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Original Study
Short title: ■■■

Prognostic Influence of Tumor Stroma on Breast Cancer Subtypes

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Micro-Abstract

Breast tumors are categorized into 4 major subtypes to predict the prognosis, using 3 standard immunohistochemical markers. Despite these classifications, numerous patients have died of relapsed disease. We performed an immunohistochemical study with 247 tumors of patients, and we demonstrated a significant relationship between the expression of matrix metalloproteinases/tissue inhibitors of metalloproteinases by tumor stroma and relapse-free survival, in order to improve the prognostic evaluation of all breast cancer subtypes.

Abstract

Introduction

The objective of the present work was to evaluate the impact of the phenotype of both intratumoral mononuclear inflammatory cells (MICs) and cancer-associated fibroblast (CAFs), assessed as to their expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) on prognosis in different breast cancer subtypes.

Materials and Methods

A total of 247 tumors of patients with primary ductal invasive breast cancer were categorized into 1 of 4 major subtypes, using the 3 standard immunohistochemical markers (estrogen receptor [ER], progesterone receptor [PR], and human epidermal growth factor receptor/Neu 2 [HER2] receptor status). An immunohistochemical study was performed using tissue arrays and specific antibodies against MMP-9, MMP-11, and MMP-14, and TIMP-1 and TIMP-2.

Results

MMP-11 expression by MICs was significantly and strongly associated with prognosis in all breast cancer subtypes. There were other significant associations with poor prognosis in luminal A tumors: expressions of MMP-9, MMP-11, and TIMP-2 by CAFs, in luminal B tumors: MMP-14 expression by MICs and TIMP-2 expression by MICs, in HER-2-positive tumors: expression of MMP-9 by MICs, and in triple negative breast cancers: expression of TIMP-1 by MICs.

Conclusion

Characterization of both tumor stromal CAFs and MICs, with regard to the expression of MMPs and TIMPs, improve the prognostic evaluation of all breast cancer subtypes.

Keywords: Cancer-associated fibroblasts; Intratumoral inflammatory cells; MMPs; Molecular subtype; TIMPs

Introduction

Breast cancer is the most common cancer in females worldwide and is the second leading cause of cancer death in women. Despite advances in early detection and comprehensive treatments for breast cancer, approximately 30% of patients with early-stage breast cancer still experience recurrent disease.[1] Breast cancer is a heterogeneous disease characterized by distinct intrinsic subtypes. Clinically, breast cancers are categorized into 1 of 4 major subtypes, using the 3 standard immunohistochemical markers, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER/Neu 2), to facilitate targeted therapy.[2] These included luminal A (ER-positive [ER^+], PR-positive [PR^+], HER2-negative [HER^-]), luminal B (ER^+ and/or PR^+, HER2-positive [$HER2^+$]; or ER^+, PR-negative [PR^-], $HER2^-$), $HER2^+$ (ER^-, PR^-, $HER2^+$), and triple negative breast cancer (TNBC) (ER^-, PR^-, $HER2^-$).[3]

However, despite these breast cancer categories used to predict the prognosis and determine the treatment modalities, numerous patients have died of relapsed disease. Thus, novel markers to improve prognosis and decide upon therapeutic strategy is expected. For these purposes, a number of research studies have been done to identify novel biomarkers based on intrinsic characteristics of cancer cells, such as cell cycle regulators, oncogenes, and tumor suppressor genes that are critically involved in carcinogenesis.[4,5] Nevertheless, it is remarkable to consider that the malignant phenotypes of tumors not only are determined by cancer cells themselves but also by their surrounding tumor microenvironments.[6,7] These microenvironments include various cell types, such as fibroblasts, lymphocyte, inflammatory cells, epithelial cells, endothelial cells, and mesenchymal stem cells. In this context, we had identified biologic markers useful to categorize patients into different subgroups based on their tumor stroma characteristics. We have found the existence of different subpopulations of mononuclear inflammatory cells (MICs) and cancer-associated fibroblasts (CAFs), characterized on the basis of expression of matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs).[8-12] These distinct stromal subsets display unique prognostic significance, such that breast carcinomas containing either MIC or CAF cells with a molecular profile of high MMP/TIMP expression had a high rate of development of distant metastases as compared with those tumors with a low expression profile. MMPs are able to impact tumor cell behavior in vivo by several means: (1) direct degradation of the stromal connective tissue and basement membrane components, favoring invasion and metastasis of cancer cells[13]; (2) cleavage of membrane-bound growth factors or cytokines as well as their receptors[14-16]; (3) cleavage of pro-apoptotic factors and induction of a more aggressive phenotype via generation of apoptotic resistant cells; (4) stimulation of tumor angiogenesis through the ability of MMPs to mobilize or activate pro-angiogenic factors[17]; or (5) cleavage of cell adhesion molecules, such as cadherins, leading to an increased cell motility occurring in epithelial mesenchymal transition (EMT).[18,19] On the other hand, although the activity of MMPs is specifically inhibited by TIMPs, it is now assumed that TIMPs are multifactorial proteins also involved in the induction of proliferation and the inhibition of apoptosis.[20,21]

In the present work, we investigated the prognostic importance of the phenotype of MICs and CAFs, with regard to the expression of MMP-9, MMP-11, and MMP-14, and TIMP-1 and TIMP-2, at tumor stroma from breast carcinomas with different subtypes. Our results indicate a key importance of cellular stroma phenotype in all breast cancer subtypes.

