

## MMP-9 and MMP-2 gelatinases and TIMP-1 and TIMP-2 inhibitors in breast cancer: correlations with prognostic factors

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### Abstract

The goal of our study was to analyse the prognostic values for some matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in breast cancer. We evaluated the activity and the expression levels of MMP-9, MMP-2, TIMP-1 and TIMP-2 in malignant *versus* benign fresh breast tumor extracts. For this purpose, gelatinzymography, immunoblotting and ELISA were used to analyse the activity and expression of MMPs and TIMPs. We found that MMP-9 expression level and activity are increased in malignant tumors. In addition, MMP-9/TIMP-1 and MMP-2/TIMP-2 ratio values obtained by us were significantly different in malignant tumors compared to benign tumors. We suggest that the abnormal MMP-9/TIMP-1 balance plays a role in the configuration of breast invasive carcinoma of no special type and also in tumor growth, while altered MMP-2/TIMP-2 ratio value could be associated with lymph node invasion and used as a prognostic marker in correlation with Nottingham Prognostic Index. Finally, we showed that in malignant tumors high expression of estrogen receptors is associated with enhanced activity of MMP-2 and increased bcl-2 levels, while high expression of progesterone receptors is correlated with low TIMP-1 protein levels.

**Keywords:** breast cancer • MMP-9 • MMP-2 • TIMP-1 • TIMP-2 • clinicopathological markers

### Introduction

The matrix metalloproteinases (MMPs) are a family of structurally and functionally related proteinases, initially characterized by their ability to degrade

the extracellular matrix (ECM) [1]. Nowadays, at least 20 enzymes that share considerable homology within their major domains (signal peptide, propeptide, catalytic, hinge and hemopexin-like domains) were included in MMPs family [2]. Most of MMPs are synthesised and secreted as partially activated latent forms, requiring, for full activation, removal of the entire propeptide domain by proteinases including other MMPs [3]. The expression of

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MMPs is tightly regulated at different levels: transcription, secretion and activation [1, 4]. Factors including inflammatory cytokines, hormones, growth factors and oncogene products induce MMPs expression [1, 5, 6]. In addition, cell-matrix and cell-cell interactions affect MMPs gene transcription [7]. The activity of MMPs is controlled by interaction with the tissue inhibitors of metalloproteinases (TIMPs) [8]. Currently, four TIMPs have been identified, most of them being capable to bind and inhibit the activity of all members of MMPs family. The members of TIMP family are known to have other different roles, like growth factor properties, stimulation of cell migration, and stimulation or inhibition of apoptosis [9–11].

Based on their substrate specificity and domain structure, the MMPs were divided into four main subgroups. One of these subgroups is represented by gelatinases, also known as type IV collagenases, which degrade gelatine and types IV, V, VII, IX and X collagen. This subgroup of MMPs includes two distinct members, known as gelatinase A (MMP-2) and gelatinase B (MMP-9). Due to their specificity for the substrate (collagen IV, particularly abundant in basal membrane), MMP-2 and MMP-9 were the most involved in tumor initiation, growth and metastasis, especially in breast cancer [12]. A large number of publications presented various studies about the role of gelatinases (MMP-2/MMP-9) and their natural inhibitors (TIMP-1/TIMP-2) in breast cancer, some of the results being contradictory [13–20]. Therefore, the aim of this study was also to corroborate our results with previously reported data, in order to introduce new valuable prognostic markers in breast cancer.

This study used freshly isolated tissues specimens from the malignant and benign breast tumors analysing the activity and the expression level of gelatinases and their natural inhibitors, in correlation with prognostic factors for breast cancer.

## Material and methods

### Patients and tissue specimens

The study was performed on freshly isolated tumor samples from thirty-two consecutive patients with primary invasive breast carcinomas (IC) (13 of special type and

19 of no special type, based on Miller and Sainsbury classification [21, 22]) and sixteen consecutive patients with benign breast tumors (eight fibroadenomas, six intraductal papillomas, one fibroadenoma and intraductal papilloma and one sclerocystic mastose). All the patients were hospitalized and had undergone surgery at “Prof. Al. Trestioreanu” Institute of Oncology, Bucharest, between 2002 and 2003. The patients with malignant tumors were aged from 37 to 78 years (mean age 53 years) and those with benign tumors were aged from 16 to 62 years (mean age 31 years). Table 1 shows the prognostic factors of the patients with IC and the arbitrary scores assigned to each of the analysed marker, based on increased aggressiveness.

Freshly isolated breast tumor extracts from patients who underwent surgery were mechanically disaggregated, washed with 150 mM NaCl and homogenized for 30 minutes on ice in Triton lysis buffer (50 mM HEPES buffer pH 7.5, 1% Triton-X 100, 250 mM NaCl, 10% glycerol, 1 mM PMSF and 10 µg/ml leupeptin). The homogenates were centrifuged at 1200 rpm for 10 minutes at +4°C and the supernatants were recentrifuged at 15000 rpm for 15 minutes at +4°C. Finally, the resulted supernatants were analyzed for total protein content, using a commercial BCA Protein Assay Reagent (Pierce) and the aliquotes were frozen at –80°C until processing.

