Artigo Original

THE INTERACTION BETWEEN AROMATASE, METALLOPROTEINASE 2,9 AND CD44 IN BREAST CANCER

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SUMMARY

Objective. This study intends to verify the expression levels and correlation of aromatase, matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9) and CD44 in ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC) when both are found in the same breast.

Methods. One hundred and ten cases were evaluated by tissue microarray (TMA) and immunohistochemically screened with anti-aromatase polyclonal antibodies, anti-MMP-2 monoclonal antibodies, anti-MMP-9 policlonal antibodies and anti-CD44 monoclonal antibodies.

Results. Aromatase was expressed in IDC and DCIS in 63 (57.3%) and 60 (67%) of the cases respectively; MMP-2 was similarly expressed in IDC and DCIS in 15 (13.60%) cases; MMP-9 was positively expressed in IDC and DCIS in 83 (75.50%) and 82 (74.50%) cases, respectively; CD44 was positively expressed in IDC and DCIS in 49 (44.50%) and 48 (42.60%) of the cases, respectively; all of them were highly correlated ($p<0,001$). The correlation analysis found positive, statistically significant correlation, in IDC between aromatase and MMP-2 ($p<0.001$) and between aromatase and MMP-9 ($p=0.034$). Positive correlation between aromatase and MMP-2 ($p<0.001$) and between MMP-9 and CD44 ($p=0.030$) were found in DCIS.

Conclusion. These results allow us to conclude that aromatase through local estrogen synthesis in breast tissue plays an important role in breast carcinogenesis, mainly influencing MMP-2 and MMP-9 which are important participants in tumor cell invasion and dependence of their connection to CD44 for action.

Key words: Aromatase. Matrix metalloproteinase 2. Matrix metalloproteinase 9. Antigens CD44. Carcinoma ductal breast.

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Introduction

It is well established that estradiol (E2) plays an important role in the genesis and progression of breast cancer[1,2,3]. Almost 70% of breast cancer occurs in postmenopausal women and approximately 75% are estrogen dependent showing that aromatase is very important for carcinogenesis in these women[4,5].

Estrogen metabolism is mediated by a number of enzymes. Aromatase is a cytochrome P-450 family coded by the CYP19 gene that catalyses the conversion of androstenedione into oestrone (E1) and testosterone into E2 through hydroxylation, oxidation and removes the C-19 carbon and aromatization of the steroid A ring [6,7]. This estrogen synthesis, which is the main form of estrogen production in postmenopausal women, can occur in peripheral tissues, but is found mainly in the local mammary tissue [7].

A large number of studies have shown that local production of estrogen in breast cancer tissue is higher than in normal breast counterparts due to the presence of very high levels of aromatase [8 - 12].

An essential process in forming distant metastases is degradation of the extracellular matrix (ECM) allowing tumor cells to invade local tissue, intravasate and extravasate blood vessels and form new metastatic colonies. This process is primarily

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influenced by activity of tumor secreted proteinases [13,14]. Metalloproteinases (MMPs) are the largest class of proteinases in the human genome [15]. MMPs are proteolytic enzymes that can degrade the structural elements of the ECM and release cell-bound inactive precursor forms of growth factors, degrade cell-cell and cell-ECM adhesion molecules and activate others MMPs [16]. The main MMPs involved in breast cancer are metalloproteinase-2 (MMP-2) and metalloproteinase 9 (MMP-9) that have the capacity to degradate type IV collagen which is present on basal membrane and has the function of separating epithelial cells from adjacent stromal [17,18]. The high expression of MMP-2 and MMP-9 in tumors is associated with elevated numbers of distant metastases and poor prognosis [19,20].

Di et al. (2005) [21], through PCR analysis of breast cancer tissues, found a positive correlation between aromatase, MMP-2, and MMP-9 ($p<0.01$). Lu et al. (2007) [22] demonstrated a positive correlation between aromatase, MMP-2, and MMP-9 in breast cancer tissues positive for hormonal receptors ($p<0.05$).

CD44 is a broadly distributed transmembrane glycoprotein that plays a critical role in a variety of cellular behaviors, including adhesion, migration, invasion and survival. CD44 mediates cell-cell and cell-matrix interactions a primarily through its affinity for hyaluronic acid (HA), a glycosaminoglycan constituent of extracellular matrices, but also potentially through its affinity for other ligands [23]. Okamoto et al. (2002) [24] showed increased levels of soluble CD44 in plasma from patients with some tumors, reflecting the increase in proteolytic activity and matrix remodeling that is associated with tumor growth and metastasis. Abraham et al. (2005) [25] found a relation between breast cancer cells that express CD44 and a greater incidence of metastasis.

