeostasis) to MMTV-PyMT mice results in

significantly reduced tumor latency, more invasive tumors with a significant increase in lung metastases [99]. These studies highlight the importance of collagen signaling in breast cancer progression.

LOX (Lysyl oxidase)

LOX is a copper dependent amine oxidase that catalyzes the covalent cross-linking of collagens and elastin in the microenvironment, which dictates the mechanical properties of the ECM. Breast cancer patients with high LOX expression have reduced overall survival [100]. LOX expression is upregulated in invasive breast cancer cell lines compared to poorly invasive ones [101], and the expression of LOX in breast cancer cells is required for metastasis formation in an orthotypic mouse model of breast cancer, as well as a tail vein lung metastasis colonization model [100].

While LOX is expressed in breast tumor cells, Peyrol et al. [102] report the highest levels of LOX expression were observed in CAFs and myoepithelial cells surrounding DCIS and the desmoplastic stroma facing the invasive front of breast tumors. Mouse mammary fat pads that are cleared and pre-conditioned with human fibroblasts [103] overexpressing LOX produce stiffer matrices due to increased deposition of fibrillar collagen, and promote the growth and invasion of a pre-malignant human mammary epithelial cell line. Interestingly, treating a cohort of these mice with a LOX inhibitor at the time of tumor cell inoculation (2 weeks after pre-conditioning of the gland) does not alter mammary tumor growth, suggesting the direct effect of LOX activity in breast cancer progression may be solely through its ability to alter ECM stiffness, indirectly affecting epithelial cell behavior and tumorigenic potential [104].

LOXL2 (Lysyl-oxidase like 2)

LOXL2, another family member of LOX proteins, has also been reported to localize to desmoplastic stroma of DCIS lesions [105], and its expression is enriched in areas positive for SMA and collagen I expression, with limited expression in disease-free breast tissue [106]. Using xenograft mouse models to study both LOX and LOXL2 upregulation during tumor desmoplasia have been difficult, given that most xenograft mouse models lack the associated host stromal response to the same extents as human breast cancers. In a compelling recent study, Barry-Hamilton et al. [106] utilized a breast cancer cell line and tumor transplantation model to generate desmosplasia similar to that seen of human breast tumors. After tumors were established, treatment with a monoclonal antibody to LOXL2 significantly attenuated tumor desmoplasia, reduced the number of both αSMA positive and CD31 positive cells, and reduced both primary tumor growth and metastatic spread. Collectively, these studies in mice highlight the promise of both LOX and LOXL2 as potential stromal biomarkers in breast cancer. Clinical studies correlating stromal LOX and LOXL2 expression with patient prognosis warrant further investigation.

Gene expression/microRNA associated biomarkers

Serial analysis of gene expression (SAGE) and single nucleotide polymorphism (SNP) analyses reveal the most dramatic and consistent modifications in gene expression occur within the fibroblast and myoepithelial cell fractions sorted from primary human breast tumors [10]. These stromal changes in gene expression are likely the result of epigenetic modifications; strong evidence supporting the notion that breast tumor stroma is genetically unstable is lacking [107, 108]. Epigenetic reprogramming of tumor associated stromal cells (CAFs in particular) may be mediated by histone modifications, DNA methyltransferases, chromatin modifying factors, and microRNAs [107, 109, 110]; however, the precise molecular mechanisms demonstrating such reprogramming remain to be determined.

Methylated genes

One of the first observations suggesting that CAFs may be epigenetically modified came from explanting CAFs isolated from digested human breast tumor tissues. These resected CAFs retain their tumor promoting phenotype when they are explanted in vitro and devoid of tumor cell secreted factors [15, 18, 21, 111]. These data suggest that CAFs permanently retain a memory of co-evolution with tumor cells possibly over several decades, inferring they may have permanently remodeled chromatin and epigenetic marks. This hypothesis is supported by epigenome profiling data, demonstrating that CAFs isolated from human breast tumors have differences in DNA methylation compared to fibroblasts isolated from reduction mammoplasty tissues [10], and CAFs isolated from human gastric carcinomas have globally reduced DNA methylation compared to normal gastric myofibroblasts [112].

