A phenotype from tumor stroma based on the expression of metalloproteases and their inhibitors, associated with prognosis in breast cancer

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Abbreviations: bFGF, fibroblast growth factor; CAF, cancer-associated fibroblast; CI, confidence interval; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; HER2, human epidermal growth factor receptor 2; HGF, hepatocyte growth factor; IGF, insulin growth factors; IGFBP, IGF binding protein; IL, interleukin; MIC, mononuclear inflammatory cell; MMP, matrix metalloprotease; NFκB, nuclear factor kappa B; PgR, progesterone receptor, TA, tissue array; TGFβ, transforming growth factor β; TIMP, tissue inhibitors of metalloproteases.

The objective of the present work was to evaluate the impact of the phenotype of both mononuclear inflammatory cells (MICs) and cancer-associated fibroblast (CAFs) in early breast cancer patients, specifically assessed as to their expression of MMP/TIMP relative to their position within the tumor (i.e., localization at the tumor center or invasive front) and the occurrence of distant metastases. An immunohistochemical study was performed using tissue arrays and specific antibodies against matrix metalloproteinase (MMP)−1, −2, −7, −9, −11, −13 and −14, tissue inhibitors of metalloproteinase (TIMP)−1, −2 and −3, both at tumor center and at invasive front, in 107 patients with primary ductal invasive breast tumors. Data were analyzed by unsupervised hierarchical clustering analysis. Our results indicated that MMP-11 expression by MICs, and TIMP-2 expression by CAFs at either the tumor center or the invasive front, were the most potent independent prognostic factors for predicting the clinical outcome of patients. Using the unsupervised hierarchical clustering analysis, we found well-defined clusters of cases identifying subgroups of tumors showing a high molecular profile of MMPs/TIMPs expression by stromal cells (CAFs and MICs), both at the tumor center and at the invasive front, which were strongly associated with a higher prevalence of distant metastasis. In addition, we found combinations of these clusters defining subpopulations of breast carcinomas differing widely in their clinical outcome. The results presented here identify biologic markers useful to categorize patients into different subgroups based on their tumor stroma, which may contribute to improved understanding of the prognosis of breast cancer patients.

Introduction

Breast cancer is the most common malignancy in women. Despite early diagnosis, surgery, and adjuvant therapy, a considerable number of patients experience recurrence with metastatic disease. Recent advances in whole-genome technologies have generated an overwhelming amount of molecular data regarding mammary carcinoma.¹ However, in spite of all of these data, breast cancer is currently treated on the basis of the status of only 3 clinical markers of cancer cells: estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). This fact reflects the complexity and heterogeneity of breast cancer biology and is the principal reason for which a personalized treatment approach remains distant. Thus, we consider the need for finding a new approach to elucidate the mechanisms intimately involved in breast cancer progression.

Tumor initiation is typically conceptualized as the accumulation of genetic and epigenetic mutations in the epithelium that results in the recruitment of reactive stroma. Similar to other solid tumors, breast carcinomas are composed by 2 distinct compartments: the parenchyma and the stroma. There are several lines of evidence indicating that progression of tumors toward a malignant phenotype does not depend exclusively on the cell-autonomous properties of cancer cells themselves but is also deeply influenced by tumor stroma reactivity.² Two well-studied cellular component of the tumor stroma are mononuclear inflammatory cells (MICs) and cancer-associated fibroblasts (CAFs), both of which may be present within the invasive front or...
infiltrate the tumor center. In this context, we have found that different subpopulations of MICs and CAFs—characterized on the basis of expression of matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs)—exist both at the tumor center and invasive front. These distinct stromal subsets display unique prognostic significance, such that breast carcinomas containing either MICs or CAFs cells with a molecular profile of high MMPs/TIMPs expression had a high rate of development of distant metastases as compared with those tumors with a low expression profile. These are relevant findings considering that MMPs are governed by the tumor stroma and exert powerful influences on the local tissue microenvironment during tumorigenesis and progression. These enzymes are able to impact tumor cell behavior in vivo by several means: i) direct degradation of the stromal connective tissue and basement membrane components, favoring invasion and metastasis of cancer cells; ii) cleavage of membrane-bound growth factors or cytokines as well as their receptors; iii) cleavage of pro-apoptotic factors and induction of a more aggressive phenotype via generation of apoptotic resistant cells; iv) regulation of tumor angiogenesis, both positively through the ability of MMPs to mobilize or activate pro-angiogenic factors, or negatively via generation of angiogenesis inhibitors, such as angiostatin, endostatin and tumstatin, cleaved from large protein precursors; or v) cleavage of cell adhesion molecules, such as cadherins, leading to an increased cell motility occurring in epithelial mesenchymal transition (EMT). On the other hand, the activity of MMPs is specifically inhibited by TIMPs, but it is now assumed that TIMPs are multifactorial proteins also involved in the induction of proliferation and the inhibition of apoptosis.

