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COMPARATIVE ANALYSIS AND CLINICAL VALUE OF THE EXPRESSION OF METALLOPROTEASES AND THEIR INHIBITORS BY INTRATUMOR STROMAL MONONUCLEAR INFLAMMATORY CELLS AND THOSE AT THE INVASIVE FRONT OF BREAST CARCINOMAS

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ABSTRACT

Aims: Matrix metalloproteases (MMPs) and their inhibitors (TIMPs) play an essential role in the degradation of stromal connective tissue and basement membrane components. The dynamic analysis of these components might help predict tumor aggressiveness.

Methods and Results: An immunohistochemical study was performed using tissue arrays and specific antibodies against MMPs -1, 2, 7, 9, 11, 13, 14, and TIMPs -1, 2 and 3. More than 5,000 determinations on cancer specimens from 124 patients with invasive breast cancer were performed on the tumor center core as well as on the invasive front. Immunostaining for MMPs/TIMPs on mononuclear inflammatory cells (MICs) was evaluated. To identify specific groups of tumors with distinct expression profiles, data obtained from both MICs populations were analyzed by unsupervised hierarchical cluster analysis. When compared with MICs at the invasive front, intratumor MICs more frequently showed expression of MMP-7 and 14, and TIMP-3, but less frequently of MMP-9 and 11, and TIMP-2.

Conclusions: Our data led us to consider the need of further studies in order to identify subsets of MICs and other protein elements of the microenvironment as attractive targets for new therapeutic strategies against cancer.

KEY WORDS: Peritumor stroma, MMP, TIMP, leukocytes, inflammation, tumor heterogeneity.

INTRODUCTION

Tumors are composed not only of cancer cells but also of other cell types, notably stromal cells. The term stromal cells include cancer associated fibroblasts, endothelial cells, pericytes, and a variable representation of leukocytes. Leucocytes can account for as much 50% of the total tumor mass in invasive breast carcinomas. Historically, tumor-infiltrating leukocytes have been considered to be manifestations of an intrinsic defense mechanism against the development of tumors [1, 2]. However, increasing evidence indicates that leukocyte infiltration can instigate a tumor phenotype, as evidenced by the presence of angiogenesis, tumor cell growth, and invasion [3-5]. This may be due to the fact that inflammatory cells probably influence cancer promotion by secreting cytokines, growth factors, chemokines and proteases, which in turn stimulate proliferation and invasiveness of/by cancer cells [6-8]. In addition, accumulating clinical data for solid tumors show a correlation of high-density leukocytic infiltration into tumors with poor patient outcome [3, 9-11]. Nevertheless, the prognostic significance of lymphoid infiltrates at the tumor site remains controversial, perhaps because the criteria of evaluation of tumor infiltrates are not sufficiently standardized to yield reliable and reproducible results. A typical leukocyte infiltrate is made up by a variable representation of leukocytes, including macrophages, neutrophils, mast cells, and B and T lymphocytes [3, 12]. Thus, there are evidences indicating that different types of leukocyte infiltrates occur in different breast carcinomas, and the type of infiltrate is probably related to the degree of tumor dissemination [13].

The functional roles of the various leukocyte components of any tumor infiltrate remain to be fully elucidated, but evidence suggests that it may differ among tumors. Consequently, it is necessary to obtain biomarkers to assess the biological heterogeneity of these leukocytes infiltrates. Among possible biological markers metalloproteases (MMPs) could be good candidates worth studying. Indeed, MMPs play an essential role in the degradation of stromal connective tissue and basement membrane components, both of which are key elements in tumor invasion and metastasis. In addition, MMPs are able to impact *in vivo* on tumor cell behaviour as a consequence of their capacity to cleave growth factors, cell surface receptors, cell adhesion molecules, and chemokines/cytokines [14-16]. Furthermore, by cleaving proapoptotic factors, MMPs produce a more aggressive phenotype via generation of apoptotic resistant cells [17]. Also, MMPs positively regulate cancer-related angiogenesis, through their ability to

mobilize or activate proangiogenic factors [18], and negatively via the generation of angiogenesis inhibitors, such as angiostatin and endostatin, that are cleaved from large protein precursors [19]. On the other hand, it is now accepted that tissue inhibitors of metalloproteases (TIMPs) are multifactorial proteins also involved in the induction of proliferation and the inhibition of apoptosis [20, 21]. Recently, we identified a phenotype of mononuclear inflammatory cells (MICs) at the intratumor stroma of breast carcinomas, which is associated with the development of distant metastasis. These MICs were characterized by overexpression of MMP-7, 9, 11, 13 and 14, as well as of TIMP-1 and 2 [22].

The clinical relevance of the expression of MMPs and TIMPs by intratumor stromal MICs led us to consider the potential biological and clinical significance of the expression of this enzymatic system by the MICs located at the invasive front of breast carcinomas. We focussed on the invasive front because it is the area where some of the most important interactions between cancer cells and the tumor supporting stroma take place [23]. Therefore, the pattern of MMPs/TIMPs expression by MICs at the invasive front could reflect prognosis better than other tumor areas or, even, provide useful complementary information of clinical interest. Consequently, the aims of the present work were to compare the expression of MMPs and TIMPs by stromal MICs (T lymphocytes, B lymphocytes and macrophages), at two different locations of breast carcinomas: intratumoral and at the invasive front of breast carcinomas. We also studied their relationship with clinico-pathological characteristics and the prognosis. Our results demonstrate different patterns of expression of MMPs and TIMPs depending on MIC location within the tumor. We propose that the combined evaluation of these variable may provide a highly predictive tool for distant metastases.

MATERIALS AND METHODS

Patient selection, characteristics and tissue specimen handling

This study comprises 124 women with a histologically confirmed diagnosis of early invasive breast cancer (without distant metastasis at the time of the initial diagnosis) and treated between 1990 and 2003. We selected women with the following inclusion criteria: invasive ductal carcinoma, at least six histopathologically assessed axillary lymph nodes, and a minimum of five years of follow-up for those women without tumor recurrence. The exclusion criteria were the following: metastatic disease at presentation, prior history of any kind 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 TAs [24]. From patients fulfilling these criteria, we randomly selected a sample size of 124 patients according to four different groups with similar size and stratified with regard to nodal status and to the development of metastatic disease, which were the key measure variables of the study. Thus, we included an important number of events in both node-positive and node-negative patient subgroups (half of the cases that developed distant metastases during the follow-up period are included in each subgroup) in order to guarantee the statistical power of the survival analysis. Patient characteristics included in the two main groups, with or without distant metastases, are listed in Table 1. Tumor border configuration is classified as expansive (smooth, pushing border) versus infiltrative (Dissection of adipose tissue by small glands or irregular cords or clusters of tumor cells) and is assessed on low-power examination. Tumors were classified as negative for necrosis if there was not necrosis at all in the microscopic sections evaluated. When we recognized one or more focus of necrosis (independent of their size) we classified the tumor as positive for necrosis.