Materials and Methods

Patient Selection

We selected 4 groups of patients, each group having a different subtype of early invasive breast cancer of ductal-type (luminal A, luminal B, HER2$^+$, or TNBC), treated between 1990 and 2011, some of whom were previously included in our preliminary studies on the expression of MMPs and TIMPs in breast cancer, where the patients were classified by node status and development of distant metastasis.[12,22-24] 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 locoregional 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. We selected a sample size of 247 patients from our database, with distribution into the 4 key subtypes and as many as possible stratified with regard to nodal status and the development of metastatic disease. Note that approximately one-half of the cases with distant metastasis during the follow-up period occurred in each groups. Patient characteristics included in the 4 main groups are listed in Table 1. Patients underwent either modified radical mastectomy or wide resection with axillary lymphadenectomy. Postoperative radiotherapy was given to 110 (45.0%) patients. Data about the criteria for systemic adjuvant therapy of the patients were described elsewhere.[23] Overall, 75 patients received chemotherapy, 60 patients received tamoxifen, and 74 patients received both types of systemic therapy.

Table 1 Basal Demographics and Clinical Characteristics of 247 Patients With Invasive Ductal Carcinoma of the Breast Included in Our Study

Characteristics	Luminal A, N (%)	Luminal B, N (%)	HER2, N (%)	Triple Negative, N (%)
Total cases	101	69	33	44
Median age, y				
≤55	60 (59.4)	34 (49.3)	11 (52.5)	15 (34.1)
>55	41 (40.6)	35 (50.7)	19 (47.5)	29 (65.9)
Tumor size				
T1	53 (52.5)	35 (50.7)	11 (33.3)	17 (38.6)
T2	48 (47.5)	34 (49.3)	22 (66.7)	27 (61.4)
Nodal status				
N–	42 (41.6)	34 (49.3)	11 (33.3)	25 (56.8)
N+	59 (58.4)	35 (50.7)	22 (66.7)	19 (43.2)
Tumor grade				
I	25 (24.8)	19 (27.5)	3 (9.1)	13 (29.5)
II	61 (60.4)	41 (59.4)	18 (54.5)	21 (47.7)
III	15 (14.9)	9 (13.0)	12 (27.3)	10 (22.7)
Histologic grade				
Well-differentiated	35 (34.7)	19 (27.5)	2 (6.1)	11 (25.0)

Moderately differentiated	45 (44.6)	29 (42.0)	10 (30.3)	12 (27.3)
Poorly differentiated	21 (20.8)	21 (30.4)	20 (60.6)	21 (47.7)
Adjuvant radiotherapy				
No	53 (52.5)	40 (58.0)	18 (54.5)	26 (59.1)
Yes	48 (47.5)	29 (42.0)	15 (45.5)	18 (40.9)
Adjuvant systemic therapy				
TMX	34 (33.7)	24 (17.4)	0 (0.0)	2 (4.5)
QMT	13 (12.9)	12 (34.8)	15 (45.5)	35 (79.5)
QMT + TMX	45 (44.6)	27 (39.1)	2 (6.1)	0 (0.0)
No treatment	9 (8.9)	6 (8.7)	4 (12.1)	7 (15.9)

Abbreviations: HER2 = human epidermal growth factor receptor 2; QMT = chemotherapy■■; TMX = tamoxifen.

The median follow-up period in patients without metastasis was 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 (TAs)

Routinely fixed (overnight in 10% buffered formalin), paraffin-embedded tumor samples stored in our pathology laboratory files were used in this study. TA 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' TA paraffin block using a manual tissue arrayer (Beecker Instruments, Sun Prairie, WI) as described elsewhere.[23] A total of 2 cores were employed for each case, corresponding to the tumor center area. This method, with 2 cores (double redundancy) at the tumor area, has been shown to correlate well with conventional immunohistochemical staining.[23] The tumor center was defined as the area inner from 2 mm surrounding the tumors and which contained cancerous cells. From the 247 tumor samples available, 8 TA blocks were prepared, each containing 32 tumor samples maximum.

Immunohistochemistry

Immunohistochemistry was carried out on TA sections 5 mm 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). The dilution for each antibody was established based on negative and positive controls (1:200 for MMP-14 and TIMP-2; 1:100 for MMP-9 and TIMP-1; and 1:1000 for MMP-11). To enhance antigen retrieval, tissue sections were treated in a PT-Link (Dako) at 97°C for 20 minutes, in citrate buffer (pH 6.1) for TIMP-1. Antibodies against MMP-9, MMP-11, MMP-14, and TIMP-2 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 60 minutes 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.[8,9] Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 minutes. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped. 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) blinded to the clinical outcome of the patients performed the histologic 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. In a prior report, 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), to identify MICs or CAFs in the tumor samples, respectively.