The objective of the present work is to evaluate the impact of total MICs and CAFs in a population of early breast cancer patients by phenotypic characterization and assessment of their MMPs/TIMPs molecular profile at the tumor center and invasive front in relation to the occurrence of distant metastases. A total of 10 MMPs and TIMPs were analyzed using immunohistochemistry and tissue array (TA) techniques, and the data were analyzed by unsupervised hierarchical cluster analysis by each cellular type and by each tumor location. We found clusters of stromal cell phenotypes in various combinations capable of defining subpopulations of breast carcinomas differing widely in their clinical outcome.

Results

In order to characterize to expression of tumor-associated metalloproteases and their inhibitors, we performed more than 8,000 determinations in arrays of cancer specimens from 107 patients (10 protein expressions in 107 tumors, 2 areas by tumor, 2 cores by tumor area, and 2 fields by core) with primary invasive ductal carcinoma of the breast.

The majority of MMPs and TIMPs were mainly expressed by cancer cells, both at the tumor center (MMP-1: 86.0% of tumors; MMP-2: 32.7%; MMP-7: 58.9%; MMP-9: 77.6%; MMP-11: 86.9%; MMP-13: 77.6%; MMP-14: 91.6%; TIMP-1: 94.4%; TIMP-2: 84.1%; TIMP-3: 86.9%) and at the invasive front (MMP-1: 96.3% of the tumors; MMP-2: 39.3%; MMP-7: 83.7%; MMP-9: 95.2%; MMP-11: 97.1%; MMP-13: 76.7%; MMP-14: 90.2%; TIMP-1: 96.2%; TIMP-2: 93.1%; TIMP-3: 58.7%) in breast carcinomas. However, these proteins were also expressed by stromal cells in a significant percentage of tumors.

Figure 1 shows representative examples of CAFs and MICs expressing MMPs and TIMPs localized to the tumor center and the invasive front in breast carcinomas. Immunostaining for these proteins revealed a cytoplasmic location in cancer cells and tumor-associated stromal cells, including both CAFs and MICs. In neoplasms positive for CAFs and MICs expressing either MMPs or TIMPs, at least 70% of these cells showed a positive immunostaining of each evaluated field.

To confirm the expression of these proteins by each stromal cell type, we performed double-immunostaining in the tissue sections using antibodies specific for MMPs/TIMPs and specific markers (CD45 and α smooth muscle actin [α-SMA], respectively) to identify MICs or CAFs in the tumor samples (Fig. 2).

Considering the clinical importance of the stromal phenotypes according to the expression of MMPs and TIMPs, and since there is a practical interest in providing easy prognostic information for clinical use, we prospectively analyzed the possible prognostic value of all of these stromal biological markers. We found that stromal expression of several MMPs and TIMPs, at various tumor locations were associated with prognosis (Table S1). Of these, MMP-11 expression by MICs and TIMP-2 expression by CAFs (either at the tumor center or at the invasive front) were the most potent independent prognostic factors for predicting the clinical outcome. As shown in Figure 3, the expression of these proteins was related to a higher probability of recurrence in the form of distant metastases.