A desmoplastic reaction was defined as the pervasive growth of dense fibrous tissue around the tumor. The fibrous peritumoral stroma undergoes proliferation of newly formed fibroblasts in an edematous, myxomatous or highly collagenized matrix. One tumor was considered positive for peritumoral inflammation if we observed a dense chronic inflammatory infiltrate rich in plasma cells and lymphocytes in at least 50% of

the peritumoral area. For ER and PgR evaluations were used mouse anti-ER clone 1D5 at a dilution of 1/50, and anti-PR clone PgR 636 at a dilution of 1/50 (Dako, Glostrup, Denmark). Staining for ERs and PgRs was scored according to the method described by Allred et al. [25].

Patients underwent either modified radical mastectomy or wide resection with axillary lymphadenectomy. Postoperative radiotherapy was given to 47 patients (42.7%). The criteria for systemic adjuvant therapy were as follows: i) node-negative patients with ER and /or PgR positive tumors received tamoxifen (20 mg per day during five years); ii) node-negative patients with ER and PgR negative tumors received six cycles of intravenous CMF (cyclophosphamide, methotrexate and 5-fluorouracil) every 3 weeks, if their tumors were either larger than one centimeter, moderately or poorly differentiated, or if patients were younger than 35 years old; iii) node-positive patients received six cycles of intravenous FEC (5-fluorouracil, epirubicin and cyclophosphamide) every 3 weeks, plus sequential tamoxifen if they had ER and/or PgRpositive tumors. Overall, 47 patients received chemotherapy, 39 patients received tamoxifen, and 17 patients received both types of systemic therapy.

Women were treated according to the guidelines used in our institution. The study adhered to national regulations and was approved by our Institution "Ethics and Investigation Committee". The end-point was distant metastatic relapse. The median follow-up period in patients without metastases was 85 months, and 46 months in patients with metastases. In addition, in the present study we analyzed the expression of the factors in normal mammary tissues obtained from 4 women that underwent cytoreductive surgery of the breast (age range: 35-60 years).

Tissue arrays and immunohistochemistry

Breast carcinoma tissue samples were obtained at the time of surgery. Routinely fixed (overnight in 10% buffered formalin), paraffin-embedded tumor samples stored in our pathology laboratories were used. Histopathologically representative tumor areas were defined on haematoxylin and eosin-stained sections and marked on the slide. Tumor tissue array (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' tissue array paraffin block using a manual tissue arrayer (Beecker Instruments, Sun Prairie, WI, USA) as described elsewhere [26]. Collection of tissue cores was carried out under highly controlled conditions. A total of four cores were used for each case. Two of these cores in each case corresponded to the tumor central area, and the other two cores corresponded to the invasive front. This method, evaluating two cores (double redundancy) of each tumor area has been shown to correlate well with conventional immunohistochemical staining [27]. The invasive front was defined as the tumoral advancing edge. This corresponds to a 2 mm margin surrounding the tumor and containing cancerous cells. From the 124 tumor samples available, four TA blocks were prepared, each one containing 31 primary tumor samples, as well as external controls including four normal breast tissue samples from two healthy women who underwent reductive mammary surgery. These latter samples contained epithelial components on which immunostaining was not seen with any of the antibodies used.

Serial 5- μ m sections of the high-density TA blocks were consecutively cut with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slide. One section from each tissue array block was stained with H&E, and these slides were then reviewed to confirm that the sample was representative of the original tumor. Immunohistochemistry was done on these sections of TA 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, -14 and TIMP-2; 1/100 for MMP- 9, -13, TIMP-1 and -3; and 1/200 for MMP-1, -11). The negative control was DakoCytomation mouse serum diluted to the same mouse IgG concentration as the primary antibody. All the dilutions were made in Antibody Diluent, (Dako, Glostrup, Denmark) and incubated for 30 min. at room temperature. As positive controls, breast tumor cytosol samples in which we confirmed the presence of the evaluated proteins by Western blot analysis were used as positive controls as shown previously [22, 28]. A single band of the expected molecular mass was observed for each protein [22]. We also used other antibodies recognizing several factors, such as

MMP-2 (policlonal, 1/50, Abcam Cambridge UK.), MMP-13 (clone 181-15A12, 1/100, Calbiochem), MMP-11 (clone SC3-05, 1/100, MERCK KGaA Darmstadt Germany).

Tissue sections were deparaffinized in xylene and then rehydrated in graded concentrations of ethyl alcohol (100%, 96%, 80%, 70%, then water). To enhance antigen retrieval only for some antibodies, TA sections were microwave treated in a H2800 Microwave Processor (EBSciences, East Granby, CT, USA) in citrate buffer (Target Retrieval Solution; Dako) at 99°C for 16min. 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 reactivity detection system. Sections were counterstained with haematoxylin, dehydrated with ethanol and permanently coverslipped.

For each antibody preparation studied, the location of immunoreactivity, percentage of reactive area and intensity were determined. All the cases were semiquantified for each protein-stained area. An image analysis system with the Olympus BX51 microscope and soft analysis (analySIS[®], Soft imaging system, Münster, Germany) were used as follows: tumor sections were stained with antibodies according to the method explained above and counterstained with haematoxylin. There were different optical thresholds for both stains. Each core was scanned with a 400X power objective in two fields per core. Fields were selected searching for the protein-reactive areas. The computer program selected and traced a line around antibody-reactive areas (higher optical threshold: red spots), with the remaining, non-stained areas (haematoxylin-stained tissue with lower optical threshold) standing out as a blue background. Any field had an area ratio of stained (red) versus non-stained (blue). A final area ratio was obtained after averaging two fields. To evaluate immunostaining intensity we used a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no reactivity; 1, weak reactivity; 2, moderate reactivity; and 3, intense reactivity. Using an Excel spreadsheet, the mean score was obtained by multiplying the intensity score (I) by the percentage of reactivity area (PA) and the results were added together (total score: I x PA). This overall score was then averaged with the number of cores that were done for each patient. If there was no tumor in a particular core, then no score was given. In addition, for each tumor the mean score of two core biopsy samples was calculated. This scoring evaluation was based on a global evaluation of staining areas

corresponding to tumoral cells as well as to stromal cells. Nevertheless, in the present work we also evaluated the immunohistochemical staining exclusively on cancerous cells or on stromal MICs. Each evaluated field (400X power objective) contained at least ten stromal MICs. We distinguished stromal cells from cancer cells because the latter are larger in size. In addition, fibroblasts are spindle shaped whereas mononuclear inflammatory cells are rounded. On the other hand, while cancer cells are arranged forming either acinar or trabecular patterns, stromal cells are spreaded. Moreover, we used several markers to distinguish mononuclear inflammatory cells (T lymphocytes (CD 3 , CD 45Ro,CD 4,CD 8) , B lymphocytes (CD 20,CD 79a) and macrophages (CD68), all of them from Dako (Glostrup, Denmark).