Some studies have shown that CD44 glycoprotein acts as a docking site for MMP-9 on the cell surface and demonstrated that CD44 and cross-linking leads to an enhanced level and relocation of MMP-9 in human breast tumor cells accompanied by increased tumor invasion and metastasis [26 - 28].

Objective

This study intends to verify the expression levels and correlation of aromatase, MMP-2, MMP-9 and CD44 in ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC) when both are found in the same breast.

Methods

Patients

We selected one hundred and ten patients surgically treated for breast cancer from the Mastology Clinic of the Obstetrics and Gynecology Department of Santa Casa Hospital between August 2002 and January 2008. Only patients with infiltrating ductal carcinoma and ductal carcinoma *in situ* in the same surgical specimen were included. This was a cross sectional study approved by the Ethics Committee.

Histopathology and Tissue Microarray Immunohistochemistry

After study of macroscopic specimens, selected fragments were dehydrated in ethanol, cleared by xylene and embedded in paraffin for preparation of the blocks. The blocks were cut by using a microtome calibrated for 4 mm thick slabs. The histological sections were stained with hematoxylin and eosin (HE) and read using a light microscope.

DCIS was classified according to presence or absence of comedonecrosis in comedocarcinomas and non-comedocarcinomas. Criteria proposed by Dabbs et al. (1993) [29] and Elston and Ellis (1991) [30] were used for classification of DCIS and IDC using nuclear and histological grades, respectively.

Cases with IDC and adjacent DCIS were selected for preparation of the tissue microarray (TMA). Two regions from each histological type were selected for composing the microarray, thus, each case was represented by four areas, two from DCIS and two from IDC from the same patient, comprising a total of 440 regions for analysis.

The expression of aromatase, MMP-2, MMP-9, and CD44 was evaluated by immunohistochemistry analyses using specific antibodies detected by use of the chromogenic substrate diaminobenzidine. The sections were counter stained with Harris hematoxylin, followed by dehydration and mounting in Entellan with cover slips. The primary antibodies used in immunohistochemistry reactions were: rabbit polyclonal antibody anti-aromatase (MCA2077 Serotec Inc.), mouse monoclonal antibody anti-MMP-2 (Mob312 DBS), rabbit polyclonal antibody anti-MMP-9 (A0150 Dako) and mouse monoclonal antibody anti-CD44 (M7082 Dako) in the dilutions 1/70, 1/150, 1/600 and 1/200, respectively.

Evaluation of aromatase, MMP-2, MMP-9 and CD44 - expression.

Expressions of aromatase, MMP-2, MMP-9 were evaluated with scores, by two independent examiners.

We used the same criteria employed by Oliveira et al. (2006) [31] for analysis of aromatase: score 0: no stained cells observed; score 1: cytoplasm and cell membrane stained diffusely and weakly (it should have at least 10% of stained cells with strong intensity); score 2: cytoplasm granular staining of the cell membrane and moderate to strong in 10-90% of cells; score 3: more than 90% of cells stained with strong intensity.

To classify the immunohistochemical expression of MMP-2, MMP-9 and CD44 we used the same criteria used by Ellis et al. (2006) [31]: score 0 and 1: less than 10% cells stained, score 2: less than 30% of cells weakly stained or strong incomplete staining, score 3: more than 30% of cells strongly and completely stained.

We used the same criteria as Ristimäki et al. (2002) [33] and Oliveira et al. (2006)[31] to classify the immunohistochemical expression as positive or negative, considering 0 and 1 as negative and 2 or 3 as positive.

Statistical analysis

The correlation between aromatase, MMP-2, MMP-9 and CD44 was assessed according to the Spearman correlation. The Chi-Square test was used to analyze nuclear grade, histological grade, age group, tumor size, lymph node status, and hormonal status. We set 5% as the rejection level for the null hypothesis for all parameters evaluated [34]. All data were analyzed by the statistical program SPSS® (Statistical Package for Social Sciences) version 17.0 for *Microsoft Windows*.