Staining for ERs and PRs was scored according to the method described by Allred et al[25] and HER2 staining according to the criteria used for the Herceptest. Controls included breast cancer tissue with known immunoreactivity for each antibody. In addition, we established the following subtypes: luminal type A (ER^+, PR^+, $HER2^-$), luminal type B (ER^+, PR^+, $HER2^+/ER^+$, PR^-, $HER2^+/ER^+$, PR^-, $HER2^-$), $HER2^+$ (ER^-, PR^-, $HER2^+$,), and triple negative (ER^-, PgR^-, $HER2^-$).[26]

In Situ Hybridization

We detected amplification of the HER2 gene via 2-color chromogenic in situ hybridization (ISH). The staining was performed using the INFORM HER2 Dual ISH DNA Probe Cocktail (Roche Diagnostics).

Data Analysis and Statistical Methods

The χ^2 test was used to determine differences in expression percentages. For analysis of metastasis-free survival and overall survival analysis, we employed the Cox univariate method. The Cox 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 were included. The PASW 18.0 software was used for all calculations.

Results

Figure 1 shows representative examples of MICs and CAFs expressing MMPs and TIMPs localized in the tumor center in breast carcinomas. Immunostaining for these proteins revealed a cytoplasmic location in cancer cells, MICs, and CAFs. With regard to MMP-14 expression, it is of note that the immunostaining revealed both cytoplasmic and membrane location. In neoplasms with positive expression of MMPs or TIMPs by MICs or CAFs, at least 70% of these cells showed a positive immunostaining of each evaluated field.

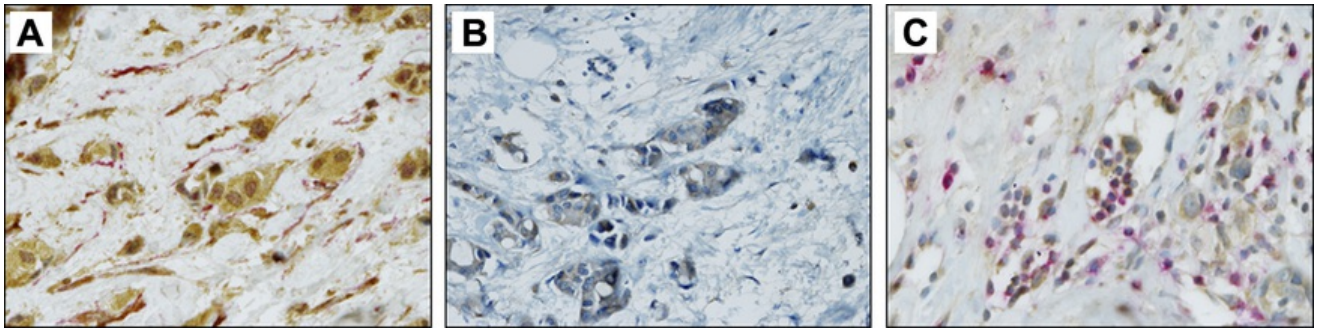

Figure 1 Human Mammary Carcinomas Contain Tumor-stromal Cells Expressing Metalloproteases and Their Inhibitors. Representative Pictures of Breast Tumors Immunostainings (200×) for **A**, MMP-9; **B**, MMP-11; **C**, MMP-14; **D**, TIMP-1; and **E**, TIMP-2. Arrows Indicates the Expression by Different Cell Types, (1) Cancer Cells, (2) MICs, and (3) CAFs

Abbreviations: CAF = cancer-associated fibroblast; MIC = mononuclear inflammatory cell; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.

The majority of MMPs and TIMPs were mainly expressed by cancer cells in breast carcinomas. However, these proteins were also expressed by stromal cells in a significant percentage of tumors. Table 2 shows the percentages of expression for each factor in each cell type from tumors. As can be seen in this Table, we did not find significant differences in factor expressions by cancer cells between tumor types. However, our results showed some significant differences, but not drastic, in some factor expressions by stromal cells. Thus, comparing among tumor types, we can determine that a lower percentage of luminal A tumors expressed MMP-14 by MICs, as well as TIMP-1 by CAFs. Luminal B tumors showed lower percentages of cases with positive MMP-14 expression by MICs or TIMP-1 by CAFs. HER2$^+$ tumors showed higher percentages of cases with positive expression of TIMP-1 by CAFs and MMP-14 by MICs, whereas, TNBC tumors showed higher percentage of cases with positive TIMP-1 expression by CAFs, or MMP-14 expression by MICs.

Table 2 Expression of Metalloproteases and Their Inhibitors in the Different Cellular Types From 247 Breast Carcinomas