Statistical analysis

Differences in percentages were calculated with the chi-square test. We consider cases with concordance between tumoral center and invasive front for each MMP or TIMP expression when in these cases one cellular type was positive or negative for the corresponding factor in both tumoral localizations. Immunostaining score values for each protein were expressed as a median (range). Correlation between score values was calculated by using the Spearman correlation test. Comparison of immunostaining values between groups was made with the Mann-Whitney or Kruskal-Wallis tests. Statistical results were corrected applying Bonferroni's correction. For metastasis-free survival analysis we used the Cox's univariate method. Cox's regression model was used to examine interactions of different prognostic factors in a multivariate analysis. In the multivariate analysis only parameters that achieve 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 an unsupervised hierarchical clustering method that, based on their similarity, organizes proteins in a tree structure. Data was reformatted as follows: "-3" designated negative staining, "3" positive staining, and missing data was left blank. We used the Cluster 3.0 program (average linkage, uncentered correlation). Results were displayed with the Treeview program [29]. The SPSS 17.0 program (SPSS Inc, Chicago, IL, USA) was used for all calculations. $P < 0.05$ was considered significant.

RESULTS

More than 10,000 determinations in cancer specimens from 124 patients with primary invasive ductal carcinoma of the breast and controls were performed on TAs (we evaluated the expression of ten different proteins, two areas by tumor, two cores by tumor area, and two fields by core). Minimal internal variance of score data between duplicate tissue cores from the same patients and the same tumor areas was detected in the TAs, showing a high agreement for each protein ($r > 0.95$ and $p < 0.0001$, for each protein). Indeed, we have previously described a validation study for MMPs and TIMPs, between the tumor center and the whole tumor section in invasive breast carcinomas [30].

Figure 1 shows representative examples of MMPs and TIMPs expression by MICs at the center of the tumor and at the invasive front of breast carcinomas. Immunostaining for these proteins shows a cytoplasmic location both in positive cancerous cells as in positive MICs. With regard to MMP-14 expression, it is noteworthy the positive immunostaining at both cytoplasmic and membrane locations. At least 70% of MICs positives for each MMPs or TIMPs showed a positive immunostaining at each evaluated field. As Table 2 shows, the expression of MMPs and TIMPs by MICs varied among tumors. MICs at the tumor center showed MMP-7 ($p < 0.0001$), MMP-14 ($p < 0.0001$), and TIMP-3 ($p < 0.0001$), positive staining than MICs at the invasive front; while MICs at the invasive front showed a higher expression MMP-9 ($p = 0.016$), MMP-11 ($p < 0.0001$), and TIMP-2 ($p = 0.013$). However, there were no significant differences in the expression of MMP-1, -2 and -13, and TIMP-1 between MICs of these two paired sets of tissue samples.

We analyzed the expressions of these factors in normal mammary tissues which were obtained from 4 women that underwent cytoreductive surgery of the breast. MICs from these normal tissue samples were negative for MMP-2, 7, 9, 11, 13 and 14; TIMP-1, 2 and 3. Only one of the 4 cases showed a weak staining for MMP-1 in a limited population of MICs.

The concordance between expressions of factors by intratumor MICs and by those at the invasive front, were of 59.6% for MMP-1, 97.5% for MMP-2, 50.9% for MMP-7,

74.2% for MMP-9, 66.7% for MMP-11, 67.5% for MMP-13, 54.2% for MMP-14, 65% for TIMP-1, 57.7% for TIMP-2, and 45.6% for TIMP-3.

The concordances between the expression of MMPs and TIMPs by MICs and by cancer cells were also analyzed at the tumor center and at the invasive front. At tumor center, the results show the following expression concordances between the two cell types: 79% for MMP-1, 68.8% for MMP-2, 62.6% for MMP-7, 37.4% for MMP-9, 45.1% for MMP-11, 59.7% for MMP-13, 62.9% for MMP-14, 31.7% for TIMP-1, 48.8% for TIMP-2, and 68.5% for TIMP-3. At the invasive front, our results show the following concordances between the two cell types: 76.6% for MMP-1, 62.3% for MMP-2, 33.9% for MMP-7, 28.9% for MMP-9, 58% for MMP-11, 48.4% for MMP-13, 39.9% for MMP-14, 43.8% for TIMP-1, 61.3% for TIMP-2, and 53.8% for TIMP-3.

We also compared the expression of MMPs and TIMPs by these two MICs populations using the global immunohistochemical staining (score values) at the tumor center (Table 3). In this tumor area, these factors were predominantly expressed, not only by MICs but also by cancer cells and, in a significant percentage, by fibroblasts, such as previously was reported by our team [22, 30]. Our results demonstrate that the expression of each protein by intratumor MICs was significantly and positively associated with their corresponding score value ($p < 0.005$, for MMP-7 and TIMP-3; and $p < 0.0001$, for MMP-1, 9, 11, 13, and 14, and TIMP-2). Expressions of MMPs and TIMPs by MICs at the invasive front showed significant associations with their corresponding score values for MMP-1, 11 and 13, and TIMP-2 ($p < 0.05$, for each one of these comparisons), and for TIMP-1 ($p < 0.005$). However, expression of MMP-2, 7 and 14, and TIMP-3, by MICs at the invasive front did not show significant associations with their corresponding score value (Table 3).

We also examined the possible relationship between the expression of MMPs or TIMPs by MICs, at the intratumor stroma (Table 4A) or at the invasive front (Table 4B), and the clinico-pathological characteristics of both patients and tumors. As shown in Table 4 (A and B), our results demonstrate a significant relationship of the expression of several MMPs or TIMPs by MICs at the tumoral center, with that at the invasive front, as well as with the clinico-pathological parameters indicative of tumoral aggressiveness (such as younger age from patients, lymph node involvement, higher

histological grade, ER-negative status, desmoplastic reaction, peritumor inflammation or advanced edge tumor). Nevertheless, we also found a significant association of the expression some TIMPs by MICs at the invasive front with parameters indicatives of less tumoral aggressiveness. For instance, TIMP-1 expression was associated with negative nodal status and non-perineural infiltration, and TIMP-3 expression with negative nodal status (Table 4 A and B).

We also analyzed the possible influence of single MMP or TIMP expression by MICs at the invasive front, or at the tumor center, on distant relapse-free survival. As shown in Table 4, multivariate analysis showed that expressions of MMP-9, -11, -13, or -14, TIMP-1 or TIMP-2, by MICs at the tumor center, were significantly associated with a high rate of distant metastases. With regard to MICs at the invasive front, these results demonstrated that expression of MMP-11, 13, or 14, and TIMP-2 by these cells, was also significantly and independently associated with a higher probability of shortened distant relapse-free survival (Table 5).