Results

The aim of our study was to assess the correlation between aromatase, MMP-2, MMP-9 and CD44 levels in ductal carcinoma *in situ* and infiltrating ductal carcinoma when both are present in the same surgical specimen. Moreover, an analysis was made to verify if there was any relation between the expression of these biomarkers and age (younger or older than 50 years), tumor size (less than or equal to 2 cm or more than 2 cm), histological grade, nuclear grade, axillary lymph node and hormonal status. Age at diagnosis ranged from 26 to 90 years with a mean age of 56.4 years, with a standard deviation of 12.81, and median of 55 years.

Evaluation of Aromatase expression

One hundred and ten cases were evaluated by immunohistochemistry with scores attributed from zero to three, according to the intensity and number of stained cells. Aromatase expression was positive in 63 cases (57.3%) in DCIS and positive in 67 cases (60%) in IDC showing a high correlation ($p < 0.001$) (Table 1). There was no statistically significant difference when the expression of aromatase with histological grade, nuclear grade, age, axillary lymph node status and hormonal status was analyzed.

Immunoreactivity to MMP-2, MMP-9 and CD44

MMP-2 expression was positive in 15 IDC and DCIS cases (13.60%); MMP-9 was positive in 83 IDC cases (75.5%) and 82 DCIS cases (74.5%); CD44 was positive in 49 IDC cases (44.5%) and 48 DCIS cases (43.6%). When performing statistical analysis a high correlation was found between the three biomarker expressions in IDC and DCIS ($p < 0.001$). As in the analysis of aromatase, there was no statistically significant difference when the expression of these biomarkers with histological grade, nuclear grade, age, axillary lymph node status and hormonal status was analyzed. (Table 1)

Table 1 - Immunohistochemical expression of aromatase, MMP-2, MMP-9 and CD44 in Invasive ductal carcinoma (IDC) and Ductal carcinoma in situ (DCIS) on 110 cases

Biomarker	Absolute number (%)
Aromatase	
idc	63 (57.3%)
dcis	67 (60%)
MMP-2	
Idc	15 (13.60%)
dcis	15 (13.60%)
MMP-9	
idc	83 (75.50%)
dcis	82 (74.50%)
CD44	
Idc	49 (44.50%)
dcis	48 (42.60%)

idc = invasive ductal carcinoma; dcis = ductal carcinoma *in situ*; MMP-2 = matrix metaloproteinase 2; MMP-9 = matrix metaloproteinase 9

Correlation between the expression of aromatase, MMP-2, MMP-9 and CD44

When only IDC regions were considered, a statistically significant positive correlation was found between aromatase and MMP-2 ($p=0.01$) and between aromatase and MMP-9 ($p=0,034$). When only the DCIS was evaluated the statistical analysis showed a statistically significant positive correlation between aromatase and MMP-2 ($p=0.001$) and between MMP-9 and CD44 ($p=0.03$). (Table 2)

There was no statistically significant correlation when the expression of aromatase, MMP-2, MMP-9 and CD44 with histological grade, nuclear grade, age, axillary lymph node status and hormonal status was analyzed.

Discussion

The estrogen function in all phases of breast carcinogenesis is well established. It is found in women of reproductive age and also in post-menopausal[1,3]. The great difference between these two phases of life is that in postmenopausal women extra-ovarian aromatase plays a fundamental role in the estrogen synthesis [7,12].

The peripheral conversion of androgens to estrogens occurs in the adipose tissue, muscle, skin and also in the breast tissue itself and especially in the latter, significant enhancement of conversion due to higher levels of aromatase expression is associated with malignant changes [10,35,36].

In our study we detected aromatase expression, by immunohistochemistry, in 57.3% of IDC cases and in 60% of DCIS cases showing a significant correlation ($p<0.01$). These results agree with the theory that high aromatase expression in tumor epithelium favors tumor formation, especially when aromatase expression is more frequent in DCIS than IDC disclosing the fundamental role of this enzyme in the initial phases of carcinogenesis. Others authors found similar results[37,31,35].

The high expression of aromatase in our study corroborates the finding of Bulun et al. (2004)[11], where in normal breast tissue, promoter I.4 acts while promoters I.3 and II act minimally. In breast cancer tissues they verified the action of promoter I.4 whereas 1.3 and II were extremely higher, and the action of promoter I.7, resulted in high estrogen concentration.

In relation to the nuclear grade our results are similar to those found by Hudelist et al. (2008)[12] ,who evaluated 96 samples of DCIS, and 104 samples of DCIS and IDC in the same samples. They found that in DCIS there was no statistically significant difference in aromatase expression between high, moderate and low grade tumors. However Silva et al. (1989)[38], differently, found higher aromatase expression in nuclear grade III ($p=0.03$).

