

Worse prognosis in breast cancer patients can be predicted by immunohistochemical analysis of positive MMP-2 and negative estrogen and progesterone receptors

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SUMMARY

Introduction: Breast cancer is the most cause of death, and approximately 90% of these deaths are due to metastases. Matrix metalloproteinase-2 (MMP-2) gelatinase activity is able to degrade a major constituent of the tumor microenvironment, type IV collagen. Two well-established proteins used as markers in clinical practice for breast cancer are the receptors for estrogen (ER) and progesterone (PR). Although the presence of these receptors has been associated with a better prognosis, loss of these proteins can occur during tumor progression, with subsequent resistance to hormone therapy.

Objective: To study the correlation among MMP-2, ER, and PR, as well as the establishment of the metastatic process in primary breast tumors.

Method: Breast cancer samples (n=44) were analyzed by immunohistochemistry for MMP-2, ER, and PR.

Results: We observed that 90% of patients who had metastases and died showed positive staining for MMP-2 (p=0.0082 for both). Using Kaplan-Meier analysis, we found that negative ER patients who were also positive for MMP-2 had even worse disease-free survival (DFS) and overall survival (OS) (p= 0.012 and p=0.005, respectively). Similar results were found in PR-negative patients for DFS (a trend p=0.077) and OS (p=0.038).

Conclusion: Regardless of our small sample size (n=44), the data obtained strongly suggest that MMP-2 in combination with already well-established markers could help to predict the emergence of metastases and death in patients with breast cancer.

Keywords: breast neoplasms, MMP-2, immunohistochemical, molecular marker.

Study conducted at Hospital Nossa Senhora das Graças and at Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil

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INTRODUCTION

Cancer is the leading cause of death in developed and developing countries and breast cancer is the second cause of cancer death among women. In Brazil, similarly to other countries, breast cancer is the most frequent and main cause of cancer death among women after lung and bronchus neoplasms.¹ The development of metastasis with the formation of secondary tumors in distant sites is responsible for over 90% of the mortality related to cancer and may occur years or decades after the primary

diagnosis or even after removal of the primary tumor and the initiation of adjuvant therapy.^{2,3} Indeed, metastasis in breast cancer can be undetectable and remain dormant for many years after the removal of the primary tumor, contributing to the poor survival and low life expectancy of these patients.⁴ Many steps are involved in this process, and other authors have described that chemokines,^{5,6} adhesion proteins,⁷ and metalloproteinases⁸⁻¹⁰ are the most important molecules for the migration of breast cancer cells and establishment of metastasis.

MMPs are involved in normal physiological processes required for development and morphogenesis. A loss of control of MMPs can result in pathological processes including inflammation, angiogenesis, cellular proliferation^{11,12} and are directly involved in the metastatic tumor cell invasion process.⁹ Among them, gelatinase A (MMP-2) is associated with a malignant phenotype, as it is responsible for the degradation of collagen fibers around tumors, thereby releasing these cells from the microenvironment in which they are established. This function is directly involved in the progress of metastasis.⁹ MMP-2 is associated with tumor cell invasion and metastasis in several cancers, including melanoma,¹³ pancreatic,¹⁴ lung,¹⁵ ovary,¹⁶ prostate, and breast¹⁷ cancer.

Hormone receptors for estrogen (ER) and progesterone (PR) are widely used in the diagnosis and treatment of patients with breast cancer. The main therapeutic approach is endocrine therapy in patients with early and advanced stages of breast cancer and has been used for over five decades.¹⁸ Authors indicate that both ER and MMP-2 have an important signaling for breast cancer establishment.¹⁹

Our study evaluated the expression of MMP-2, ER, PR, and HER2 proteins by immunohistochemistry (IHC) in association with the clinical pathological variables of patients as well as the rates of overall survival and disease-free survival in breast cancer. Studies using this approach are scarce, and our results showed that the presence of MMP-2 and absence of ER and PR in primary breast tumors confer susceptibility to metastasis and the consequent death of patients. The inclusion of MMP-2 as a molecular marker could ultimately help in characterizing the disease and guiding the treatment of patients with breast cancer.