To identify specific groups of tumors with distinct MMP/TIMP immunohistochemical expression profiles as well as their possible prognostic importance, the obtained data were evaluated by unsupervised hierarchical cluster analysis for each cellular type. This algorithm placed proteins on the horizontal axis and samples on the vertical axis based on similarity of their expression profiles. It produced a dendrogram with well-defined cluster of cases for MICs at the center of the tumor as well as for MICs at the invasive front. Therefore, the dendrogram showed a first-order division of the tumors into distinct MMP/TIMP molecular profiles, two distinct groups for MICs at the center of the tumor (with high or low MMP/TIMP profile, designated as group 1A -n=33- and group 2A -n=91-, respectively) (Figure 2a) and three distinct groups for MICs at the invasive front (with high, intermediate and low MMP/TIMP profiles, designated as group 1B -n=32-, group 2B -n=44- and group 3B -n=48-, respectively) (Figure 2b). MMP-1, 7, 9, 11, 13 and 14, and TIMP-1, 2 and 3, were identified as showing a significantly higher expression in groups 1A and 1B, compared with groups 2A and 3B, respectively (in both MICs populations). In addition, MMP-1, 11 and TIMP-2, were also identified as showing a significantly higher expression in group 2B than in group 3B (in MICs at the invasive front). Likewise, it was also remarkable our finding indicating that patients with both MICs populations belonging

both to the high molecular MMP/TIMP profile expression groups (groups 1A, 1B and 2B), had the highest probability of distant metastases; whereas patients with both MICs populations belonging to the low molecular MMP/TIMP profile groups (groups 2A and 3B) had the lowest probability of distant metastases ($p < 0.0001$) (Table 5 and Figure 3). Multivariate analysis according to Cox model demonstrated that tumor stage (II: (relative risk (RR) (confidence interval (CI)=1.8(0.9-3.6); III: 3(1.4-6.4); $p < 0.001$) and ER status (positive: 0.5(0.3-0.8), $p < 0.001$) were significantly and independently associated with distant relapse-free survival. Nevertheless, this same analysis also demonstrated that clustering for MICs populations was the most potent independent factor associated with distant relapse-free survival (groups 1A and 1B or 2B: 4.8 (2.4-9.5), $p < 0.0001$) (Table 5).

DISCUSSION

This is the first study comparing the expression of MMPs and TIMPs by stromal MICs located at both the intratumor area and at the invasive front of breast carcinomas. Our results demonstrate in a significant number of cases, differences in the expression of these biological factors between intratumor and invasive front MICs. In addition, we have found that those different MIC populations might reflect different biological tumor behaviours and, thus, reinforce complementary clinical information in breast cancer patients.

The inflammatory response to tumor growth may be induced by at least two mechanisms. On the one hand, tumor growth and invasion are associated with significant cell death, some of which occurs by apoptosis or necrosis. It is well known that necrotic cells release numerous mediators of inflammation which are a potent stimulator of macrophages [31]. On the other hand, tumor cells may induce an inflammatory response by secreting cytokines and chemokines that activate macrophages and mast cells and recruit monocytes and lymphocytes [8]. In any case, examination of primary tumors frequently reveals an inflammatory infiltrate that varies in composition and intensity [32]. In addition, the functional role of the cellular components of the inflammatory infiltrates in tumors remains to be fully elucidated, but evidence suggests that it may differ from that observed in the context of physiological tissue repair [8]. Thus, although infiltrates of MICs in the neoplastic microenvironment may be expected to be beneficial for cancer patients, yet it often fails to correlate with good prognosis. Consequently with this latter hypothesis, our results suggest that inflammation can be both cancer stimulatory and cancer inhibitory, depending on the functional status of the infiltrating cell populations.

Our results show levels of discordance in the expressions of MMPs and TIMPs by intratumor MICs and by those located at the invasive front, ranging from 2.5% for MMP-2 to 54.4% for TIMP-3. Likewise, clustering analysis shows two different groups, with low or high MMP/TIMP molecular profile, in both MICs populations, but each one of them with non-identical MMP/TIMP patterns. In addition, this latter analysis led us to identify a third group of tumors, which correspond to these cases with MICs showing an intermediate MMP/TIMP molecular profile at invasive front. Therefore, these findings led us to consider the existence of functional differences in host MICs in these

two tumor areas in a significant percentage of cases, which could represent a new contribution to the knowledge of the stromal heterogeneity of breast carcinomas. Intratumor MICs showed a positive expression of MMP-7 and 14, and TIMP-3 more frequently than MICs at the invasive front. However this latter population of MICs showed more frequent expressions of MMP-9 and 11, and TIMP-2. This different pattern of expression of MMPs and TIMPs may correspond to differences in cellular density, which is higher in the tumor center, and/or to different biological mechanisms of interaction between cancer cells and MICs in those two different tumor areas. Nevertheless, it is noteworthy that in many tumors there was a lack of concordance in the expression of MMPs or TIMPs by cancer cells and MICs, which seems to indicate that these cell types display an independent phenotype with regard to expression of those enzymes. It was also remarkable that, except for MMP-2 and TIMP-1, there were significant and positive relationships between the expression of each MMP/TIMP by intratumoral MICs and their corresponding global expression (score values) in the tumor center; whereas expression of MMPs/TIMPs by MICs at the invasive front only showed significant, but lower, relationships with score values in the tumor center for MMP-1, 11 and 13, and TIMP-1, and 2. Likewise, we found low percentages of concordance in MMPs/TIMPs expression between MICs and cancer cells, both at the tumor center and at the invasive front.

All of these findings add up to the accumulating evidence of the presence of different phenotypes of MICs infiltrating breast carcinomas at two differentiated tumor areas: tumor center and invasive front. Our results also show several significant associations between MMPs/TIMPs expression by MICs with clinico-pathological factors indicative of tumor progression, depending of the tumoral area analyzed. With regard to prognosis, we found a significant value of MMP-11, 13 and 14, and TIMP-2, by MICs both at the tumor center and at the invasive front, as independent predictive factors of distant metastases. However, MMP-9 and TIMP-2 expression by MICs at the center of the tumor, and not at invasive front, were associated with distant metastasis. As of today, we do not have a reasonable explanation for the prognostic significance of the different MIC locations. Even so, our data suggested that host stromal MICs that appear at sites of active tumor invasion may have a different activation status of biological relevance for tumor growth and progression. Likewise, it was remarkable our finding indicating a high prognostic value of the combination of several molecular profiles of MMP/TIMP

expression, based on clustering analysis, of each MICs population. Patients with high MMP/TIMP patterns in the corresponding MICs populations at the center of the tumor, as well as at the invasive front, had the highest probability of distant metastases. On the contrary, patients with low MMP/TIMP patterns in both MICs populations had the lowest risk of distant metastases. Therefore, our results indicate the importance of evaluating the expression of these factors, that are involved in tumor growth, by MICs on a tumor location basis, thus providing a valuable complementary information on tumor behavior.