Some studies have evaluated prognosis of patients that present higher aromatase expression; Silva et al. (1989)[38] showed that higher aromatase expression correlates with lower disease free survival ($p<0.05$). Eppenberger et al. (2001)[39] found that women with high aromatase expression in breast cancer have increased

Table 2 - Correlation between the expression of aromatase, MMP-2, MMP-9 and CD44 in 110 IDC and DCIS cases

		Aromatase *IDC*	Aromatase DCIS	CD44 IDC	CD44 DCIS	MMP-2 IDC	MMP-2 DCIS	MMP-9 IDC	MMP-9 DCIS
Aromatase IDC	r	1.000	.906(*)	.059	.082	.318(*)	.318(*)	.203(*)	.170
	p		.000	.542	.397	.001	.001	.034	.076
Aromatase DCIS	r	.906(*)	1.000	.034	.048	.318(*)	.318(*)	.210(*)	.170
	p	.000	.	.728	.617	.001	.001	.028	.076
CD44 *IDC*	r	.059	.034	1.000	.989(*)	-.128	-.128	.184	.200(*)
	p	.542	.728	.	.000	.182	.182	.055	.036
CD44 DCIS	r	.082	.048	.989(*)	1.000	-.105	-.105	.191(*)	.207(*)
	p	.397	.617	.000	.	.273	.273	.046	030
MMP-2 i IDC	r	.318(*)	.318(*)	-.128	-.105	1.000	1.000(*)	.019	.023
	p	.001	.001	.182	.273	.	.	.843	.815
MMP-2 DCIS	r	.318(*)	.318(*)	-.128	-.105	1.000(*)	1.000	.019	.023
	p	.001	.001	.182	.273	.	.	.843	.815
MMP-9 IDC	r	.203(*)	.210(*)	.184	.191(*)	.019	.019	1.000	.978(*)
	p	.034	.028	.055	.046	.843	.843	.	.000
MMP-9 DCIS	r	.170	.170	.200(*)	.207(*)	.023	.023	.978(*)	1.000
	p	.076	.076	.036	.030	.815	.815	.000	.

(*) Statistically significant correlation $p<0.05$. Statistical test: Spearman correlation; r = correlation coefficient; IDC = invasive ductal carcinoma; DCIS = ductal carcinoma ***in situ***; MMP-2 = matrix metalloproteinase 2; MMP-9 = matrix metalloproteinase 9

risk of relapse and death ($p<0.05$). Lu et al. (2007)[22] in the univariate survival analysis showed that aromatase is significantly and positively associated with decreased overall survival (OS) ($p=0.04$) in the estrogen receptor (ER) and/or progesterone receptor (PR), positive patients (10 year OS 100% for the aromatase-negative group compared with 85.1% for the aromatase-positive group). However, in the multivariate survival analysis, we did not find this association to be significant ($p> 0.05$). In the ER and PR negative cases, there were no significant OS differences between aromatase negative and aromatase positive patients in either univariate or multivariate analysis ($p> 0.05$).

For local invasion to occur the basal membrane must be disrupted. MMPs are proteolytic enzymes which can degrade the structural elements of the extracellular matrix. This isplays an important role in invasion by extracellular matrix remodelling, liberating and activating growth factors, and cytokines, thereby facilitating tumor invasion [40,16]. Studies have shown that in many types of cancer the MMPs expression are elevated and associated with poor prognosis [41,19,42]. In breast cancer MMP-2 and MMP-9 are the main MMPs related with this type of cancer [19].

In our study MMP-2 expression was positive in 13.6% of IDC and in DCIS cases with perfect positive correlation (cc = 1). MMP-9 was positive in 75.5% in IDC and in 74.5% in DCIS with statistically significant correlation ($p<0.01$). Di et al. (2005)[21] and Lu et al. (2007)[22] using polyclonal antibodies for immunohistochemistry analyzed only IDC and found positive values similar to ours (72.3% and 62.7% respectively). When evaluating MMP-2 they found higher values of positivity (68.7% and 58.6% respectively). Kohrmann et al. (2009) [43] evaluated normal breast tissues and IDC specimens and found higher expression of MMP-2 and MMP-9 in the tumors.

When we evaluated only IDC we found a statistically significant positive correlation between aromatase and MMP-2 ($p=0.001$) and between aromatase and MMP-9 ($p=0.034$). Similar results were obtained by Di et al. (2005) and Lu et al. (2007) with $p<0.001$ [21,22].