Factors	Luminal A			Luminal B			HER2			Triple Negative		
	Cancerous Cells	CAFs	MICs	Cancerous Cells	CAFs	MICs	Cancerous Cells	CAFs	MICs	Cancerous Cells	CAFs	MICs
MMP-9	88 (87.1)	50 (49.5)	32 (31.7)	60 (90.9)	36 (54.5)	18 (27.3)	22 (81.5)	17 (63.0)	10 (37.0)	36 (81.8)	16 (36.4)	9 (20.5)
MMP-11	98 (97.0)	67 (63.3)	31 (30.7)	64 (92.8)	47 (68.1)	24 (34.8)	24 (85.7)	17 (60.7)	11 (39.3)	39 (88.6)	36 (81.8)	19 (43.2)
MMP-14	91 (92.9)	74 (75.5)	32 (32.7)[a]	61 (89.7)	48 (70.6)	19 (27.9)[a]	22 (95.7)	22 (95.7)	13 (56.5)[a]	39 (88.6)	36 (81.8)	19 (43.2)[a]
TIMP-1	82 (83.7)	30 (30.6)[a]	14 (13.9)	60 (88.2)	25 (36.8)[a]	14 (20.6)	26 (96.3)	14 (51.9)[a]	7 (25.9)	38 (86.4)	25 (56.8)[a]	12 (27.3)
TIMP-2	89 (88.1)	68 (67.3)	45 (44.6)	66 (95.7)	51 (73.9)	34 (49.3)	27 (96.4)	19 (67.9)	13 (46.4)	41 (93.2)	25 (56.8)	22 (50.0)

Data are represented as number of positive cases (percentage).

Abbreviations: CAFs = cancer associated fibroblasts; HER2 = human epidermal growth factor receptor 2; MICs = mononuclear inflammatory cells; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.

[a] χ^2 test, $P < .05$.

The possible association between MMPs or TIMPs expression by each cell type and relapse-free survival and overall survival was investigated in all patients included in the present study. Our results did not show significant associations between the different expressions of these factors by tumor cells with prognosis, in each tumor type (data not shown). However, we found several significant associations between expression of MMPs and TIMPs by stromal cells and prognosis. Thus, as can be seen in Figure 2, MMP-11 expression by MICs was significantly and strongly associated with prognosis in all breast cancer types. In addition, there were other significant associations with poor prognosis in the different groups of breast cancer subtypes. In luminal A tumors, positive expressions of MMP-9, MMP-11, and TIMP-2 by CAFs are related to a lower relapse-free survival (RFS) (Figure 3A); in luminal B tumors: positive expression MMP-14 and TIMP-2 expression by MICs is associated with a lower RFS (Figure 3B); in HER2$^+$ tumors: positive expression of MMP-9 by MICs is related to lower RFS (Figure 3C), whereas in TNBC tumors, a shortened RFS is related to positive expressions of TIMP-1 by MICs (Figure 3D). For other possible MMP or TIMP expressions by MICs or CAFs, we found no significant associations (data not shown).