Our results are in accordance with evidences indicating that MICs can express and secrete a variety of MMPs that together have the potential to hydrolyse all known ECM proteins [33]. In the tumor context, it reduces the physical barriers that tumoral cells have to overcome as they migrate towards lymphatic and blood vessels. In addition, some of the ECM protein degradation products display bioactivity on their own. Indeed, it has been reported that the release of cryptic fragments of laminin as a result of MMP-mediated cleavage of laminin-5 γ 2 chains that mimic EGF receptor (EGFR) ligands, and induce migration and invasive potential in EGFR expressing cells [34]. In addition, it has been reported that leukocyte-derived MMP-7 cleaves and activates cell surface heparin-binding EGF into its bioactive form, which in turn enhances tumor cell migration and survival [35]. Likewise, MMP-7 can cleave E-cadherin, facilitating tumor cell disaggregation and enhancing motility [36]. Therefore, by recruiting and harnessing the inflammatory response, weakly invasive tumors cells may acquire the means to disseminate.

All of these findings led us to formulate the question of whether inflammation and lymphocytic infiltration occur in order to favour the antitumor response to breast cancer or not. Our data suggest that intratumor leukocytes from peripheral blood suffer a modification of their phenotype that allow them to penetrate the tumor architecture, from the invasive front to the tumor center. This seem to be a dynamic processes in which inflammatory cells and immunomodulatory mediators present in the tumor microenvironment polarize host immune response toward specific phenotypes impacting tumor progression. Our results demonstrate biological heterogeneity among tumors with regard to these cellular infiltrates, identifying subsets of MICs, both at tumor center and at invasive front, which seem associated with the occurrence of distant

metastasis. Therefore, our findings are in accordance with these of other authors indicating that leukocyte infiltration can promote tumoral phenotypes, such as angiogenesis, growth, and invasion [4, 5, 37].

In summary, our results reveal dynamic and reciprocal interactions between cancer cells and cells of the tumor microenvironment which may orchestrate events critical to tumor evolution towards metastasis. Likewise, our data may contribute to identify subsets of MICs and protein elements of the microenvironment as attractive targets for therapeutic strategies.

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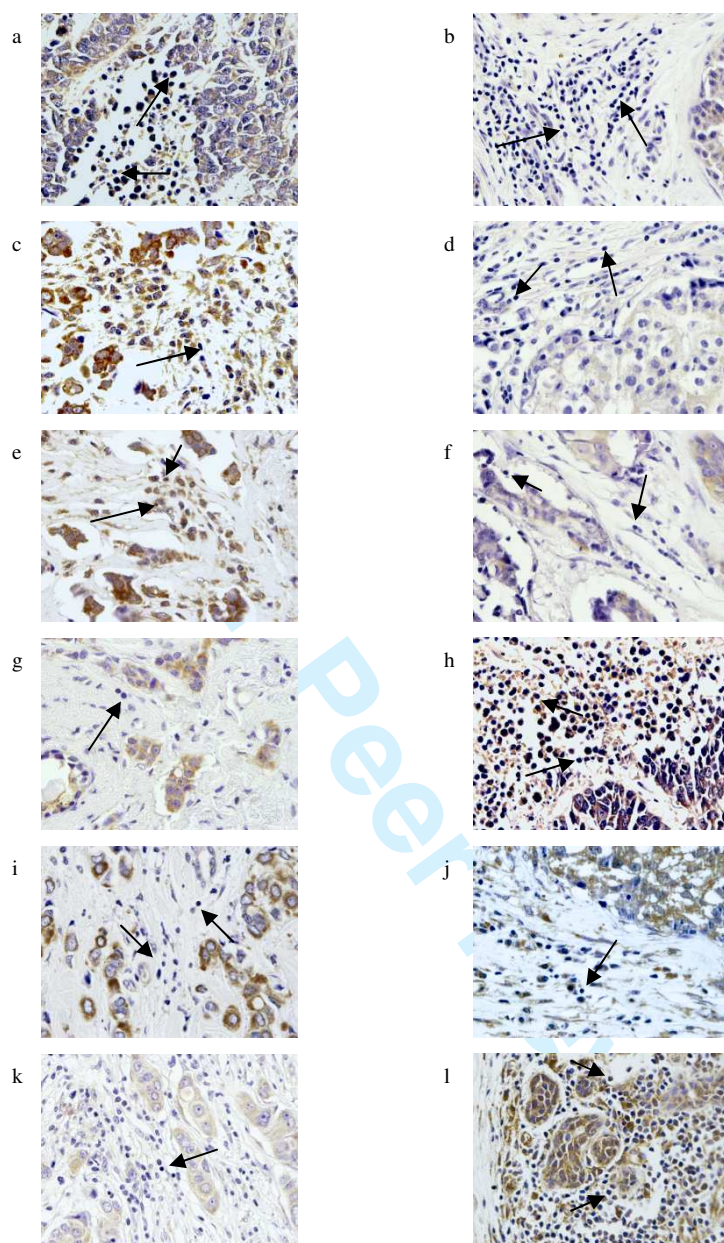


Figure 1. (400x). Comparative expresión of MMPs and TIMPs by MICs (arrows) in the center (left) of the tumor and at the invasive front (right) of breast carcinoma. a-Positive expression of MMP-7; b-No expression of MMP-7;c-Positive expression of MMP-14; d-No expression of MMP-14; e-Positive expression of TIMP-3; f-No expression of TIMP-3; g-No expression of MMP-9; h-Positive expression of MMP-9; i- No expression of MMP-11; j-Positive expression of MMP-11; k-No expression of TIMP-2; l-Positive expression of TIMP-2.