These results and others that show positive correlation between aromatase and MMP-2 and aromatase and MMP-9 increase the possibility that aromatase can elevate the invasion capacity of tumor cells by increasing the activity of MMP-2 and MMP-9. It is possible that with a higher expression of aromatase, there is a higher activity and action of MMPs for degrading the extracellular matrix and favoring disease progression.

When we evaluated only DCIS we found a statistically significant positive correlation between aromatase and MMP-2 and MMP-9 and CD44 ($p=0.030$). The positive correlation between aromatase and MMP-2 in DCIS confirms the tendency that neoplasia, which shows aromatase expression, has higher invasion potential.

A melanoma study by Yu and Stamenkovic (2000) [44] and a prostate study by Desai et al. (2007) [26] showed a fundamental connection between CD44 and MMP-9, as the latter acts in degrading the basal membrane and initiates local invasion. Ours results showing a positive correlation between MMP-9 and CD44 ($p=0.030$) corroborate findings by these authors and Peng et al. (2007) [28] who observed MMP-9 and CD44 correlation in breast cancer cells. These results are an indication that CD44 acts as a docking site for MMP-9 and that formation of the CD44/MMP-9 complex on the cellular surface probably is necessary for MMP-9 action.

Conclusion

After analysis of our results we can conclude that aromatase from local breast tissue estrogen synthesis play an important role in breast carcinogenesis, influencing mainly MMP-2 and MMP-9 which are important participants in tumor invasion and their probable dependence of their conection with CD44 to act.

These results allow us to consider that use of aromatase inhibitors (AI) acts in the prevention and treatment of breast cancer not just because of reduction in blood and local concentration of estrogen but also because of the action on important pathways for tumor progression where the CD44/MMP-9 complex plays a fundamental role. It is possible that use of some substances which inhibit MMPs isolated or associated with AI, may be a promising target for researchers of breast cancer treatment.

Conflict of interest: none

Resumo

A interação entre aromatase, metalloproteinase 2, 9 e CD44 no câncer de mama

Objetivo. O objetivo desse estudo é verificar as expressões e correlações da aromatase, metalloproteinase 2 da matriz (MMP2), metalloproteinase 9 da matriz (MMP-9) e CD44 no carcinoma ductal in situ (CDIS) e carcinoma ductal infiltrativo (CDI) quando ambos estão presentes simultaneamente na mesma mama.

Métodos. Foram avaliados 110 casos pelo método de tissue microarray (TMA) e através da utilização de anticorpos policlonais antiaromatase, anticorpos monoclonais anti-MMP-2, anticorpos policlonais anti-MMP-9 e anticorpos monoclonais anti-CD44.

Resultados. A aromatase estava expressa de forma positiva no CDI e CDIS em 63 (57,3%) e 60 (67%) casos, respectivamente. A expressão de MMP-2 estava expressa de forma positiva em 15 (13,6%) casos tanto no CDI, quanto no CDIS. A expressão da MMP-9 estava expressa de forma positiva em 83 (75,5%) e 82 (74,5%) casos de CDI e CDIS, respectivamente. A expressão de CD44 estava expressa de forma positiva em 49 (44,5%) e 48 (42,6%) casos de CDI e CDIS, respectivamente. Todos eles apresentando alta correlação ($p<0,001$). Na avaliação de correlação foi encontrada correlação positiva estatisticamente significante no CDI entre aromatase e MMP-2 ($p<0,01$) e entre aromatase e MMP-9 ($p=0,034$). Nos casos de CDIS houve correlação positiva estatisticamente significante entre aromatase e MMP-2 ($p<0,001$) e entre CD44 e MMP-9 ($p=0,030$).

Conclusão. Após analisarmos os resultados de nosso estudo, podemos concluir que a aromatase, através da síntese de estrogênio local no tecido mamário, desempenha importante papel na carcinogênese mamária, principalmente influenciando a atuação da MMP-2 e da MMP-9, grandes responsáveis pela invasão celular tumoral que, por sua vez, provavelmente dependem de sua ligação a CD44 para poder desempenhar suas funções. [Rev Assoc Med Bras 2010; 56(4): 472-7]

Unitermos: Aromatase. Metaloproteinase 2 da matriz. Metaloproteinase 9 da matriz. Antígenos CD44. Carcinoma ductal de mama.

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