METHOD

Patients

In this study, breast cancer tumors from patients embedded in paraffin (n=44) were used for the immunohistochemistry analysis. The samples were obtained from patients undergoing surgery for the removal of the primary tumor at Nossa Senhora das Graças Hospital, Curitiba, Paraná state, Brazil. All patients signed an informed consent for the retention and analysis of tissues for research purposes according to the Ethics Committee. The age of the patients ranged from 27 to 84 years old (average 57.8±14.7). The histological types included invasive ductal carcinoma (IDC 77%) and invasive lobular carcinoma (ILC 23%). The histological grade (SBR) was determined according to the Bloom-Richardson criteria. TNM stage was determined according to the classification of the World Health Organization (WHO). The clinicopathological data of the patients are shown in Table 1.

Ethics Committee

All patients received and signed a legal consent form releasing their samples for scientific research purposes. The National Committee of Ethics in Research (CONEP) approved this study under the case number 25000.007020/2003-93. The institutional approval was given by the Ethics Committee on Research with Human Beings at the Federal University of Paraná (UFPR) under the registration number 7220-251/2003 (20/02/2003).

Immunohistochemical analysis (IHC)

For each sample, a morphological assessment was carried out using a 5 µm tissue section stained with hematoxylin-eosin, and the sections were used for MMP-2, ER, and PR immunostaining using the avidin-biotin immunoperoxidase technique (Polyvalent immunoperoxidase kit; Omnitag, Lipshaw, MI, USA). Endogenous peroxidase was inactivated by incubation with H₂O₂/methanol, and each slide was treated with an antigen unmasking solution (sodium citrate 10 mM plus Tween 20). After blocking, the slides were washed in PBS with 1% bovine serum albumin and first incubated with the primary antibody and then incubated with a universal secondary biotinylated polyvalent antibody. The primary antibodies used were as follows: polyclonal rabbit anti-MMP-2/72 kDa Collagenase IV (MyBioSource®, dilution 1:400); monoclonal mouse anti-estrogen receptor Clone 1D5 (Dako®); monoclonal mouse anti-progesterone receptor Clone PgR636 (Dako®). The chromogenic reaction was developed by incubation with a freshly prepared solution 3-amino-9-ethylcarbazol (AEC) and counterstained with hematoxylin before mounting. The cutoff considered in the analysis of MMP-2 was 10% of the cells, as this value is used for the breast cancer markers ER and PR in the medical literature and in clinical practice. For negative controls, instead of the primary antibody, the breast tumor sections were treated with rat IgG at the same concentration used for all antibodies. HER2 detection was performed according to the HercepTest™ protocol (DAKO CYTOMATION code K5204) (Figure 1). HER2 was analyzed in terms of membrane staining on a 0-3+ scale according to the manufacturer's recommendations. In patients with 2+ HER2 expression, an additional chromogenic *in situ* hybridization test, HER2FISH pharmaDx™ (DAKO CYTOMATION code K5331), was performed. Two pathologists interpreted the slides in an independent manner. The slides were observed using a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE) equipped with automated scanning VSlide (Metasystem).

TABLE 1 Clinicopathological features of the 44 patients with primary breast carcinomas according to immunohistochemical status of MMP-2, ER and PR proteins.