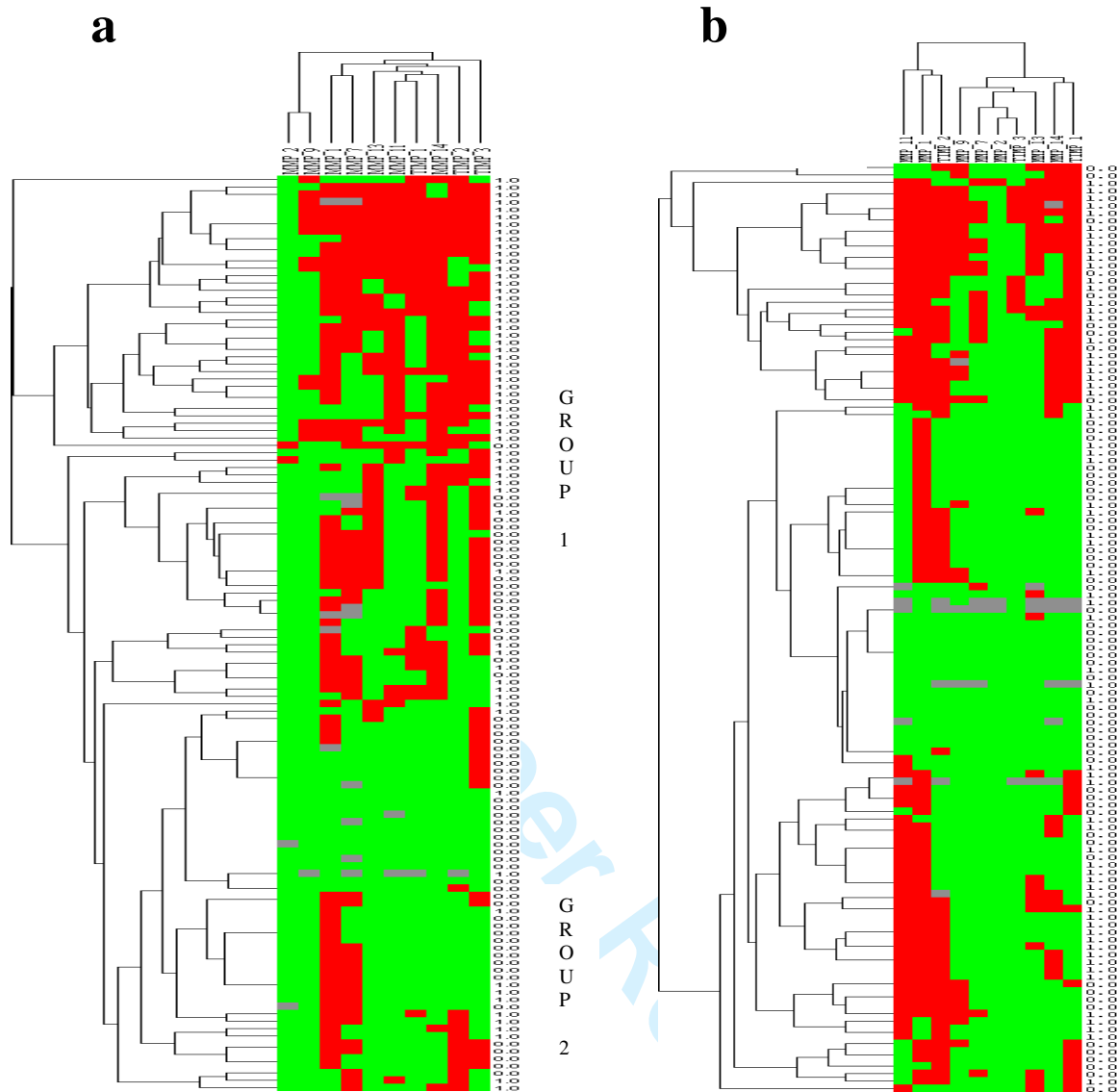


Figure 2: Hierarchical clustering analysis of global MMPs/TIMPs expression in the different cell types of breast cancer as measured by immunohistochemistry on TMA. Graphical representation of hierarchical clustering results in monocites in the center of the tumor (**a**), and monocites at the invasive front (**b**). 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 (1 and 2) are shown in both monocites populations.

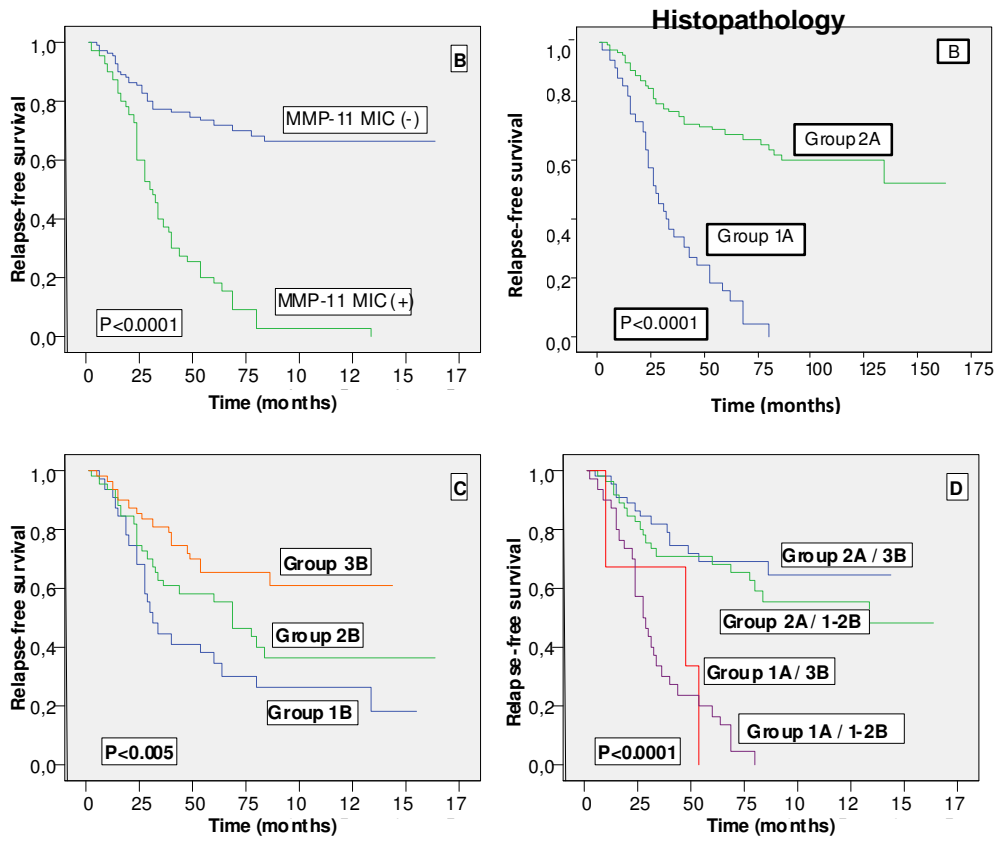


Figure 3. Kaplan-Meier survival curves as function of the immunostaining expression by monocytes at tumoral center of MMP-11 (A); as function of two major clusters of tumors (Group1 and Group 2) shown in monocytes at tumoral center (B), in monocytes at invasive front (C) and in combination of the different cluster groups (D).

TABLE 1 Basal characteristics of 124 patients with invasive ductal carcinoma of the breast.