Although the precise mechanisms demonstrating how these epigenetic alterations sustain and contribute to CAF phenotype are lacking, some may be inferred from studies using fibrotic fibroblasts. Fibrotic fibroblasts share several characteristics of both myofibroblasts and CAFs: both are "activated" fibroblast states that are retained in vitro, characterized by enhanced matrix deposition, αSMA expression, and induced by transforming growth factor beta-1 (TGFβ1) [19, 113–115]. A study by Bechtel et al.

[116] showed that fibrotic fibroblasts have 12 methylated genes that are non-methylated in disease-free fibroblasts. Hypermethylation of one of these genes in particular, the *RASAL1* gene, is associated with the fibrotic fibroblast state, and is regulated by TGFβ.

Stromal tumor suppressor genes

Genetic mouse models that use a fibroblast specific promoter (FSP-Cre) to ablate genes of interest in fibroblasts have implicated both TGFβ and phosphatidylinositol 3-kinase (PI3K) signaling as mechanisms by which stromal fibroblasts suppress breast cancer initiation and progression. Co-implantation of 4T1 mouse mammary tumor cells with fibroblasts that lack expression of the TGFβ type II receptor ($Tgfbr2^{fspKO}$ fibroblasts) under the renal kidney capsules of nude mice results in larger, highly vascular primary tumors and increased formation of spleen, liver and lung metastases [117]. Fibroblast mediated tumor suppression by autocrine TGFβ signaling is not specific to mammary fibroblasts, as the $Tgfbr2^{fspKO}$ mice form intraepithelial neoplasias in the prostate and invasive squamous cell carcinomas of the forestomach [118].

Utilizing mouse mammary fat pad transplantation techniques to stringently determine the effect of mammary fibroblast specific loss of the tumor suppressor gene *Pten*, Trimboli et al. [119] showed that *ErbB2* driven mammary tumor incidence was significantly enhanced upon stromal *Pten* deletion. These tumors had extensive ECM remodeling and immune cell infiltration, and increased angiogenesis. Intriguingly, immunohistochemical analysis for PTEN expression in patients with invasive breast carcinoma showed absent or low expression in half of the tissue samples examined [119], although it remains unclear whether this reduction in PTEN expression was specific to the tumor stroma.

microRNAs

microRNAs are a class of short noncoding regulatory RNAs that are involved in stem cell maintenance, developmental programming and cell fate specification, as well as various disease pathogeneses [120–123]. Their altered expression (both in tumor stroma as well as tumor cells) has been implicated in several types of cancers, including breast cancer [124–129]. However, the contribution of specific microRNAs to tumor desmoplasia and CAF phenotype remains largely unknown. However, studies involving fibrotic fibroblasts have elucidated specific microRNAs that govern this activated fibroblast state, and provide speculation for similar regulatory mechanisms in CAFs. In particular, miR21 is induced by TGFβ in myofibroblasts, fibrotic fibroblasts and vascular smooth muscle cells, all of which have similar gene expression profiles and contractile properties [130–133]. In vivo, miR21 contributes to fibrotic disease in mouse models of idiopathic pulmonary fibrosis and myocardial disease [131, 132]. Because miR21 is overexpressed in both tumor cells and breast tumor stroma [125], future studies should address if attenuation of miR21 function suppresses not only tumor cell growth and invasion [134–136], but also attenuates tumor desmoplasia and the presence of CAFs. Intriguingly, overexpression of miR21 in human breast tumors significantly correlates with dual overexpression of TGFβ and poor patient outcome [137].

Conclusions

The stromal contribution to the initiation and progression of breast tumors has been highly substantiated within the last decade. Stromal gene expression signatures can predict patient prognosis and response to neoadjuvant chemotherapy [12, 138], and tumor associated stroma serves as a potential drug target [29, 30, 139]. Identifying stromal-specific markers, including immune cell distinctions, ECM alterations, and gene expression changes in breast fibroblasts and adipocytes could not only significantly impact patient prognosis and treatment plans, but also implicate their use as novel targets for adjuvant therapy. In particular, CAFs may be an attractive target, given their genomic stability and their abundance in tumor associated stroma.

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