### Expression levels of estrogen and progesterone receptors and bcl-2

Estrogen receptors (ER), progesterone receptors (PR), and bcl-2 expression levels were analyzed as described by Bussolati and Gugliotta [23]. Briefly, the tissue sections were incubated with primary antibodies anti-ER, 1D5 clone (1:100), anti-PR, 1A6 clone (1:100) or anti-bcl-2, 124 clone (1:40) (DAKO). The detection was performed using avidin-biotin peroxidase complex and diaminobenzidine as a chromogen. The tissue sections counterstained with Meyer’s haematoxylin were mounted.

The immunoreactivity for ER and PR was evaluated by Histochemistry Score System (HSCORE), calculated based on algorithm  $HSCORE = \sum [(I + 1) \times PC]$ , where I signifies the staining intensity and PC signifies the percentage of cells that stain at each intensity, respectively. Staining intensity was classified based on an arbitrary scale from 0 to 3 as follows: **0** for negative staining, **1** for mild reactivity, **2** for moderate reactivity and **3** for strong reactivity.

**Table 1** Prognostic Factors of the patients with primary invasive breast carcinomas (IC)

Prognostic Factors		Patients (No)	Arbitrary Scores
<b>Tumor Stage</b>	I	3	1
	IIA	18	1.75
	IIB	7	2
	IIIA	1	2.75
	IIIB	3	3
<b>Histological Grade</b>	1	4	1
	2	23	2
	3	5	2
<b>Histological Type (HT)</b>	special	13	1
	no special	19	2
<b>Tumor Size (<math>\Phi</math>T)</b>	$\Phi$ T<2 cm	15	the highest $\Phi$ T value
	$\Phi$ T>2 cm	17	the highest $\Phi$ T value
<b>Node invasion (N)</b>	absent	18	1
	present	14	3
<b>NPI</b>	<3.4	10	NPI value
	>3.4	22	NPI value

The bcl-2 expression level was evaluated based on the percentage of immunopositive tumor cells, and scored as follows: **0** for the absence of staining (0% bcl-2 immunopositive tumor cells), **1** for mild staining (<5% bcl-2 immunopositive tumor cells), **2** for moderate staining (5–70 % bcl-2 immunopositive tumor cells) and **3** for strong staining (>70% bcl-2 immunopositive tumor cells).

### Nottingham prognostic index

Nottingham Prognostic Index (NPI) with Yorkshire group correction [24] was calculated for each patient using the algorithm:  $NPI = (0.2 \times \text{tumor diameter in cm}) + \text{lymph node stage} + \text{histological grade}$ . Node invasion, tumor diameter and histological grade were arbitrarily scored as shown in Table 1. An NPI score of less than 3.4 suggested a good prognosis.

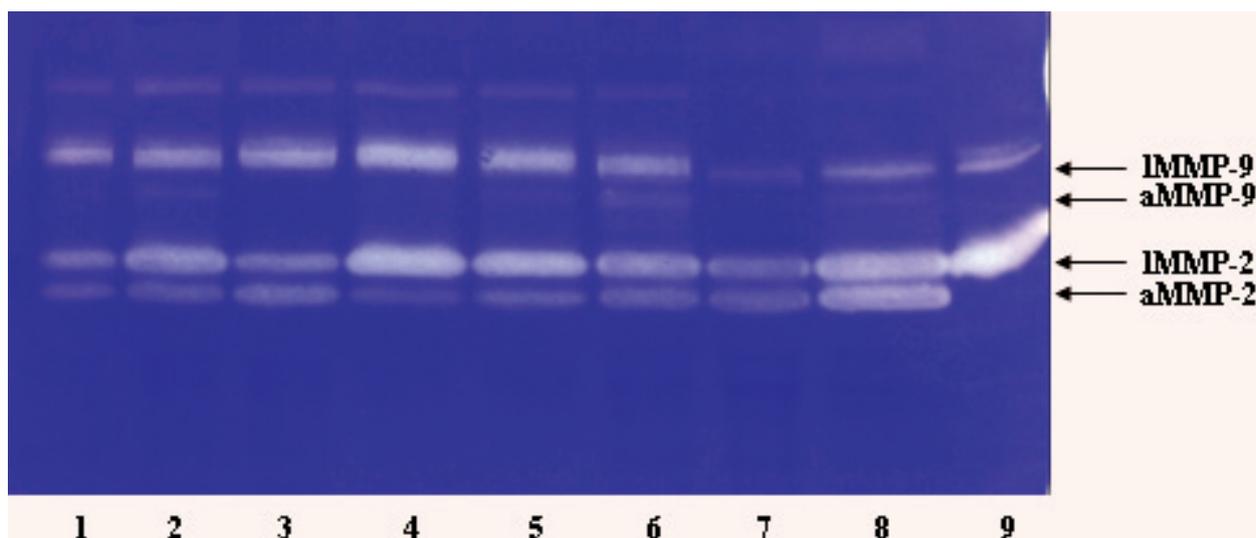
### Gelatinase activity in tumor samples

Sixty  $\mu$ g of total protein from tumor extracts were analysed for gelatinase activity using zymography on 8% SDS-PAGE containing 0.1% gelatine, well known as a semiquantitative assay [25]. The zymograms were scanned and quantified using Total Lab V1.11 software and the values were expressed as Arbitrary Units (AU).