Variables	Samples (%)	MMP-2		p	Estrogen receptor (ER)		p	Progesterone receptor (PR)		p
		Negative	Positive		Negative	Positive		Negative	Positive	
Stage										
I	11 (25)	6 (55)	5 (45)	0.1172	2 (18)	9 (82)	0.0263	2 (18)	9 (82)	0.0641
II	20 (45)	6 (30)	14 (70)		4 (20)	16 (80)		6 (30)	14 (70)	
III/IV	13 (30)	2 (15)	11 (85)		8 (62)	5 (38)		8 (62)	5 (38)	
Tumor size										
pT1	10 (23)	4 (40)	6 (60)	0.5998	0	10 (100)	0.0112	1 (10)	9 (90)	0.0994
pT2	24 (54)	8 (33)	16 (67)		10 (42)	14 (58)		11 (46)	13 (54)	
pT3/pT4	10 (23)	2 (20)	8 (80)		4 (40)	6 (60)		4 (40)	6 (60)	
SBR										
I	11 (25)	6 (55)	5 (45)	0.1172	2 (18)	9 (82)	0.0263	2 (18)	9 (82)	0.0641
II	20 (45)	6 (30)	14 (70)		4 (20)	16 (80)		6 (30)	14 (70)	
III	13 (30)	2 (14)	11 (86)		8 (62)	5 (38)		8 (62)	5 (38)	
Lymph node status										
Positive	23 (52)	7 (30)	16 (70)	1.0000	9 (39)	14 (61)	0.3419	9 (39)	14 (61)	1.0000
Negative	21 (48)	7 (33)	14 (67)		5 (24)	16 (76)		12 (57)	9 (43)	
HER-2										
Positive	13 (30)	6 (46)	7 (54)	0.2878	4 (31)	9 (69)	1.0000	4 (31)	9 (69)	0.7385
Negative	31 (70)	8 (26)	23 (74)		10 (32)	21 (68)		12 (39)	19 (61)	
MMP-2										
Positive	30 (68)	-	-		14 (47)	16 (53)	0.0015	15 (50)	15 (50)	0.0071
Negative	14 (32)	-	-		0	14 (100)		1 (7)	13 (93)	
Progesterone receptor (PR)										
Positive	28 (64)	13 (46)	15 (54)	0.0071	0	28 (100)	<0.0001	-	-	
Negative	16 (36)	1 (6)	15 (94)		14 (88)	2 (12)		-	-	
Estrogen receptor (ER)										
Positive	30 (68)	14 (47)	16 (53)	0.0015	-	-		2 (6)	28 (94)	<0.0001
Negative	14 (32)	0	14 (100)		-	-		14 (100)	0	
Metastasis										
Positive	20 (43)	2 (5)	18 (90)	0.0082	9 (45)	11 (55)	0.1121	9 (45)	11 (55)	0.3520
Negative	24 (57)	12 (50)	12 (50)		5 (21)	19 (89)		7 (32)	17 (68)	
Death										
Positive	20 (45)	2 (10)	18 (90)	0.0082	10 (50)	10 (50)	0.0253	10 (50)	10 (50)	0.1196
Negative	24 (55)	12 (50)	12 (50)		4 (27)	20 (73)		6 (33)	18 (67)	
Histological type										
IDC	34 (77)	12 (35)	22 (65)	0.4618	10 (29)	24 (71)	0.7013	10 (29)	24 (71)	0.1331
ILC	10 (23)	2 (20)	8 (80)		4 (40)	6 (60)		6 (60)	4 (40)	

The bold represents p-value<0.05.

MMP-2: matrix metalloproteinase-2; SBR: Scarff-Bloom-Richardson grade; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma.

Statistical analysis

Statistical analyses were performed using SPSS software (version 18.0, SPSS Inc., Chicago, IL, USA). Associations among the clinical pathological parameters were evaluated using chi-square (χ^2) or Fisher's exact test. Values were considered significant if $p < 0.05$. For the analysis of survival, time was calculated using the date of disease diagnosis and the occurrence of death or metastasis. Statistical tests by Kaplan-Meier analysis were considered significant if $p < 0.05$.²⁰ For this analysis, the time interval was 125 months, and the data were collected between 2004 and 2014.

RESULTS

Immunohistochemical data analyses

A total of 44 patients, with a mean age of 57.8 years old (range 27 to 84), were enrolled in the study. The clinical data available were stage, tumor size, tumor grade (SBR – Scarff-Bloom-Richardson grade), lymph node, metastasis, death and histological type, as well as IHC staining for human epidermal growth factor receptor-2 (HER2), MMP-2, estrogen receptor (ER) and progesterone receptor (PR), as shown in Table 1. Representative slides of breast tumor samples with negative or positive staining are presented in Figure 1.

MMP-2 expression in tumor cells was positive in 68% of patients (Table 1) but not associated with the factors of stage, tumor size, SBR, lymph node status, HER2 or histological type. In contrast, the presence of MMP-2 was significantly associated with PR ($p = 0.0071$), ER ($p = 0.0015$), metastasis ($p = 0.0082$), and death ($p = 0.0082$).