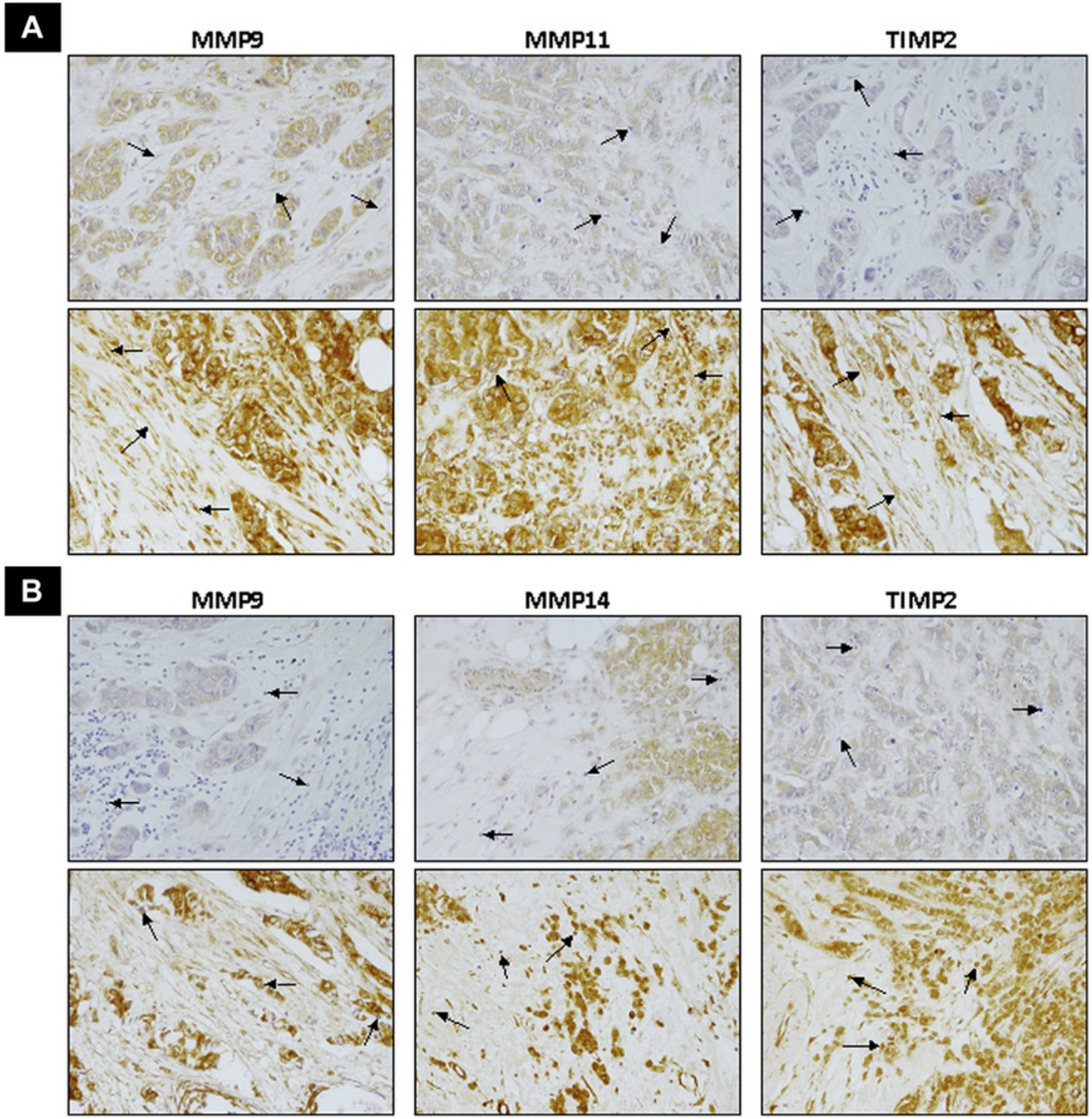

Figure 2 Prognostic Significance of Stromal Expression of MMP11 in Breast Carcinomas. Kaplan-Meier Survival Curves for Relapse-free Survival as a Function of Expression of Matrix MMP-11 in Patients With Luminal A Tumors (**A**); Luminal B Tumors (**B**); HER2-Positive Tumors (**C**), and Triple Negative Tumors (**D**) by MICs

Abbreviations: HER2 = human epidermal growth factor receptor 2; MICs = mononuclear inflammatory cells; MMP = matrix metalloproteinase; TNBC = triple negative breast cancer.

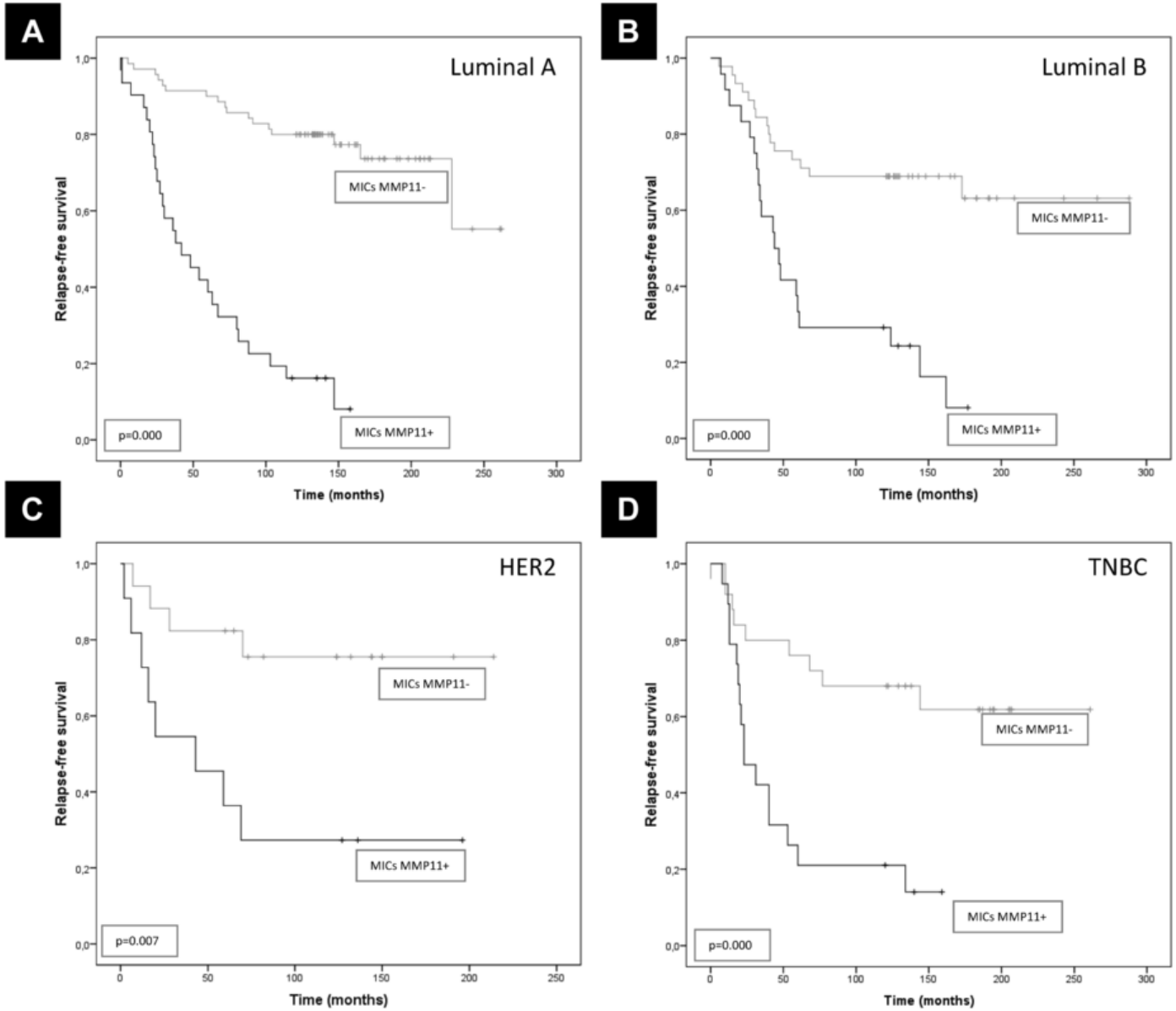

Figure 3 Prognostic Significance of Stromal Expression of Metalloproteases and Their Inhibitors in Luminal A Tumors (**A**); Luminal B Tumors (**B**); HER2-Positive Tumors (**C**); and TNBC (**D**)
Abbreviations: HER2 = human epidermal growth factor receptor 2; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase; TNBC = triple negative breast cancer.