### Expression levels of MMPs and TIMPs in tumor samples

The expression levels of MMP-9, TIMP-1 and TIMP-2 in tumor samples were analysed using quantitative ELISA kits and MMP-2 expression level was evaluated by semiquantitative immunoblotting method.

ELISA kit Quantikine® (R & D Systems) for human MMP-9 and ELISA System (Biotrak, Amersham



**Fig. 1** Gelatine zymography of breast tumours extracts. Breast tumor extracts were resolved in SDS-PAGE 0.1 % gelatine zymography. Subsequently, the gel was incubated in a 2.5 % Triton X-100 solution and then in substrate buffer containing 5 mM CaCl<sub>2</sub>. The lytic gelatinase activity was identified after Coomassie Brilliant Blue R staining as a clear band on a blue background. Figure presents gelatinase activities in benign (lines 1, 3, 4, 5) and malign tumour extracts (lines 2, 6, 7, 8), and a positive control for the activities of IMMP-9 and IMMP-2 (line 9).

Biosciences) for TIMP-1 and TIMP-2 were used. Tumor samples were diluted 1:25 for MMP-9 and 1:40 for TIMP-1 and TIMP-2 and analysed using the manufacturer's protocol.

Sixty µg of total protein from each tumor extract were subjected to 8% SDS-PAGE gel electrophoresis under reducing conditions and analysed by immunoblotting with 1 µg/ml monoclonal antibody specific to pro/active forms of human MMP-2 (R & D Systems) followed by anti-mouse IgG peroxidase conjugate (1:20000 dilution) and Enhancement Chemiluminescence (ECL) detection (Amersham Pharmacia Biotech). The images were scanned and quantified using Total Lab V1.11 software and the levels of MMP-2 were expressed as Arbitrary Units (AU).

### Statistical analysis

The significance of the differences between malignant and benign tumors was examined using Student's t-test and Student's t-test with Bonferroni correction. The significance level of 0.05 by Student's t-test ( $p < 0.05$ ) was adjusted to 0.01 after Bonferroni correction ( $P_c < 0.01$ ). Spearman's coefficient was calculated in order to establish the correlation of both the activity and expression level of gelatinases, and the expression level of TIMP-1

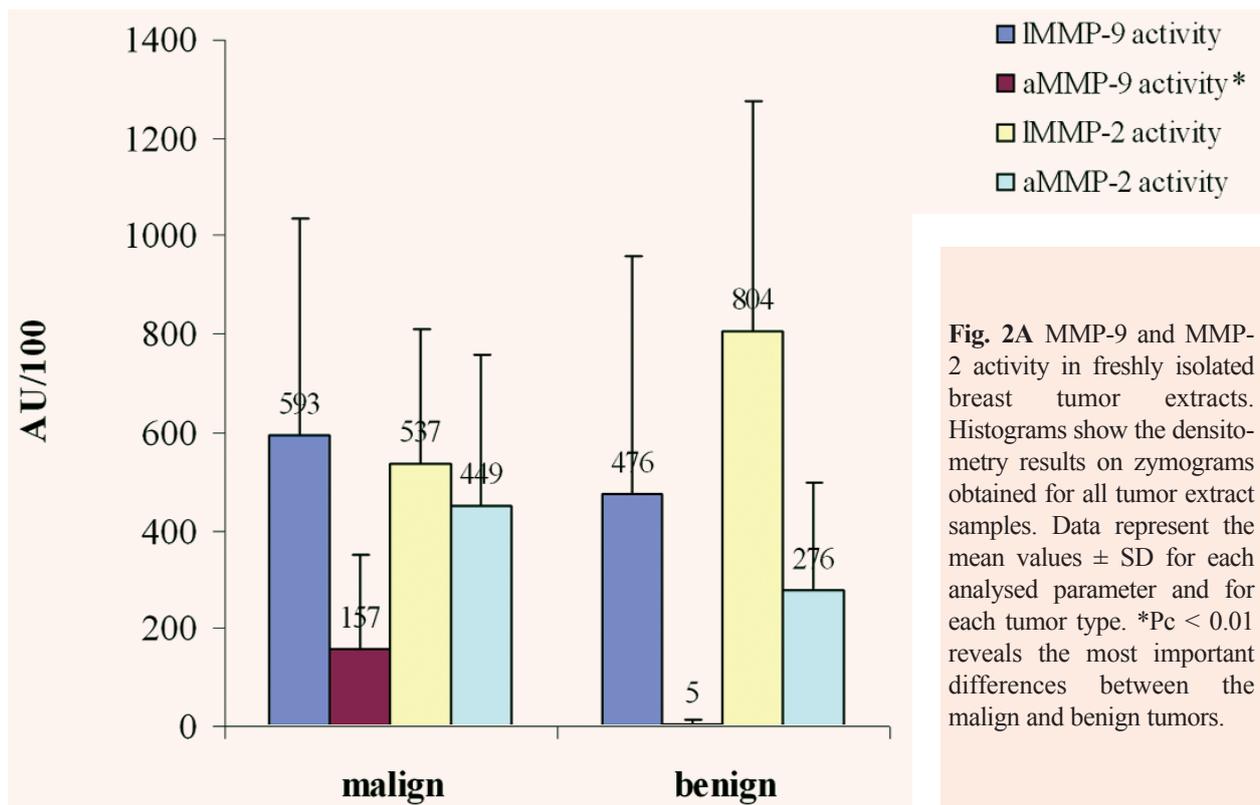
and TIMP-2 with prognostic factors for breast cancer. The correlation coefficient  $|r| > 0.5$ , with a probability  $P < 0.01$ , was considered as significant.