To identify specific groups of tumors with distinct MMP/TIMP immunohistochemical expression profiles, the obtained data were subsequently evaluated by unsupervised hierarchical clustering analysis for each cell type. This algorithm places proteins on the horizontal axis and samples on the vertical axis based on similarity of their expression profile, and if appropriate, generates dendrograms with well-defined clusters of cases for each cellular type (Fig. 4). In the case of CAFs, the dendrogram shows a first-order division of the tumors into 2 distinct MMP/TIMP molecular profiles, both at the tumor center (designated group F1A (n = 64) and group F1B (n = 43) and at the invasive front (designated group F2A (n = 51) and group F2B (n = 56) (Fig. 4A and 4B, respectively). Among MICs, we also found a first-order division of tumors into 2 distinct MMP/TIMP molecular profiles at the tumor center (designated group M1A (n = 59) and group M1B (n = 48) (Fig. 4C). However, we identified 3 distinct groups for MICs at the invasive front with high, intermediate, and low MMP/TIMP profiles (designated group M2A = 23), group M2B = 57); and group M2C (n = 27), respectively) (Fig. 4D). Nevertheless, this method did not produce a dendrogram with well-defined clusters of cases according to MMP/TIMP profiles among cancer cells, either at the tumor center or at the invasive front (Figure S1).

Table 2 shows MMPs and TIMPs expressions in the different clusters of breast carcinomas with distinct MMP/TIMP
molecular profiles in CAFs and in MICs located at either the tumor center or the invasive front. As shown in this table, MMP−1, −11, −13, −14 and TIMP−1 and −2, were identified as the most frequently expressed factors in the groups of tumors with high MMPs/TIMPs molecular profiles relative to those with low expression profiles.

We also studied the possible relationship between these cluster subgroups and patient outcome, and found that the cluster subgroups with high MMP/TIMP molecular profiles, either CAFs or MICs, located at both the tumor center and the invasive front, were associated with both poor relapse-free and overall survival (Figs. 5A-D). Also, we further investigated the possible impact of the different combination of these molecular MMP/TIMP profiles on prognosis. We found that among our patient data, there was a subgroup of tumors (n = 23) showing the highest molecular profile of MMPs/TIMPs expression by stromal cells (both CAFs and MICs) in both tumor locations, which were strongly associated with recurrence in the form of distant metastases (P < 0.001; Fig. 5E). On the contrary, tumors displaying the lowest MMPs/TIMPs profile (n = 11) had an excellent clinical outcome, whereas the third subgroup (n = 73) showed an intermediate molecular profile and an intermediate clinical outcome (Fig. 5E).

Regarding the possible biological relevance of these combinations of cluster subgroups (low, intermediate and high molecular

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**Figure 1.** Human mammary carcinomas contain tumor stromal cells expressing metalloproteases and their inhibitors. Representative pictures of mammary cancer patient tissue array immunostaining for the different matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) analyzed in breast cancer patients (200X), both at tumor center and at invasive front. (A) MMP-1, (B) MMP-2, (C) MMP-7, (D) MMP-9, (E) MMP-11, (F) MMP-13, (G) MMP-14, (H) TIMP-1, (I) TIMP-2 and (J) TIMP-3.
profile of MMP/TIMP expression in CAFs and MICs, at the tumor center and the invasive front, we investigated their possible relation with the clinicopathological characteristics from patients and tumors. Our data demonstrated no significant associations between patients or tumors characteristics and MMPs/TIMPs molecular profile (Table S2).

Cox's multivariate analysis demonstrated that histological grade (grade II: (relative risk (RR) (confidence interval (CI)) = 1.6 (0.8–3.0); Grade III: 3.8 (1.9–7.6) was the only clinical characteristic significantly and independently associated with distant relapse-free survival. Moreover, the combination of cluster groups of stromal cells at the tumor center and at the invasive front (intermediate expression profile: 2.9 (0.7–12.1); high expression profile: 10.5 (2.4–46)) was the most potent predictive factor significantly and independently associated with distant relapse-free survival.