Expression of ER in tumor cells was found in 68% of patients (Table 1). The presence of ER was not related to lymph node status, HER2, metastasis or histological type, but there was an association with the variables stage ($p = 0.0263$), tumor size ($p = 0.0112$), SBR ($p = 0.0263$), MMP-2 ($p = 0.0015$), PR ($p < 0.0001$), and death ($p = 0.0253$).

PR-positive tumor cells were found in 64% of patients (Table 1). The presence of PR was not associated with stage, tumor size, SBR, lymph node status, HER2, metastasis, death or histological type, but an association with MMP-2 ($p = 0.0071$) and ER ($p < 0.0001$) was observed.

A significant association was found between some of the clinical features and IHC data (Table 1). All patients negative for MMP-2 on IHC were positive for ER ($p = 0.0015$), and 93% of them were also PR positive ($p = 0.0071$) (Table 1). Ninety percent (90%) of the patients that developed metastases and/or died were MMP-2 positive ($p = 0.0082$, for both analysis). In addition, 94% of the PR-negative and 100% of the ER-negative patients were MMP-2-positive

($p = 0.0071$ and $p = 0.0015$, respectively). These results suggested that these proteins at the same time could be useful for metastasis or death prognosis in breast cancer. To confirm this hypothesis, statistical tests were performed using the Kaplan-Meier method to assess disease-free survival and overall survival in these samples. The patients who showed positive staining for MMP-2 in IHC assays had lower disease-free survival ($p = 0.004$) (Figure 2 A) and overall survival rates ($p = 0.007$) (Figure 2 B). Interestingly, ER-negative patients who were simultaneously positive for MMP-2 had worse disease-free survival and overall survival rates ($p = 0.012$ and $p = 0.005$, respectively) (Figure 2 C and D, respectively). Similar results were found for the presence of MMP-2 and the absence of PR with regard to the trends of disease-free survival ($p = 0.077$) and overall survival ($p = 0.038$) (Figure 2 E and F, respectively).

DISCUSSION

Breast cancer is the leading cause of cancer-related death in women worldwide.² Breast cancer metastases may be undetectable and remain dormant for many years after the removal of the primary tumor, emerging years later as an incurable disease.⁷ Although certain molecular markers of proliferative disease are already used in the diagnosis and prognosis of breast cancer, including estrogen receptor (ER), progesterone receptor (PR), and HER2 overexpression, new metastasis markers must be investigated for future use. MMP-2 is a protein associated with metastasis and is extensively studied in several cancers.¹¹⁻¹⁶ ER and MMP-2 have shown a molecular relationship with E2 or estradiol binding in the E domains of ER, present in the plasma membrane. This interaction could activate MMP-2 and MMP-9 with subsequent transactivation of EGFR. This mechanism after ER and MMP-2 interaction supports the proliferation and survival of tumor cells in the absence of nuclear ER.^{21,22}

In our work, we evaluated the presence of MMP-2 using IHC and its correlation with ER and PR as prognostic factors for metastasis in breast cancer. The ER analyses showed a significant association with factors such as stage, tumor size, SBR grade, MMP-2, PR staining, and death (Table 1), while PR showed significance regarding MMP-2 and ER staining (Table 1). We observed that 90% of patients who had metastases and died were MMP-2 positive ($p = 0.0082$). Additionally, 94 and 100% of patients negative for PR or ER, respectively, were positive for MMP-2 (Table 1).

Other works report weak or non significant impact of ER on overall survival in lymph node-negative populations, and suggest that another molecular marker can