Multivariate analysis according to the Cox model demonstrated that tumor stage was significant and independently associated with RFS in luminal A cases (tumor stage II: relative risk [RR], 0.33; 95% confidence interval [CI], 0.13-0.84; tumor stage III: RR, 0.35; 95% CI, 0.17-0.70; $P = .007$) and HER2$^+$ cases (tumor stage II: RR, 0.00; 95% CI, 0.00-0.00; tumor stage III: RR, 0.28; 95% CI, 0.00-0.27; $P = .011$). Histologic grade is also significant and independently associated with RFS in HER2$^+$ cases (RR, 0.17; 95% CI, 0.04-0.74; $P = .0001$).

Moreover, this same analysis also demonstrated that MMP11 expression by MICs was significant and independently associated with RFS in all tumor types: luminal A (RR, 6.44; 95% CI, 3.28-12.64; $P = .0001$); luminal B (RR, 4.23; 95% CI, 2.02-8.82; $P = .0001$); HER2$^+$ (RR, 70.26; 95% CI, 4.04-1221.62; $P = .004$); and TNBC (RR, 4.27; 95% CI, 1.77-10.29; $P = .0001$). In addition, we found that TIMP-2 expressed by CAFs was significant and independently associated with RFS in HER2$^+$ cases (RR, 0.07; 95% CI, 0.01-0.56; $P = .013$).

Discussion

The application of gene expression profiling has reshaped our understanding of breast cancer biology. Four main intrinsic molecular subtypes of breast cancer (luminal A, luminal B, HER2-enriched, and TNBC) have been classified over the last 15 years, and each of these subtypes has different features, clinical behaviors, and treatment response profiles.[27] However, in each one of these categories, there is heterogeneity in clinical behavior. Our results did not show significant differences in factor expressions by cancer cells between tumor types. However, we found some significant differences, but not drastic, in some factor expressions by stromal cells. In summary, the factors studied were often higher expressed by MICs or CAFs in HER2$^+$ or TNBC tumors compared with luminal B and, especially, luminal A tumors. These findings seem are in accordance with the different biological aggressiveness of these respective tumors. Nevertheless, our results demonstrate that phenotype characteristics from stroma influence prognosis in all of these breast cancer subtypes.

Luminal A breast cancers are associated with the most favorable short-term prognosis owing to a favorable response to endocrine therapy.[28] However, it was of note that, in this tumor type, many stromal factors were associated with prognosis (such as expressions of MMP-11 by MICs, and MMP-9, TIMP-1, and TIMP-2 by CAFs). It seems to indicate the relevancy of the stroma reactivity despite of the low biological aggressiveness from breast carcinomas, which is in accordance with our previous study that demonstrated a dynamic role of CAFs in tumor progression of breast carcinoma.[29]

Luminal B disease is defined by an aggressive clinical behavior and has a similar prognosis to non-luminal cancers (including the HER2-enriched and TNBC subtypes).[30] In fact, it has been reported that 48.1% of the patients with recurrence and metastasis had luminal B subtype.[31] These tumors should be treated with a more aggressive therapy, which has not always been demonstrated to be effective owing to the molecular and clinical heterogeneity of this breast cancer subtype.[32] In addition, luminal B constitutes the most heterogeneous molecular subtype, both clinically and molecularly. Unfortunately, the immunohistochemistry correlate of the luminal B subtype still remains imprecise, and it has now become of paramount importance to define a classification scheme capable of segregating luminal tumors into clinically meaningful subgroups that may be used clinically to guide patient management. In fact, although many of the luminal B tumors are ER$^+$/HER2$^-$/high Ki-67, expression profiles also classify the ER$^+$/HER2$^+$ tumors as luminal B, and these patients receive a different therapy regimen (that incorporates targeted anti-HER2 therapy) compared with other luminal B breast cancer subtypes.[33] For all these reasons, one major challenge in the management of luminal B tumors is to discriminate those patients that would benefit from cytotoxic drugs or anti-targeted therapy from those that would not. Our data indicate that the expression of MMP-11, MMP-14, and TIMP-2 by MICs impact on prognosis in luminal B tumors, which may be to contribute to a better prognostic characterization of these tumors.