**Discussion**

Here, we have found that tumor stroma phenotype varies in concordance with the expression of certain MMPs and TIMPs by CAFs and MICs, both in tumor center and in the invasive front. These findings led us to identify different subgroups of tumors differing widely in their stromal phenotype and in regards to patient prognosis. There was a subgroup of tumors showing a stromal molecular profile of abundant MMP/TIMP expression in each one of these tumor locations, which was strongly associated with higher recurrence of distant metastases. By contrary, tumors with stromal phenotypes displaying low molecular profiles had an excellent clinical outcome. Thus, these findings suggest a diabolic link between stromal cell phenotypes and disease recurrence, with stromal cells appearing along the tumor locations that ultimately may promote the escape of cancer cells from the primary tumor and favor metastatic spread in breast cancer patients. Although cancer cells also express high levels of MMPs and TIMPs, it seems that the stromal expression of these proteins is particularly crucial to tumor progression. Indeed, mammary cancer cells expressed proteins involved in extracellular matrix degradation, however the response of the stroma appears to depend on their crosstalk with cancer cells, and this reciprocal communication is what opens the door to tumor cell invasion and the development of metastatic disease.

Our results are in agreement with prior studies reporting that stromal characteristics may dictate tumor outcome in

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**Table 1.** Basal demographics and clinical characteristics of 107 patients with invasive ductal carcinoma of the breast included in our study.

| CHARACTERISTICS             | Without recurrence | With recurrence |
|----------------------------|--------------------|-----------------|
| Total cases                | 45 (100)           | 62 (100)        |
| Menopausal status          |                    |                 |
| Premenopausal              | 17 (37.8)          | 16 (25.8)       |
| Postmenopausal             | 28 (62.2)          | 46 (74.2)       |
| Tumor size                 |                    |                 |
| T1                         | 25 (55.6)          | 27 (43.5)       |
| T2                         | 20 (44.4)          | 35 (56.5)       |
| Nodal status               |                    |                 |
| N (-)                      | 25 (55.6)          | 27 (43.5)       |
| N (+)                      | 20 (44.4)          | 35 (56.5)       |
| Histological grade         |                    |                 |
| Well Dif. (I)              | 19 (42.2)          | 14 (22.6)       |
| Mod. Dif. (II)             | 22 (48.9)          | 27 (43.5)       |
| Poorly Dif. (III)          | 4 (8.9)            | 21 (33.9)       |
| Estrogen Receptor          |                    |                 |
| Negative                   | 17 (37.8)          | 35 (56.5)       |
| Positive                   | 28 (62.2)          | 27 (43.5)       |
| Progesterone Receptor      |                    |                 |
| Negative                   | 21 (46.7)          | 40 (64.5)       |
| Positive                   | 24 (53.3)          | 22 (35.5)       |
| Adjuvant radiotherapy      |                    |                 |
| No                         | 33 (73.3)          | 33 (53.2)       |
| Yes                        | 12 (26.7)          | 29 (46.8)       |
| Adjuvant systemic therapy  |                    |                 |
| Chemotherapy               | 15 (33.3)          | 27 (43.5)       |
| Tamoxifen                  | 18 (40.0)          | 14 (22.6)       |
| Chemotherapy + Tamoxifen   | 8 (17.8)           | 8 (12.9)        |
| No treatment               | 4 (8.9)            | 13 (21)         |
| HER2 Status                |                    |                 |
| Negative                   | 38 (84.4)          | 52 (83.9)       |
| Positive                   | 7 (15.6)           | 10 (16.1)       |
| Basal-like phenotype       |                    |                 |
| Non basal-like             | 33 (73.3)          | 41 (66.1)       |
| Basal-like                 | 12 (26.7)          | 21 (33.9)       |
many cases of breast cancer. Normal fibroblasts are the most abundant cell type in the connective tissue and are responsible for the synthesis and turnover of the extracellular matrix. However, CAFs are different from normal fibroblasts, including a more rapid proliferation rate, as well as several actions promoting tumor phenotypes such as survival, proliferation, general metabolic reprogramming, angiogenic shift, ECM remodeling, EMT activation, the acquisition of stem cell traits, metabolic reprogramming toward a reverse Warburg phenotype, or inflammatory cell recruitment. All of these actions are due to the fact that CAFs produce a repertoire of growth factors and cytokines that influence the behavior of the epithelium, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin growth factors (IGFs), IGF binding protein (IGFBPs), fibroblast growth factor (bFGF) or transforming growth factor β (TGFβ). In addition, there are data indicating that CAFs contribute to both chemotherapy and endocrine resistance. It is also known that CAFs are capable of evoking a proinflammatory response. After activation, CAFs initiate a pro-inflammatory response including the secretion/expression of interleukin (IL)-1β, IL-6, IL-8, the chemokine SDF-1/CXCL12, and nuclear factor kappa (NF-kB), which may induce inflammation by recruiting components of the immune system. Thus, CAFs may orchestrate a distorted architecture of the host tissue and a functional “corrupted” stroma which in turn helps the metastatic spread. Nevertheless, these CAFs are heterogeneous populations and their relative composition greatly differs among tumors. The origin of CAFs is matter of debate and many origins have been proposed, such as resident normal fibroblasts, derivation from mesenchymal stem cells or transdifferentiation of epithelial and endothelial cells. Possibly due to divergent origins, there is marked heterogeneity in marker expression among CAFs subpopulations, or in CAFs originating from different tumors. We consider that our results identify biological markers of CAFs, which confer tumor aggressiveness and may help define functionally distinct fibroblast-like cell populations in breast carcinomas.