CHARACTERISTICS	WITHOUT RECURRENCE	WITH RECURRENCE
	N (%)	N (%)
TOTAL CASES		
AGE (YEARS)		
<57	28 (47.5)	38 (58.5)
>57	31 (52.5)	27 (41.5)
MENOPAUSAL STATUS		
PREMENOPAUSAL	18 (30.5)	18 (27.7)
POSTMENOPAUSAL	41 (69.5)	47 (72.3)
TUMORAL SIZE		
T1	31 (52.5)	27 (41.5)
T2	28 (47.5)	38 (58.5)
NODAL STATUS		
N-	28 (47.5)	28 (43.1)
N+	31 (52.5)	37 (56.9)
HISTOLOGICAL GRADE (SBR)		
I	20 (33.9)	14 (21.5)
II	26 (44.1)	35 (53.8)
III	13 (22)	16 (24.6)
NOTTINGHAM PRONOSTIC INDEX		
<3.4	28 (47.5)	17 (26.2)
3.4-5.4	24 (40.7)	35 (53.8)
>5.4	7 (11.9)	13 (20)
ESTROGEN RECEPTORS		
NEGATIVE	18 (30.5)	35 (53.8)
POSITIVE	34 (57.6)	26 (40)
PROGESTERONE RECEPTORS		
NEGATIVE	22 (37.3)	41 (63.1)
POSITIVE	30 (50.8)	20 (30.8)
ADJUVANT RADIOTHERAPY		
NO	44 (74.6)	33 (50.8)
YES	15 (25.4)	32 (49.2)
ADJUVANT SYSTEMIC THERAPY		
CHEMOTHERAPY	18 (30.5)	29 (44.6)
ADJUVANT TAMOXIFEN	24 (40.7)	15 (23.1)
CHEMOTHERAPY+SEQUENTIAL TAMOXIFEN	10 (16.9)	7 (10.8)
NO TREATMENT	7 (11.9)	14 (21.5)

Table 2. Expression of MMPs and TIMPs by monocytes at invasive front and at tumoral center of invasive ductal carcinomas of the breast.

FACTOR	TUMORAL CENTER	INVASIVE FRONT	p VALUE
	N. POSITIVE CASES (%)	N .POSITIVE CASES (%)	
MMP-1	81 (68.1)	87 (73.1)	N.S.
MMP-2	2 (1.7)	1 (0.8)	N.S.
MMP-7	60 (53.6)	17 (15.2)	0.0001
MMP-9	13 (10.8)	28(23.3)	0.016
MMP-11	39 (33.3)	66 (56.4)	0.0001.
MMP-13	40 (33.3)	29 (24.2)	N.S.
MMP-14	61 (51.7)	35 (29.7)	0.0001
TIMP-1	33 (27.5)	47 (39.2)	N.S.
TIMP-2	44 (37.3)	64 (54.2)	0.013
TIMP-3	67 (54.5)	10 (8.1)	0.0001

TABLE 3 Relationship between MMPs and TIMPs expressions by monocytes at tumoral center or at invasive front and global expressions of MMPs and TIMPs (score values) in tumoral center. Data are represented as median of score values (range).

FACTOR	TUMORAL CENTER (SCORE VALUES)			INVASIVE FRONT (SCORE VALUES)		
	MONOCYTES (-)	MONOCYTES (+)	P	MONOCYTES (-)	MONOCYTES(+)	P
MMP-1	45 (0-202)	150 (35-285)	0.0001	128 (0-277.5)	144.9 (0-285)	0.031
MMP-2	0 (0-246)	60.5 (56-65)	0.141	0 (0-246)	0 (0-0)	0.420
MMP-7	68.6 (0-248)	132 (20-270)	0.001	129.1 (0-262.5)	119 (0-270)	0.618
MMP-9	70 (0-273)	156 (56-264)	0.0001	72 (0-237)	72 (0-273)	0.754
MMP-11	138 (0-279)	248.36 (65-276.8)	0.0001	128 (0-279)	165 (0-277)	0.031
MMP-13	56.2 (0-234)	69.1 (49-192.3)	0.0001	59.4 (0-180.3)	67.2 (0-234)	0.038
MMP-14	77 (0-261)	88.9 (61-258.5)	0.0001	83 (0-261)	85 (0-258.5)	0.616
TIMP-1	144 (0-282)	138 (62-285)	0.582	134 (0-270)	168 (0-285)	0.001
TIMP-2	79 (0-243)	144 (52-243)	0.0001	72 (0-243)	127 (0-243)	0.017
TIMP-3	65.7(0-271.3)	136.4(30.5-272.4)	0.002	110.2 (0-272.4)	150.5 (47-271.3)	0.163

Columns represent tumors with monocytes with negative (-) v.s. positive (+) immunostaining for each factor.

TABLE 4A. Significant associations between expressions of MMP or TIMPs by monocytes at tumoral center and clinico-pathological characteristics from breast carcinomas.

FACTORS AND CHARACTERISTICS	N TOTAL OF CASES	N (%) OF POSITIVE CASES	P
TUMORAL CENTER			
MMP-1			
NODAL STATUS			
N-	54	31 (57.4)	0.023
N+	65	50 (76.9)	
DESMOPLASTIC REACTION			
YES	80	59 (73.8)	0.057
NOT	39	22 (56.4)	
MITOSIS			
<10	67	40 (59.7)	0.043
>10	49	38 (77.6)	
MMP-7			
NODAL STATUS			
N-	52	20 (38.5)	0.004
N+	63	41 (65.1)	
MMP-9			
PERITUMORAL INFLAMATION			
YES	47	8 (17)	0.012
NOT	72	6 (8.3)	
MMP-11			
AGE			
<57	65	27 (41.5)	0.028
>57	57	13 (22.8)	
PERITUMORAL INFLAMATION			
YES	47	23 (48.9)	0.011
NOT	71	17 (23.9)	
MMP-13			
DESMOPLASTIC REACTION			
YES	84	37 (44)	0.001
NOT	40	6 (15)	
EDGE OF ADVENCING TUMORAL			
EXPANSIVE	53	9 (17)	0.0001
INFILTRATING	68	34 (50)	
PERINEURAL INFILTRATION			
YES	11	7 (63.6)	0.035
NOT	113	36 (31.9)	
MMP-14			
NODAL STATUS			
N-	56	20 (35.7)	0.0001
N+	68	46 (67.6)	
TUMORAL SIZE			
T1	58	25 (43.1)	0.034
T2	66	41 (62.1)	
DESMOPLASTIC REACTION			
YES	84	53 (63.1)	0.001
NOT	40	13 (32.5)	
EDGE OF ADVENCING TUMORAL			
EXPANSIVE	53	22 (41.5)	0.011
INFILTRATING	68	44 (64.7)	
PERITUMORAL INFLAMATION			
YES	47	33 (70.2)	0.022
NOT	73	33 (45.2)	
MENOPAUSE			
PREMENOPAUSE	36	14 (38.9)	0.041
POSTMENOPAUSE	88	52 (59.1)	
TUMORAL NECROSIS			
YES	14	12 (85.7)	0.041
NOT	107	54 (50.5)	
TIMP-2			
PERITUMORAL INFLAMATION			
YES	47	24 (51)	0.046
NOT	72	22 (30.6)	
TIMP-3			
AGE			
<57	66	42 (63.6)	0.036
>57	58	26 (44.8)	
DESMOPLASTIC REACTION			
YES	84	53 (63.1)	0.007
NOT	40	15 (37.5)	
EDGE OF ADVENCING TUMORAL			
EXPANSIVE	53	23 (43.4)	0.019
INFILTRATING	68	44 (64.7)	