HER2 is a proto-oncogene located on chromosome 17.[34] When HER2 is overexpressed, it allows cell growth, survival, and cell differentiation through a signal transduction cascade mediated by the activation of PI3K/Akt and the Ras/Raf/MEK/MAPK pathways.[35] There are approximately 2 million HER2 proteins presented on the surface of HER2$^+$ cancer cells, which is around 100 times more than on a normal cell.[36] Overexpression of the HER2 receptor is generally associated with poor prognosis in patients with breast cancer.[37] In addition, HER2 positivity may result in increased resistance to endocrine therapy and non-response to non–anthracycline-, non–taxane-containing chemotherapy.[38] About 1 in 5 women diagnosed with breast cancer worldwide will have HER2$^+$ breast cancer.[39] In our study population, HER2$^+$ tumors were the most infrequent type of the invasive breast cancers. In fact, retrospectively, only 33 cases retain all the inclusion criteria. Although this protein acts as an important biomarker and target of therapy for about 30% of patients with breast cancer,[40] our results point out that stromal factors, such as expressions of MMP-9 or MMP-11 by MICs, might be new predictive factors in HER2$^+$ tumors.

TNBC is a heterogeneous group of tumors characterized by the lack of expression of hormonal receptors and the absence of HER2 overexpression that accounts for approximately 15% to 20% of all breast cancer diagnoses.[41] TNBC is characterized by an aggressive natural history and worse disease-specific outcomes compared with other breast cancer subtypes.[42] Despite initial responses to chemotherapy, early and higher rates of distant, typically visceral, recurrences are observed for TNBC, with an early peak of distant recurrences at 3 years after diagnosis. The majority of deaths occur in the first 5 years following initial diagnosis.[42-45] Anthracycline- and taxane-based chemotherapy traditionally has been the mainstay of therapy for TNBC. However, given the trade-off of greater toxicities in the early setting and mixed results in the metastatic setting, it would be ideal to be better able to identify patients most likely to benefit from this treatment strategy. In addition, clinical data had already indicated the existence of heterogeneous treatment responses and long-term outcomes.[42,43,46] Unfortunately, predictive factors that allow the identification of patients who will present a pathologic complete response, at the time of diagnosis, do not exist. Our results show that expressions of MMP-11 or TIMP-1 by MICs may be of prognostic importance in this tumor subtype and, therefore, of clinical value.

Globally, our results support the many reports on the importance of the cell components from tumor stroma, such as inflammatory cells and CAFs, in breast cancer progression. In addition, the data of the present work are also in concordance with several data indicating the biological importance of the MMPs/TIMPs, investigated in the present study, in tumor aggressiviness. Expression of MMP-11 (also known as stromelysin-3) by MICs was significantly and strongly associated with prognosis in all breast cancer subtypes. Previously, we found that 32% of breast carcinomas analyzed were infiltrated by MICs exhibiting a high MMP expression profile, which was associated with a high rate of distant metastasis (97.6%). But MMP-11 was the most frequently expressed factor of all these (as it was found in 85.7% of MICs exhibiting a high MMP/TIMP profile expression but only in 4.6% of MICs exhibiting a low MMP profile).[8] As a follow-up to this study, we found a positive and significant relationship between the MMP-11 expression by intratumoral MICs and genetic expression of 19 recognized intratumoral factors associated with inflammation and tumor progression, specially interleukin (IL)-1, IL-5, IL-6, IL-17, and NFκB.[47,48] In addition, we recently found that the characterization of the tumor stroma regarding to the MMP11-positive status by MICs is associated with a type of CAFs that contribute even more to tumor progression. This seems to be because of the fact that these CAFs overexpress some molecular factors of biological importance in tumor progression, such as CXCL12, TIMP-1, VEGFA, S100A, and HGF.[29] Therefore, these data suggest that immune cells and CAFs act, in turn, on tumor cells to increase their proliferative and migratory/invasive properties. All of these data seem to indicate that MMP11 expression by MICs is especially associated with tumor progression and metastasis. MMP-11 belongs to the MMP extracellular enzyme family.[49] Now, our results on different populations of patients with different breast cancer subtypes confirm our previous reports pointing to a great prognostic impact of MMP-11 expression by intratumoral MICs from breast carcinomas.[12,22,23] In addition, in a study conducted by Boulay et al,[50] it was demonstrated that tumorigenesis induced by MMP-11 is not a result from increased cancer cell proliferation, but from decreased cancer cell death through apoptosis and necrosis, indicating that the cellular function of MMP-11 is to favor cancer cell survival in the stromal environment. On the other hand, there are data indicating that MMP-11 may alter the stromal microenvironment of human carcinomas to stimulate tumor angiogenesis.[51,52]

MMP-9 (gelatinase B) is related to tumor invasion and metastasis by its special capacity to degrade the type IV collagen found in basement membranes[53] and to induce angiogenesis.[16] High MMP-9 expression correlates significantly with tumor aggressiveness and poor prognosis.[54-56] Thus, our results demonstrated that MMP-9 expression is related with poor prognosis in both luminal A and HER2$^+$ cases, as HER2 overexpression improved the transcription of MMP-9.[57,58]

MMP-14 (membrane type 1 MMP, or MT1-MMP) is a key metalloprotease involved in the degradation of extracellular matrix, in the activation of pro-MMP-13[59] and pro-MMP-2[60] on the cell surface, and plays crucial roles in molecular carcinogenesis, tumor cell growth, invasion, and angiogenesis.