Immune cell infiltration within a solid tumor is a naturally occurring event, which can sometimes lead to blocking of cancer progression and thus limit or even prevent the generation of metastasis. In general, this occurs in early stages of tumor progression as a result of host immunosurveillance. However, unfortunately in many cases, the host is unable to generate sufficient antitumor immune responses due to the immunosuppressive abilities of tumors that circumvent or dampen immunity, thus leading to tumor escape from host immunosurveillance. Additionally, it remains a distinct possibility that MICs favor tumor progression. Accumulating evidences indicate that diverse immune cells exert pro-tumor functions, as they secrete cytokines, growth factors, chemokines and proteases that stimulate angiogenesis as well as proliferation, migration and invasive potential of cancer cells. In this sense, the data from our present study, similarly to our
previous reports, indicate the existence of a phenotype of MICs showing a high molecular profile of MMPs/TIMPs in the tumor center that is associated with a high metastatic rate. These results suggest that leukocytes from peripheral blood undergo phenotypic modification to infiltrate tumors from the invasive front to the tumor center. This seems to be a dynamic process in which inflammatory cells and immunomodulatory mediators present in the tumor microenvironment polarize

Table 2. Expression of MMPs and TIMPs in 3 different cluster groups resulting from the combination of stromal cells in the tumor center and in the invasive front. Data are expressed as number of positive cases (%).

| Low MMP/TIMP molecular profile by CAFs and MIC at TC and IF (N = 11) | Intermediate MMP/TIMP molecular profile by CAFs and MIC at TC and IF (N = 73) | High MMP/TIMP molecular profile by CAFs and MIC at TC and IF (N = 23) |
|---|---|---|
| **CAFs** | **MICs** | **CAFs** | **MICs** | **CAFs** | **MICs** | **CAFs** | **MICs** |
| MMP-1 | 8 (72.7) | 7 (63.6) | 3 (27.3) | 0 (0) | 54 (74.0) | 44 (60.3) | 67 (91.8) | 55 (75.3) | 23 (100) | 19 (82.6) | 23 (100) | 22 (95.7) |
| MMP-2 | 3 (27.3) | 1 (9.1) | 1 (9.1) | 0 (0) | 15 (20.5) | 1 (1.4) | 6 (8.2) | 1 (1.4) | 9 (39.1) | 0 (0) | 3 (13.0) | 0 (0) |
| MMP-7 | 8 (72.7) | 6 (54.5) | 2 (18.2) | 0 (0) | 47 (64.4) | 29 (39.7) | 14 (19.2) | 12 (16.4) | 20 (87.0) | 18 (78.3) | 9 (39.1) | 6 (26.1) |
| MMP-9 | 0 (0) | 0 (0) | 2 (18.2) | 0 (0) | 9 (12.3) | 7 (9.6) | 23 (31.5) | 16 (21.9) | 8 (34.8) | 5 (21.7) | 14 (60.9) | 9 (39.1) |
| MMP-11 | 5 (45.5) | 0 (0) | 6 (54.5) | 3 (27.3) | 47 (64.4) | 16 (21.9) | 50 (68.5) | 32 (43.8) | 22 (95.7) | 18 (78.3) | 20 (87.0) | 21 (91.3) |
| MMP-13 | 3 (27.3) | 1 (9.1) | 2 (18.2) | 0 (0) | 35 (47.9) | 19 (26.0) | 29 (39.7) | 16 (21.9) | 19 (82.6) | 17 (73.9) | 16 (69.6) | 11 (47.8) |
| MMP-14 | 9 (81.8) | 1 (9.1) | 1 (9.1) | 1 (9.1) | 56 (76.7) | 34 (46.6) | 28 (38.4) | 16 (21.9) | 23 (100) | 23 (100) | 19 (82.6) | 12 (52.2) |
| TIMP-1 | 8 (72.7) | 2 (18.2) | 1 (9.1) | 0 (0) | 26 (35.6) | 14 (19.2) | 28 (38.4) | 23 (31.5) | 18 (78.3) | 13 (56.5) | 19 (82.6) | 16 (69.6) |
| TIMP-2 | 3 (27.3) | 3 (27.3) | 1 (9.1) | 1 (9.1) | 25 (34.2) | 22 (30.1) | 26 (35.6) | 41 (56.2) | 18 (78.3) | 16 (69.6) | 16 (69.6) | 18 (78.3) |
| TIMP-3 | 4 (36.4) | 4 (36.4) | 1 (9.1) | 0(0) | 42 (57.5) | 36 (49.3) | 5 (6.8) | 4 (5.5) | 20 (87.0) | 17 (73.9) | 4 (17.4) | 6 (26.1) |