## Results

### Activities and levels of MMP-9, MMP-2, TIMP-1 and TIMP-2 in malignant versus benign breast tumors

Tumor extracts were analysed for gelatinase activity by gelatine zymography. The lytic activity was present at different apparent molecular weights (92, 86, 72 and 66 kDa respectively), corresponding to latent MMP-9 (IMMP-9), fully activated MMP-9 (aMMP-9), latent MMP-2 (IMMP-2) and fully activated MMP-2 (aMMP-2) forms. A representative example of gelatine zymography performed on four malignant and four benign tumor tissue extracts is illustrated in Fig. 1.

Densitometry analysis of all zymograms revealed that the activity of IMMP-9 form was almost comparable between malignant and benign tumor extracts, while the activity of aMMP-9 form was more increased in malignant than in benign tumor extracts



**Fig. 2A** MMP-9 and MMP-2 activity in freshly isolated breast tumor extracts. Histograms show the densitometry results on zymograms obtained for all tumor extract samples. Data represent the mean values  $\pm$  SD for each analysed parameter and for each tumor type. \* $P_c < 0.01$  reveals the most important differences between the malign and benign tumors.

( $P_c = 0.0001$ ) (Fig. 2A). Further analyses showed an enhanced activity of IMMP-2 form in benign specimens compared to malignant tumor samples ( $p = 0.048$ ), while the gelatinolytic activity of aMMP-2 form was higher in malignant specimens compared to benign tumor samples ( $p = 0.031$ ) (Fig. 2A).

When the expression levels of MMP-9, -2 and TIMP-1, -2 were analysed, some interesting results were obtained. The MMP-9 expression level was significantly higher in malignant than in benign tumor samples ( $P_c = 0.0002$ ). Although the malignant tumors showed an increased level of MMP-2 compared to benign tumors, the result of statistical analysis was under the significance level ( $p = 0.039$ ). In contrast, the levels of TIMP-1 and TIMP-2 were comparable between malignant and benign tumor extracts (Fig. 2B).

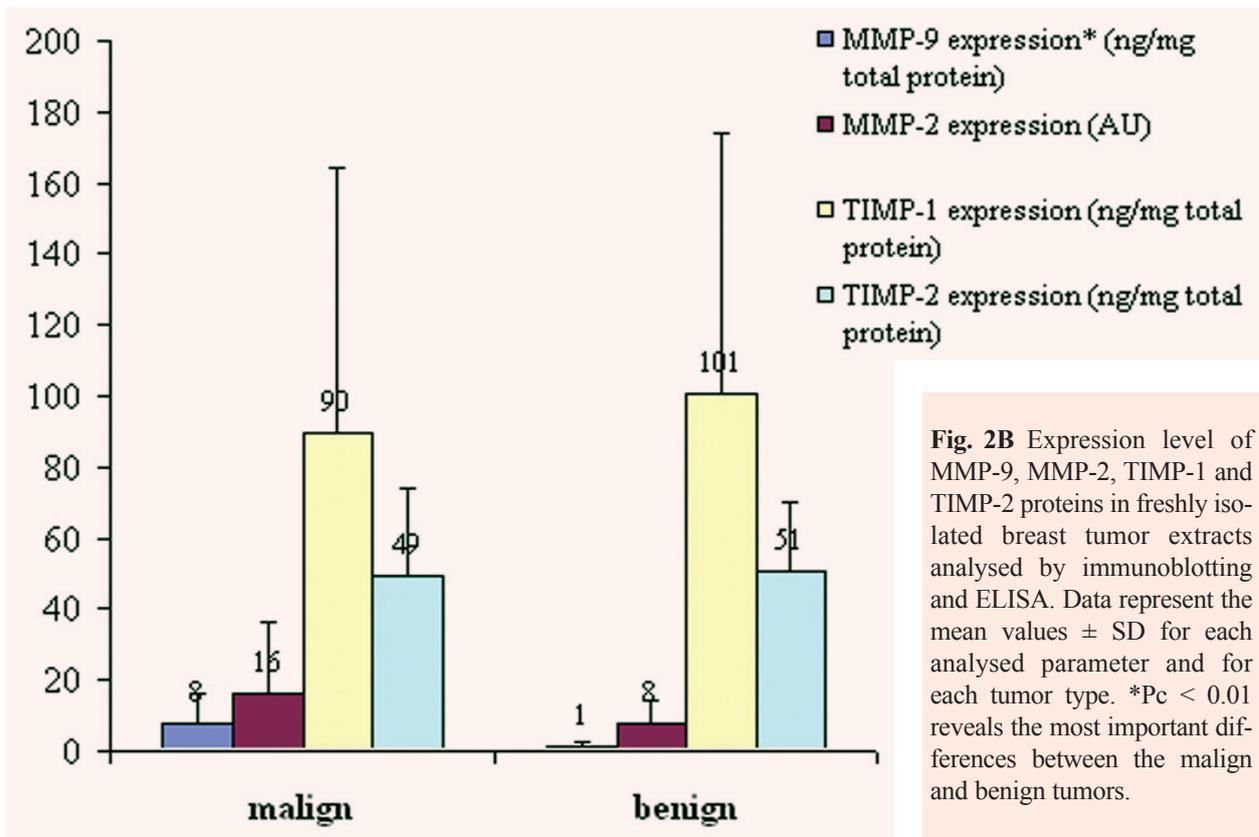
To evaluate the relative inhibition of the MMPs activity, we calculated MMP-9/TIMP-1 and MMP-2/TIMP-2 ratio value for each tumor sample. Our analysis showed that both ratio values were increased in malignant tumor samples, clearly differentiating between the malignant and benign tumors ( $P_c = 0.002$  and  $P_c = 0.007$ , respectively) (Fig. 2C).