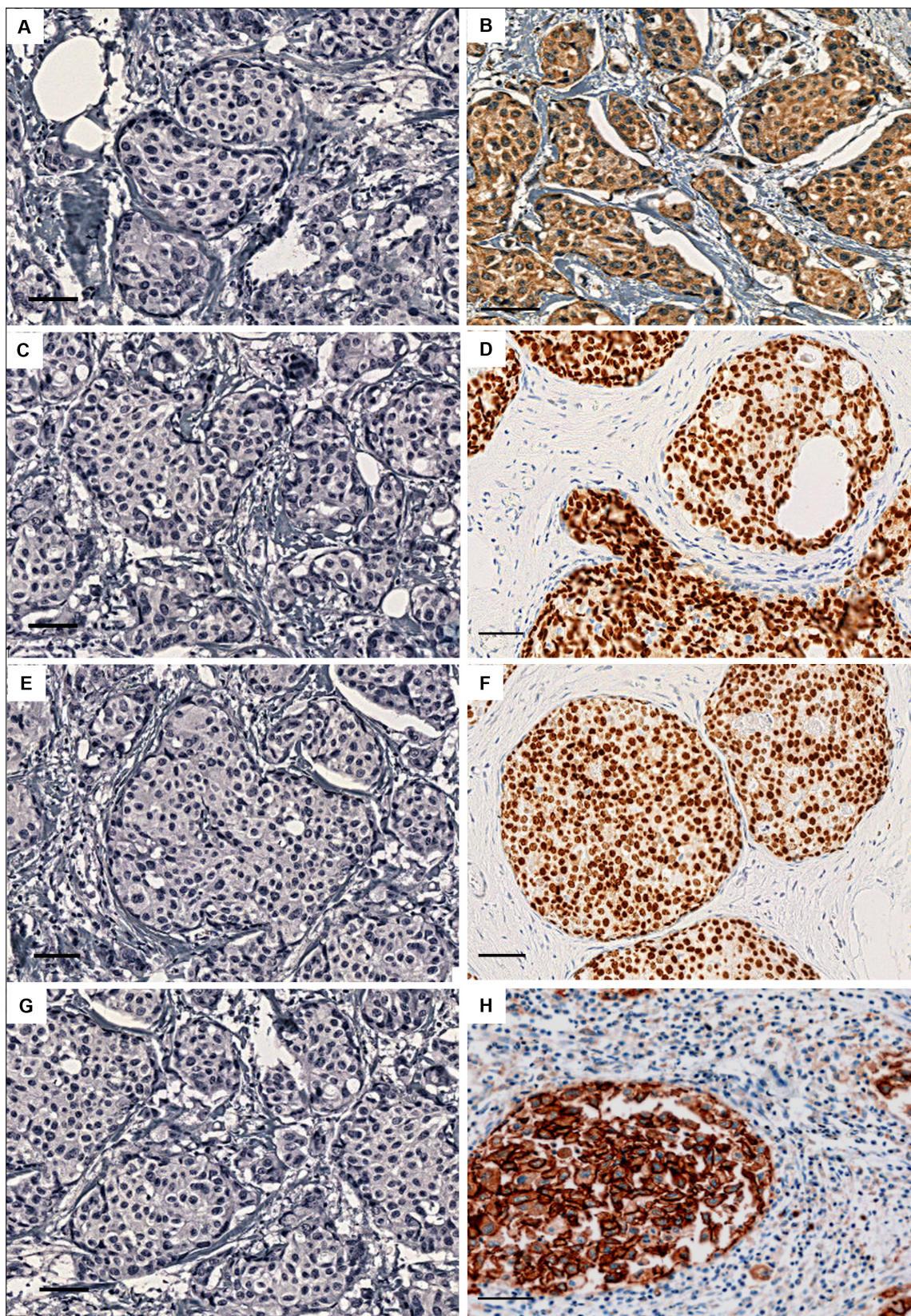


FIGURE 1 Representative examples of immunostaining for the protein markers tested in breast cancer. Negative or positive staining, respectively: MMP-2 (A, B), ER (C, D), PR (E, F), and HER2 (G, H). All the slides were analyzed in x 200 microscope objective and the scale is 50 μ m.