CAF, cancer-associated fibroblasts; IF, invasive front; MIC: mononuclear inflammatory cell; MMP, matrix metalloprotease; TC: tumor center; TIMP, tissue inhibitors of metalloproteases.

*P < 0.05 refers to low vs. intermediate and high molecular profile.

**P < 0.001 refers to low vs. intermediate and high molecular profile.

Figure 4. Clustering analysis of human mammary carcinoma patient expression of metalloproteases and their inhibitors. Hierarchical clustering analysis of global expression patterns of matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) among different cells types present in breast tumor specimens (n=107) as measured by immunohistochemistry on tissue microarrays from the tumor center and the invasive front. Graphical representation of hierarchical clustering results in cancer-associated fibroblasts (A, B) and mononuclear inflammatory cells (C, D). Rows: tumor samples; columns: MMPs/TIMPs. Protein expressions are depicted according to a color scale: red, positive staining; green, negative staining; gray, missing data. Two major clusters of tumors (F1A and F1B) were shown in fibroblast-like cells both in the tumor center (A), and (F2A and F2B) in the invasive front (B). For mononuclear inflammatory cells (MICs), we found 2 major cluster of tumors (M1A and M1B) in the tumor center (C), whereas 3 distinct clusters of tumors (M2A, M2B and M2C) were found at the invasive front (D).
host immune response toward specific phenotypes impacting on tumor progression.

Our results demonstrate the biological heterogeneity among tumors with regard to the molecular profile of MMPs/TIMPs expression by stromal cells, both at the tumor center and at the invasive front. In order to improve clinical prognosis on the basis of stromal functionally, we investigated the outcome impact of expression of each MMPs/TIMPs by each key stromal cellular type and in each tumor location. Thus, we identified several biological markers with strong statistical impact on both relapse-free and overall survival, such as MIC expression of MMP-11 and CAF expression of TIMP-2 at either the tumor center or at the invasive front.