### Correlation between gelatinases and their tissue inhibitors and breast cancer prognostic factors

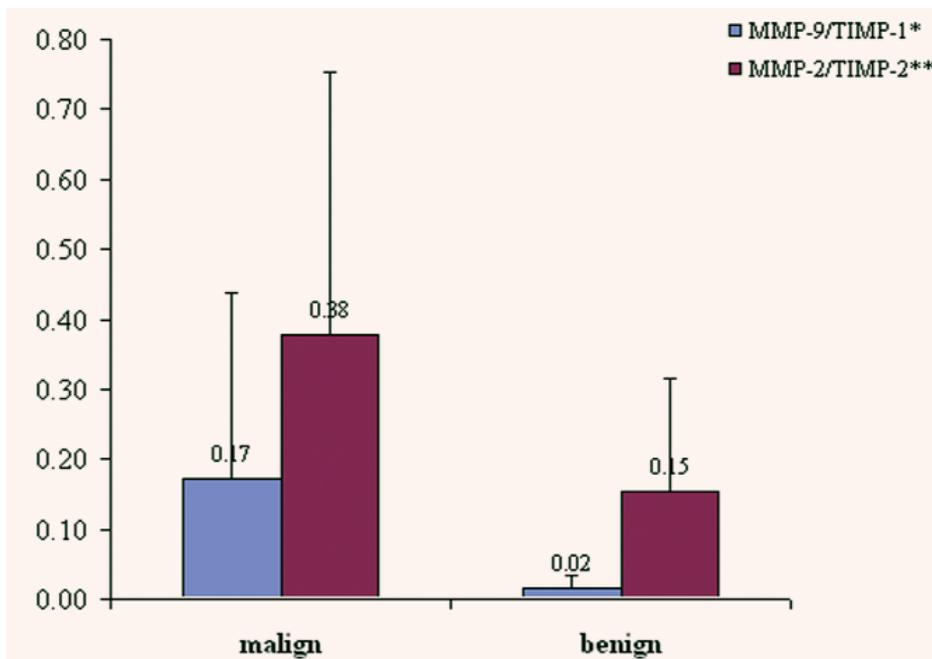
Afterwards, we correlated the activity and the expression of gelatinases and their natural inhibitors with accepted and used prognostic factors for breast cancer (tumor stage, histological type, histological grade, tumor size, nodal status and NPI) [21, 24]. A positive correlation was found between the score assigned for the histological type and each of the following: the activity of aMMP-9 form, the expression of MMP-9 and the MMP-9/TIMP-1 ratio value. When IC tumors were divided in special and no special types, based on Miller and Sainsbury classification [21, 22], the activities of MMP-9 forms, the expression level of MMP-9 and the MMP-9/TIMP-1 ratio values were markedly different between these tumor types (Table 2).

Unfortunately, due to the small number of cases within some groups (see Table 1), we could not use statistical analysis to identify the correlation between MMPs and TIMPs and tumor stage or histological grade.

A weak positive correlation between MMP-9/TIMP-1 ratio value and tumor size was found (Table 3). In the large tumors, the MMP-9/TIMP-1



**Fig. 2B** Expression level of MMP-9, MMP-2, TIMP-1 and TIMP-2 proteins in freshly isolated breast tumor extracts analysed by immunoblotting and ELISA. Data represent the mean values  $\pm$  SD for each analysed parameter and for each tumor type. \*Pc < 0.01 reveals the most important differences between the malign and benign tumors.