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TABLE 4B. Significant associations between expressions of MMP or TIMPs by monocytes at invasive front and clinico-pathological characteristics from breast carcinomas

FACTORS AND CHARACTERISTICS	N TOTAL OF CASES	N (%) OF POSITIVE CASES	P
INVASIVE FRONT			
MMP-1			
AGE			
<57	66	53 (80.3)	0.040
>57	58	37 (63.8)	
MMP-7			
NODAL STATUS			
N-	55	13 (23.6)	0.029
N+	66	6 (9.1)	
MMP-9			
PROGESTERONE RECEPTORS			
POSITIVE	48	7 (14.6)	0.049
NEGATIVE	62	19 (30.6)	
TUMORAL NECROSIS			
YES	14	4 (28.5)	0.034
NOT	104	24 (23.1)	
MMP-11			
PERITUMORAL INFLAMMATION			
YES	46	33 (71.7)	0.028
NOT	70	33 (47.1)	
ESTROGEN RECEPTORS			
POSITIVE	57	26 (45.6)	0.038
NEGATIVE	52	34 (65.4)	
MITOSIS			
<10	65	31 (47.7)	0.033
>10	52	35 (67.3)	
MMP-13			
NODAL STATUS			
N-	54	18 (33.3)	0.034
N+	66	11 (16.7)	
TIMP-1			
NODAL STATUS			
N-	55	29 (52.7)	0.004
N+	66	18 (27.3)	
DESMOPLASTIC REACTION			
YES	81	26 (32.1)	0.003
NOT	40	21 (52.5)	
PERINEURAL INFILTRATION			
YES	11	1 (9.1)	0.034
NOT	110	46 (41.8)	
SBR			
I	33	13 (39.4)	0.015
II	60	17 (28.3)	
III	28	17 (60.7)	
TIMP-2			
AGE			
<57	64	41 (64.1)	0.026
>57	55	24 (43.6)	
PERITUMORAL INFLAMMATION			
YES	46	33 (71.7)	0.010
NOT	69	30 (43.5)	
TIMP-3			
NODAL STATUS			
N-	55	10 (18.2)	0.0001
N+	68	0 (0)	
MENOPAUSE			
PREMENOPAUSE	36	0 (0)	0.034
POSTMENOPAUSE	87	10 (11.5)	

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TABLE 5: Cox's univariate(HR) and multivariate(RR) analysis of the relationship between MMPs and TIMPs expression and relapse-free survival in Monocytes.

FACTOR		N of Patients	Event Frequency	HR (95%CI)	RR (95%CI)
MMP-1	Monocytes (-)vs(+) at tumoral center	38/81	17/47	1.61 (0.92-2.8)	
	Monocytes (-)vs(+)at invasive front	34/90	12/53	1.83 (0.97-3.43)	
MMP-2	Monocytes (-)vs(+) at tumoral center	120/2	64/1	0.78(0.1-5.64)	
	Monocytes (-)vs(+) at invasive front	121/1	63/1	1.04 (0.14-7.61)	
MMP-7	Monocytes (-)vs(+) at tumoral center	54/61	25/38	1.47(0.88-2.44)	
	Monocytes (-)vs(+) at invasive front	102/19	52/11	1.09 (0.59-2.11)	
MMP-9	Monocytes (-)vs(+) at tumoral center	109/14	50/14	3.4 (1.84-6.3)*	3.23(1.71-6.1)*
	Monocytes (-)vs(+) at invasive front	93/28	46/17	1.44 (0.82-2.51)	
MMP-11	Monocytes (-)vs(+) at tumoral center	82/40	25/39	5.41(3.21-9.1)*	5.18(3.02-8.87)*
	Monocytes (-)vs(+) at invasive front	53/66	16/47	3.36(1.89-5.95)*	3.02 (1.66-5.5)*
MMP-13	Monocytes (-)vs(+)i at tumoral center	81/43	35/30	2.03(1.23-3.3)**	2.2(1.3-3.7)**
	Monocytes (-)vs(+) at invasive front	91/29	40/23	2.27(1.35-3.8)**	2.5(1.43-4.36)*
MMP-14	Monocytes (-)vs(+) at tumoral center	58/66	16/49	4.4 (2.43-7.97)*	4.44(2.36-8.35)**
	Monocytes (-)vs(+) at invasive front	83/35	33/28	2.9(1.7-4.8)*	3.26(1.89-5.6)*
TIMP-1	Monocytes (-)vs(+) at tumoral center	90/33	36/28	2.52(1.54-4.18)*	2.14(1.27-3.61)**
	Monocytes (-)vs(+) at invasive front	74/47	36/27	1.23 (0.74-2.04)	
TIMP-2	Monocytes (-)vs(+)i at tumoral center	76/47	24/40	3.78(2.26-6.3)*	4.3(2.5-7.38)*
	Monocytes (-)vs(+) at invasive front	54/65	20/41	2.01 (1.17-3.44)***	2.25 (1.28-3.96)**
TIMP-3	Monocytes (-)vs(+) at tumoral center	56/68	28/37	1.12(0.68-1.83)	
	Monocytes (-)vs(+) at invasive front	113/10	56/8	1.94 (0.92-4.09)	
Cluster Group at tumoral center					
Group 1A vs. Group 2A		32/91	33/33	5 (3-8.5)	4.7 (2.7-8)*
Cluster Group at invasive front					
Group 3B (low)		32	23	1	1
Group 2B (intermediate)		44	25	2.7 (1.4-5.1)	1.6 (0.8-3.1)
Group 1B (high)		48	17	1.8 (0.9-3.3)	2.2 (1.1-4.4)**
Combination of Cluster Groups*					
Group 2A and Group 3B		44	14	1	1
Group 2A and Group 1B or 2B		47	19	1.3 (0.6-2.6)	1.1 (0.5-2.3)
Group 1A and Group 3B		3	3	5.5 (1.5-19.4)	9.9 (2.6-37.6)
Group 1A and Group 1B or 2B		30	29	5.8 (3-11.3)	4.8 (2.4-9.5)*

Abbreviations: HR, hazard ratio; RR, relative risk; CI, confidence interval; MIC, mononuclear inflammatory cells.

*p<0.001; **p<0.005; ***p<0.01; ****p<0.05