**Figure 5.** Survival analysis of human mammary carcinoma patients stratified according to expression of metalloproteases and their inhibitors. Kaplan-Meier survival curves (relapse-free survival and overall survival) as a function of the 2 major clusters of tumors (high and low profile of matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) expression) in fibroblast-like cells at the tumor center (A) and at the invasive front (B), or in mononuclear inflammatory cells (MICs) at the tumor center (C) or at the invasive front (D). Survival curves as a function of the combination of the different cluster subgroups both in fibroblast cells and MICs, in tumor center and at the invasive front (E).
invasive front. The finding that MICs express relatively abundant MMP-11 (stromalysin-3) is a strong indicator of metastatic rate, in accordance with our previous reports, an expression previously reported to be associated with a high inflammatory molecular profile (highest intratumoral levels of IL-1, IL-5, IL-6, IL-17, IFNβ and NFKβ) in breast carcinomas. An interesting question is how both CAFs and MICs maintain their defined functional state over a prolonged period of time. At least, 3 distinct mechanisms are possible: (i) genetic mutations, such as reported in studies describing p53 and PTEN mutations in the stromal compartment of breast cancer patients, although other studies indicate that the stromal population is genetically stable in breast cancer; (ii) epigenetic alterations, or persistent environmental effects due to the presence of signals originating from cancer cells. Therefore, we consider that our findings show different functional stages in the tumor-associated stromal cellular components that influence disease outcome and may be an excellent platform to assess the nature of these signals. The unsolved question is, if the tumor stromal cells merely respond to signals from the carcinoma cells or respond to autocrine signals to modulate tumor progression. Regardless, in the present study, we identify phenotypes of the more frequent stromal cells broadly influencing tumor prognosis. Therefore, this finding is of great clinical interest and also suggests that these populations of host stromal cells could be possible targets for inhibition of tumor progression and metastasis. This is especially relevant if we consider that the stromal compartment surrounding the tumors is generally considered as genetically stable compared to the rapidly dividing carcinoma cells. In addition, there are many pieces of evidence indicating that the tumor microenvironment is fertile ground for the development of novel therapies with the potential to augment existing treatment and prevention options. In fact, some new potential drugs targeting stromal cells, either CAFs or tumor-associated macrophages occurring in breast cancer, have already been developed and are under investigation in pre-clinical and clinical trials.

Patient selection
This study comprised 107 women with a histologically confirmed diagnosis of early invasive breast cancer of ductal-type treated between 1990 and 2001, some of which were previously included in our preliminary studies on the expression of MMPs and TIMPs in breast cancer. We selected women with the following inclusion criteria: invasive ductal carcinoma, at least 6 histopathologically-assessed axillary lymph nodes, and a minimum of 10 years of follow-up in those women without tumor recurrence. The exclusion criteria were the following: metastatic disease at presentation, prior history of any type of malignant tumor, bilateral breast cancer at presentation, having received any type of neoadjuvant therapy, development of loco-regional recurrence during the follow-up period, development of a second primary cancer, and absence of sufficient tissue in the paraffin blocks used for manufacturing the tissue arrays. Randomly selected a sample size of 107 patients, in accordance to 4 different groups of similar size and stratified with regard to nodal status and the development of metastatic disease, which were key variables in our study. Thus, we include a sufficient number of both node-negative and node-positive patients to comprise each subgroup among patients without and with disease recurrence for securing the statistical power of the survival analysis. Note that approximately half of the cases with distant metastasis during the follow-up period occurred in each of the node-negative and node-positive subgroups. Patients’ characteristics included in the 2 main groups, with or without distant metastases (recurrence), are listed in Table 1. Patients underwent either modified radical mastectomy or wide resection with axillary lymphadenectomy. Postoperative radiotherapy was given to 41 patients (38.3%). Data about the criteria for systemic adjuvant therapy of the patients were described elsewhere. Overall, 42 patients received chemotherapy, 32 patients received tamoxifen, and 16 patients received both types of systemic therapy.

The median follow-up period in patients without metastasis was of 187 months, and 52 months in patients with metastatic disease. The study adhered to national regulations and was approved by our Institution’s Ethics and Investigation Committee.

Tissue arrays
Routinely fixed (overnight in 10% buffered formalin), paraffin embedded tumor samples stored in our pathology laboratory files were used in this study. TAs blocks were obtained by punching a tissue cylinder (core) with a diameter of 1.5 mm through a histologically representative area of each ‘donor’ tumor block, which was then inserted into an empty ‘recipient’ tissue array paraffin block using a manual tissue arrayer (Beecker Instruments, Sun Praerie, Winconsin, USA) as described elsewhere. A total of 4 cores were employed for each case. Two of these cores in each case corresponding to tumor center area, and another 2 cores corresponding to invasive front. This method, with 2 cores (double redundancy) of each tumor area has been shown to correlate well with conventional immunohistochemical staining. The invasive front was defined as the area within 2 mm surrounding the tumors and which contained cancerous cells. From the 107 tumor samples available, 8 tissue array blocks were prepared, each containing 32 tumor samples maximum.