**Fig. 2C** Ratio MMP-9/TIMP-1 and MMP-2/TIMP-2 values calculated to evaluate the relative inhibition of the MMPs activity. Data represent the mean values  $\pm$  SD for each ratio and for each tumor type. \*Pc < 0.01 reveals the most important differences between the malign and benign tumors.

ratio values were higher than in small tumors; therefore, the MMP-9/TIMP-1 ratio values statistically differentiated between these two types of tumors (Pc = 0.013).

A relevant positive correlation between MMP-2/TIMP-2 ratio and node invasion was identified (Table 4). The patients with node invasion presented an increased level of MMP-2 and a reduced TIMP-2

**Table 2** Correlations and differences when Histological Type (HT) of tumor was used as an evaluation parameter

Group	IMMP-9 activity (AU/100)	aMMP-9 activity (AU/100)	MMP-9 Expression (ng/mg protein)	TIMP-1 expression (ng/mg protein)	MMP-9/TIMP-1
all IC	r = 0.490 § P = 0.005	r = 0.663 P = 0.0002	r = 0.563 P = 0.001	no correlation	r = 0.662 P < 0.0001
G1*	352 ± 250‡	26 ± 32	1.90 ± 1.05	126.9 ± 88.2	0.02 ± 0.02
G2**	669 ± 283	209 ± 138	11.53 ± 9.68	63.5 ± 52.3	0.28 ± 0.31
G1 vs. G2	0.003***	0.00003	0.001	0.032	0.002

\* special type, \*\* no special type, § correlation with HT (r; P), ‡average ± SD, \*\*\* t-test (Pc)

**Table 3** Correlations and differences when Tumor Size (ΦT) was used as an evaluation parameter

Group	TIMP-2 expression (ng/mg protein)	MMP-2/TIMP-2
all IC	r = - 0.513§ P = 0.002	r = 0.543 P = 0.001
G7*	63.62 ± 25.86‡	0.18 ± 0.13
G8*	40.84 ± 21.31	0.43 ± 0.38
G7 vs. G8	0.038***	0.016

\*ΦT<2 cm, \*\*ΦT>2 cm, §correlation with ΦT (r; P), ‡average ± SD, \*\*\*t-test (Pc)

**Table 4** Correlations and differences when Node invasion (N) status was used as an evaluation parameter

Group	MMP-2 expression (AU)	TIMP-2 expression (ng/mg total protein)	MMP-2/TIMP-2
all IC	no correlation §	no correlation	r = 0.532 P = 0.001
G5*	10.2 ± 7.36‡	56.16 ± 26.35	0.20 ± 0.14
G6**	18.39 ± 13.12	39.09 ± 22.62	0.60 ± 0.43
G5 vs. G6	no difference***	no difference	0.011

\*N absent, \*\*N present, § correlation with N (r; P), ‡average ± SD, \*\*\*t-test (Pc)

level. Therefore, the mean value of MMP-2/TIMP-2 ratios was three times higher in patients with than in patients without node invasion, the ratios statistically differentiating between these groups of patients.

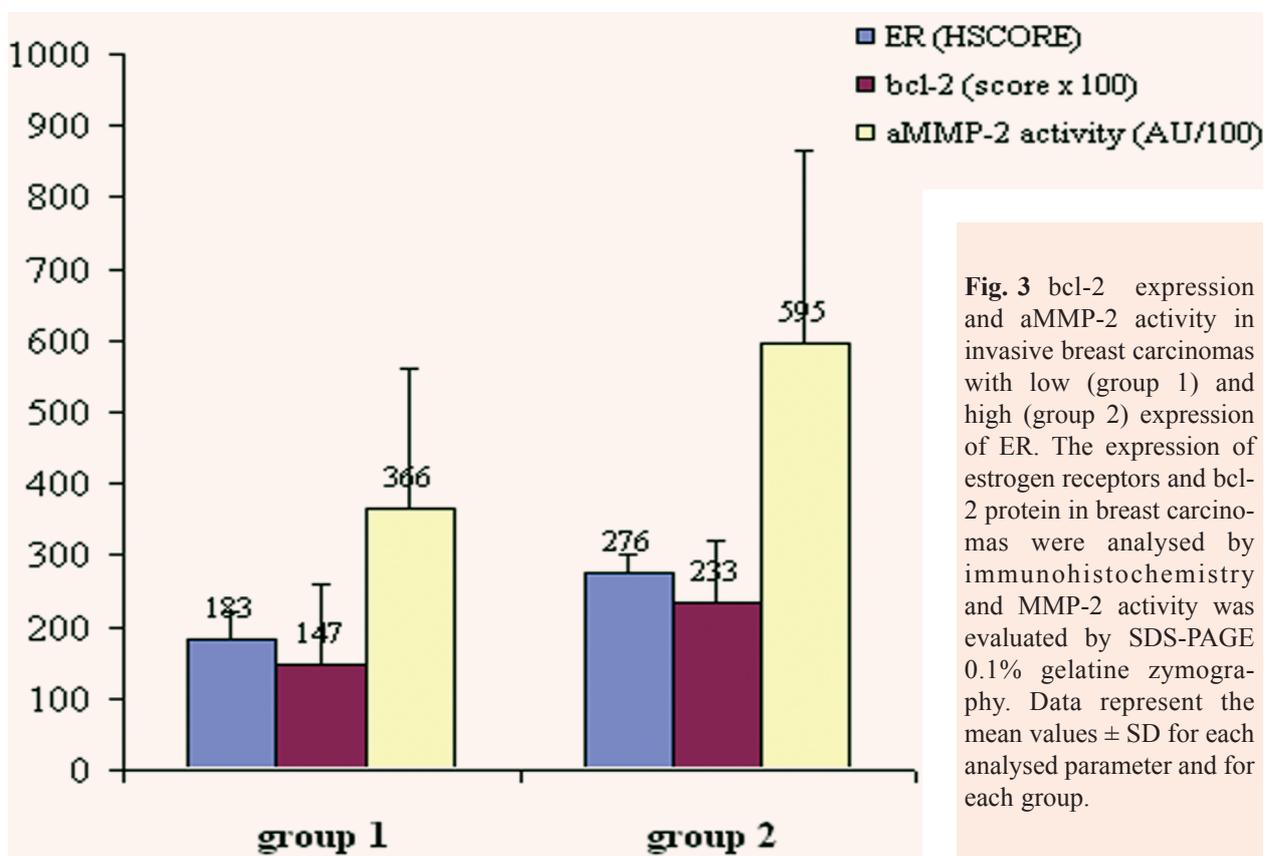
**Table 5** Correlations and differences when Nottingham Prognostic Index (NPI) was used as an evaluation parameter