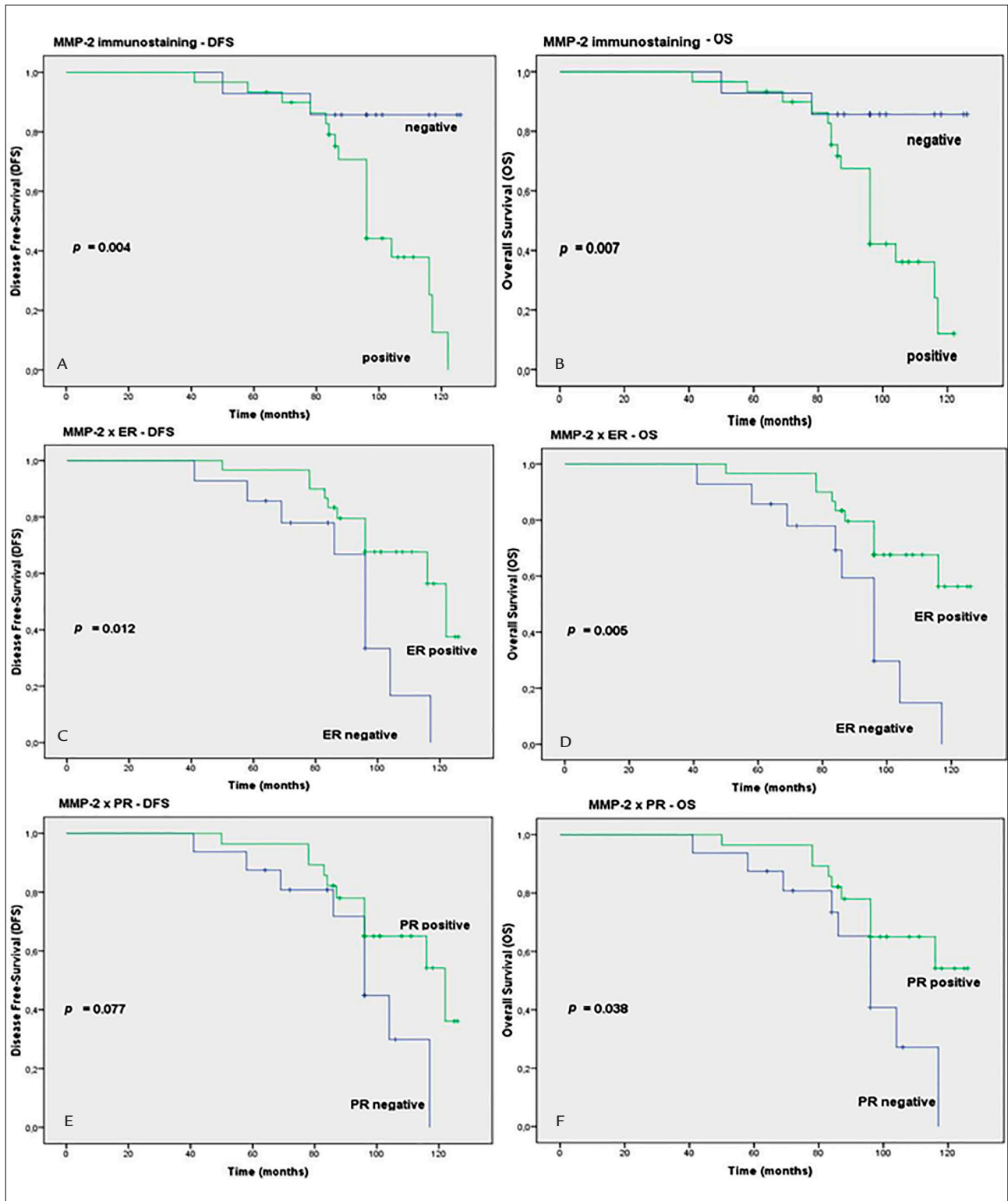


FIGURE 2 Kaplan-Meier curves for disease-free survival (DFS) or overall survival (OS) according to the MMP-2 IHC staining and its correlation with ER and PR. MMP-2 staining and the correlation with (A) DFS and (B) OS are shown. The DFS relationship between MMP-2 positive and ER is shown in (C) and OS relationship between MMP-2 positive and ER is shown in (D). The DFS relationship between MMP-2-positive and PR is shown in (E) and the OS relationship between MMP-2-positive and PR is shown in (F). MMP-2: matrix metalloproteinase-2; ER: estrogen receptor; PR: progesterone receptor.

better predict survival in breast cancer patients.²³ Our Brazilian sample does not have ER or PR significantly associated with lymph node, except if MMP-2 is present.