Immunohistochemistry
Immunohistochemistry was carried out on TA sections 5 μm thick, fixed in 10% buffered formalin and embedded in paraffin...
using a TechMate TM50 autostainer (Dako, Glostrup, Denmark). Antibodies for MMPs and TIMPs were obtained from Neomarker (Lab Vision Corporation, Fremont, CA, USA). The dilution for each antibody was established based on negative and positive controls (1:50 for MMP-2, −7, −13 and −14; 1:100 for MMP-9, TIMP-1, −2 and −3; 1:200 for MMP-1; and 1:400 for MMP-11). To enhance antigen retrieval, tissue sections were treated in a PT-Link (Dako) at 97°C for 20 min in citrate buffer (pH 6.1) for MMP-1, −14, TIMP-1 and −3, or in ethylenediamine tetraacetic acid (EDTA) buffer (pH 9) for MMP-13 and TIMP-2. Antibodies against MMP-2, −7, −9 and −11 do not require antigen retrieval. The negative control was DakoCytomation mouse serum diluted at the same concentration as the primary antibody used. All dilutions were made in Antibody Diluent (Dako, Glostrup, Denmark) and incubated for 30 min to 2 h at room temperature. Breast tumor samples in which we confirmed the presence of the evaluated proteins by Western blot analysis, were used as positive controls, as described previously.3,5 Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently overslipped.

Double-immunostaining methods were performed with the BenchMark ULTRA Staining System (Ventana-Roche, Rotkreuz, Switzerland). Paraffin-embedded tissue sections were pre-treated at 95°C for 52 min in buffer (pH 8), and incubated with the following primary antibodies: MMP-11 (1:1000 at 40°C for 80 min) and the CD45 marker (ready-to-use (Roche), 36 min) specific for MICs, or TIMP-2 (1:100 at 40°C for 60 min) and the α-SMA marker (ready-to-use (Roche), 40 min) specific for fibroblast. For each antibody preparation studied, the location of immunoreactivity in each cell type was determined. In each case, immunoreactivity was classified into 2 categories depending upon the percentage of cells stained (negative: 0–10% positive cells: positive: >10% positive cells) in each cell type (cancer cells, CAFs and MICs). We studied both cores that were carried out for each patient and averaged the results. In the event that no tumor was present in a particular core, then the results of the other core analyzed was given. Two certified pathologists (LOG and NB) blinded to the clinical outcome of the patients performed the histological examination. We distinguished stromal cells from cancer cells on the basis of cell size (the latter cells are larger in size). Stromal cell subsets were distinguished primarily by morphology (CAFs are spindle shaped cells whereas MICs are round cells). Additionally, whereas cancer cells are arranged forming either acinar or trabecular patterns, stromal cells are scattered throughout the tissue.

Staining for ERs and PgRs was scored according to the method described by Allred et al.50 and HER-2 staining according to the criteria used for the Herceptest. Controls included breast cancer tissue with known immunoreactivity for each antibody. Ki-67 and p53 were scored according to the percentages of stained cells. A case was considered positive if at least 15% or 25% of cells were stained, respectively. In addition, we established the following subtypes: luminal type A (ER+, PgR+, HER2), luminal type B (ER+, PgR+, HER2+), HER2+ (HER2+, ER-, PgR-), and basal-like (ER-, PgR-, HER2+)51.

Data analysis and statistical methods
The significance of observed differences in percentages were calculated using the χ² test. For analysis of metastasis-free survival and overall survival analysis, we employed Cox’s univariate method. Cox’s regression model was used to examine the interactions of different prognostic factors in a multivariate analysis. In the multivariate analysis only the parameters that achieved statistical significance for distant relapse-free survival in the univariate analysis (as well as the type of systemic therapy) were included. Expression profiles were analyzed by unsupervised hierarchical clustering, a method that organizes the patterns of protein expression in a tree structure based on similarity. Data were reformatted as follows: –3 designated negative staining, 3 indicated positive staining, and missing data were left blank. We used the Cluster 3.0 program (average linkage, Pearson’s correlation). Results were displayed with the Treeview program. The PASW 18.0 software was used for all calculations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.

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