Group	TIMP-2 expression (ng/mg protein)	MMP-2/TIMP-2
all IC	$r = - 0.513$ § $P = 0.002$	$r = 0.543$ $P = 0.001$
G7*	$63.62 \pm 25.86$ ‡	$0.18 \pm 0.13$
G8*	$40.84 \pm 21.31$	$0.43 \pm 0.38$
G7 vs. G8	0.038***	0.016

\*NPI<3.4, \*\*NPI>3.4, § correlation with NPI (r; P), ‡average  $\pm$  SD, \*\*\*t-test (Pc)

The expression and activity of gelatinases and their tissue inhibitors in malignant tumor samples were next correlated with NPI (Table 5). The TIMP-2 level was negatively correlated with NPI, whereas MMP-2/TIMP-2 ratio values and NPI were positively correlated. The tumors with favorable prognosis expressed higher levels of TIMP-2 and lower MMP-2/TIMP-2 ratio values compared to tumors with poor prognosis.

The relationship between the expression levels of hormone receptors and bcl-2 and the activity/expression of MMPs and TIMPs in malignant samples was also investigated. Based on the mean value of HSCORE, the tumors were divided in groups with high (HSCORE > 200) and low (HSCORE < 200) expression of ER and PR. Statistical analysis demonstrated that the activity of aMMP-2 tends to a positive correlation with ER



**Fig. 3** bcl-2 expression and aMMP-2 activity in invasive breast carcinomas with low (group 1) and high (group 2) expression of ER. The expression of estrogen receptors and bcl-2 protein in breast carcinomas were analysed by immunohistochemistry and MMP-2 activity was evaluated by SDS-PAGE 0.1% gelatine zymography. Data represent the mean values  $\pm$  SD for each analysed parameter and for each group.

expression ( $r = 0.431$ ,  $P = 0.010$ ). Indeed, in tumors expressing high levels of ER, the activity of aMMP-2 form ( $64238 \pm 31326$  AU) was higher than in tumors expressing low levels of ER ( $35798 \pm 20004$  AU) ( $P_c = 0.018$ ). When TIMP-1 levels were correlated with PR expression, a clear negative correlation was obtained ( $r = -0.543$ ,  $P = 0.003$ ). Using the expression level of PR as an evaluation parameter, our data showed that the tumors with high expression of PR had a more reduced level of TIMP-1 ( $50 \pm 15$  ng/mg total protein) than the tumors with low expression of PR ( $116 \pm 88$  ng/mg total protein) ( $P_c = 0.006$ ). This observation suggested that the expression level of PR could be involved in the expression of TIMP-1 in malignant tumors.

When the tumors were grouped based on bcl-2 expression, the activity of aMMP-2 was significantly higher in tumors with bcl-2 expression than in tumors without bcl-2 expression ( $56956 \pm 31891$  AU,  $29213 \pm 8816$  AU respectively) ( $P_c = 0.002$ ).

Corroborating ER expression, bcl-2 expression and the activity of aMMP-2 form in breast cancer, another important aspect was revealed. Thus, the tumors with high ER expression level presented increased bcl-2 expression and aMMP-2 activity (Fig. 3).