MMP-2 was associated with many aspects of breast cancer prognosis including unique associations with ER/PR tumor status as well as with survival. Our analysis used a Kaplan-Meier test to demonstrate that the presence of MMP-2 was connected to disease-free survival (DFS) ($p=0.004$) (Figure 2 A) and overall survival (OS) ($p=0.007$). Additionally, both DFS and OS were worse in the presence of MMP-2 and absence of ER ($p=0.012$ and $p=0.005$, respectively) (Figure 2 C and D). Ranogajec et al. (2012) studied MMP-2 in breast cancer, and OS was significantly shorter in patients with positive MMP-2.²⁴ Recently, Min et al.¹⁷ correlated the MMP-2 expression pattern with ER and PR, but did not show a correlation with DFS or OS. In another study that used single nucleotide polymorphism analysis (SNP), the authors suggest that MMP-2 can influence patient survival as an independent factor, attenuating the metastatic potential and augmenting survival rates. These authors also suggest that the MMP-2 gene is an important pathway for tumor progression and invasiveness in ER+/PR+ and ER+/PR- samples.¹² Although based on a smaller sample, our study shows that ER-positive patients did not express MMP-2, while MMP-2-positive patients are ER-negative. A lack of MMP-2 could possibly become an important factor in certain subgroups of breast carcinoma while selecting the adjuvant therapy. Our results support the thesis that MMP-2 analysis in connection with ER could be auxiliary for disease diagnosis.

Several authors report a weak or non-significant impact of ER on OS in node-negative populations.

In this work, for the first time, we establish the relevance of a simultaneous evaluation of MMP-2, ER and PR using IHC to predict the onset of metastases and death, i.e., a poor prognosis in breast cancer patients.

CONCLUSION

Our results demonstrate the importance of evaluating the relationship between proliferative markers widely used in clinical practice with a possible new marker of metastasis in breast cancer. To summarize, our data show that the IHC analysis of MMP-2 could be included as a new molecular biomarker in breast cancer, improving the prognostic value for metastasis and survival when associated with the traditional ER and PR markers. The clinical importance of this new marker can possibly be extended to create new patient subgroups for better disease characterization.

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RESUMO

Pacientes com câncer de mama de pior prognóstico podem ser estimadas por análise imuno-histoquímica de MMP-2 positiva e receptores de estrógeno e progesterona negativos

Introdução: o câncer de mama é a segunda causa de morte no mundo, sendo 90% dessas mortes decorrentes de metástases. A metaloprotease de matriz 2 (MMP-2) possui atividade de gelatinase capaz de degradar o principal constituinte do microambiente tumoral, o colágeno do tipo IV. Há duas proteínas bem estabelecidas utilizadas como marcadores na prática clínica para o câncer de mama, os receptores de estrógeno (RE) e de progesterona (RP). Embora a presença desses receptores tenha sido associada a um melhor prognóstico, a perda delas pode ocorrer durante a progressão do tumor, com subsequente resistência à terapia hormonal.

Objetivo: analisar a correlação entre as proteínas MMP-2, RE e RP por imuno-histoquímica e estabelecer o processo metastático em tumores de mama primários.

Método: amostras de tumor de mama ($n=44$) foram analisadas por imuno-histoquímica para MMP-2, receptor de estrógeno e progesterona.

Resultados: observou-se que 90% das pacientes que tinham metástases e morreram apresentaram coloração positiva para a MMP-2 ($p=0,0082$ para ambos). Usando a análise de Kaplan-Meier, verificou-se que as pacientes RE negativas, também positivas para MMP-2, apresentaram sobrevida livre de doença (SLD) e sobrevida global (SG) ($p=0,012$ e $p=0,005$, respectivamente) piores quando comparadas às pacientes MMP-2 negativas. Resultados semelhantes foram encontrados em pacientes RP negativas para SLD ($p=0,077$) e SG ($p=0,038$).

Conclusão: embora o número de amostras avaliadas tenha sido baixo ($n=44$), esses dados iniciais permitem inferir que a MMP-2 em combinação com os marcadores já bem estabelecidos poderia ajudar na previsão do surgimento de metástase e morte em pacientes com câncer de mama.

Palavras-chave: neoplasias da mama, MMP-2, imuno-histoquímica, marcador molecular.

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