The positive relationship between TIMP expression by tumor-stromal cells and cancer progression could look paradoxical, because both TIMP-1 and TIMP-2 are well-known inhibitors of MMP activity. However, is also known that TIMPs are multifunctional proteins that, in addition to their MMP-inhibitory effect, also promote the proliferation of some cell types, and their anti-apoptotic effects may favor tumor expansion during the onset and early growth of the primary tumor.[20,21,61,62]

Conclusion

In summary, our data point to the importance of the phenotype of both intratumoral MICs and CAFs, with regard to the expression of MMPs and TIMPs, in order to improve the prognostic evaluation of all breast cancer subtypes. As it was shown, CAFs plays an important role in regard to MMPs and TIMPs expression in luminal A breast tumors, whereas in luminal B, HER2$^+$, and TNBC tumors, the expression of these factors by MICs is more determining regarding relapse-free survival.

Clinical Practice Points

- Clinically, breast cancers are categorized into 4 major subtypes (luminal A, luminal B, HER2+, and triple negative breast cancer), using the 3 standard immunohistochemical markers: ER, PR, and HER/Neu 2, to facilitate targeted therapy.
- However, despite these breast cancer categories used to predict the prognosis and determine the treatment modalities, numerous patients died of relapse disease.
- Thus, novel markers to improve prognosis and decide therapeutic strategy are expected.
- The malignant phenotypes of tumors not only are determined by cancer cells themselves but also by their surrounding tumor microenvironment, which includes fibroblasts and inflammatory cells.
- In this context, we had identified biologic markers useful to categorize patients into different subgroups based on their tumor stroma characteristics.
- We found that CAFs play an important role in regard to MMP and TIMP expression in luminal A breast tumors, whereas in luminal B, HER2+, and TNBC tumors, the expression of these factors by MICs is more determining regarding the RFS.

Disclosure

The authors declare that they have no conflicts of interest.

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Supplemental Data

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Appendix

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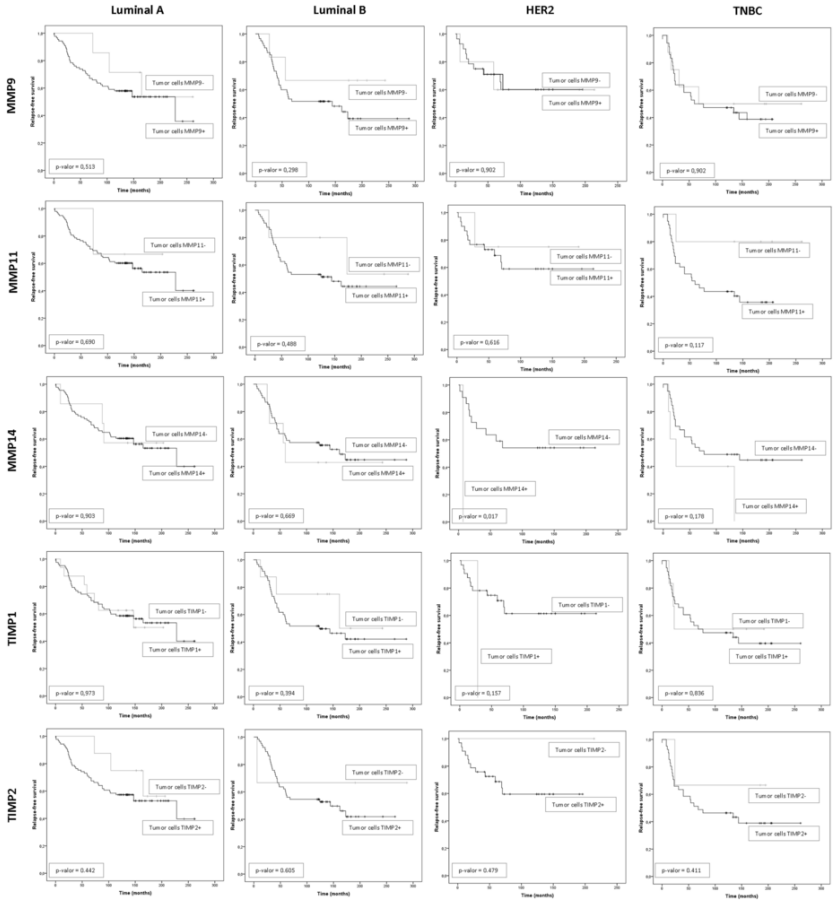

Supplemental Figure 1 Prognostic significance of expression of MMPs and TIMPs in breast carcinomas. Kaplan–Meier survival curves for relapse-free survival as a function of expression of MMP-9, MMP-11, MMP-14, TIMP-1 and TIMP-2 in patients with Luminal A tumors, Luminal B , HER-2 positive tumors, and Triple negative tumors by tumor cells cells.■

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Query: Caption says Figure 1, but this appears to correspond to Figure 2.
Answer: This Caption correspond to Figure 2. Caption of Figure 1 is: **Figure 1:** Representative pictures of double immunohistochemical staining for MMP11 and the different specific markers for each cell type. **(A)** α-SMA staining (red) positive expression by CAFs in combination with MMP11 (brown), **(B)** Desmin negative expression by CAFs and MMP11 positive expression. **(C)** CD45 staining (red) specific for MICs in combination with MMP11 (brown).

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