## Discussion

Our results showed that MMP-9 and MMP-2 were expressed in both malignant and benign tumors, but to different extent. Thus, the expression and activity of MMP-9 and MMP-2 tended to be greater in malignant than in benign tumors. However, only the expression of total MMP-9 protein and the activity of aMMP-9 form significantly differentiated the two types of breast tumors, suggesting that these are particularities associated with malignancy. Generally, our results are in line with the previous reports, showing that the levels of gelatinases in human breast malignant tumors are increased compared to normal breast tissues [26–29], fibroadenomas [30] or hyperplasia [31].

Contrary to MMPs, the levels of TIMP-1 and TIMP-2 determined in our investigation showed an even distribution regardless of the tumor type. TIMP-1 and TIMP-2 expression constitutes a subject of contradictions. Nakopoulou *et al.* [32] and Jones *et al.* [33] found a comparable immunore-

activity for TIMP-2 in cancer cells and fibroblasts, while Garbett *et al.* [34] reported an enhanced expression of TIMP-2 in tumor cells compared to fibroblasts and inflammatory cells. The level of TIMP-1 was found either significantly lower [35] or significantly higher [36] in carcinomas *versus* fibroadenomas.

It has been well demonstrated that in physiological conditions the balance between MMPs and TIMPs is tightly regulated. Because the balance of these proteins is critical to matrix destruction, the ratio of MMP-9/TIMP-1 and MMP-2/TIMP-2 may adequately reflect the proteolytic potential. Giannelli *et al.* showed that the imbalance between MMPs and TIMPs is responsible for cancer metastasis [18]. Therefore, the ratios MMP-9/TIMP-1 and MMP-2/TIMP-2 were evaluated for all the studied tumor extracts, and we found that both ratios were increased, more in malignant than in benign tumor tissues. This imbalance between MMPs and TIMPs suggested a strong proteolytic potential of gelatinases in malignant tumors.

Despite a large number of studies, the conflicting relationship between MMPs and TIMPs and the prognostic factors for breast cancer still stands. For the first time we identified an association between MMPs and TIMPs and the subtypes of invasive breast carcinomas. We found that invasive carcinomas of no special type, with the most increased aggressiveness, display the highest levels of MMP-9 expression and activity, the lowest level of TIMP-1 and the highest ratio values for MMP-9/TIMP-1. These results suggested that MMP-9 and TIMP-1 imbalance could be involved in the configuration of invasive carcinoma of no special type. We have noticed in our study a relationship between MMP-9/TIMP-1 imbalance and the size of the tumor. This association was also reported by other groups of researchers, showing positive correlations of MMP-9 and TIMP-1 with the tumor size [36–38].

MMP-2 and TIMP-2 could play an important role in lymph node invasion. Onisto *et al.* [39] showed a positive correlation between MMP-2/TIMP-2 mRNA balance and node invasion, suggesting that the evaluation of this ratio has a higher prognostic value than the evaluation of MMP-2 and TIMP-2 expression alone. We also found a significant positive correlation between lymph node metastasis and MMP-2/TIMP-2 ratio value, attribut-

ed to increased MMP-2 and decreased TIMP-2 levels. Recently, it was demonstrated that IMMP-2, aMMP-2, IMMP-9 and aMMP-9 levels are significantly higher in tumor tissues than in adjacent normal tissue of breast cancer patients [26]. In addition, it was established that MMP-2, specifically, contributes to cancer cell migration by a mechanism involving MMP-2 interaction with collagen. To exclude potential overlapping effects of MMP-9, additional experiments of authors showed that MMP-2 also contributed to migration of MMP-9<sup>-/-</sup> cells. The results provide evidence that MMP-2 is an important determinant of cancer cell behavior [40].

We found an evident negative correlation between TIMP-2 and NPI score, therefore the MMP-2/TIMP-2 ratio positively correlated with NPI. Our observation is supported by data from a previous report showing that TIMP-2 expression is associated with a better overall survival of the patients [32]. While our data showed a negative correlation between TIMP-2 and NPI, Baker *et al.* demonstrated a negative correlation between TIMP-1 and NPI [41].

The prognostic value of hormone receptors in breast cancer was largely discussed [42–46]. Based on previous reports, we investigated the relationship between MMPs or TIMPs and hormone receptors. We found a significant negative correlation between TIMP-1 and PR, TIMP-1 level being a discriminating factor between tumors with high and low PR expression. The negative correlation between TIMP-1 and the expression of PR has not yet been reported, although McCarthy *et al.* [36] have shown a negative correlation of TIMP-1 with the expression of ER.

Another tendency of positive correlation between the activity of aMMP-2 and ER expression level was found, the activity of aMMP-2 form being different in tumors with high ER levels *versus* low ER levels. Regarding this relationship between MMP-2 and ER, Razandi *et al.* [47] have demonstrated that estradiol, *via* estrogen receptors, rapidly stimulates the signal transduction cascade from plasma membrane to a G protein, finally leading to MMP-2 and MMP-9 activation.

The role of bcl-2 in breast cancer progression, as anti-apoptotic molecule and mediator of MMPs and TIMPs, was also previously acknowledged [48–50]. We also found a significantly higher activity of aMMP-2 form in malignant tumors expressing high levels of bcl-2 than in malignant tumors

with low bcl-2 expression. Recently, it was demonstrated the anti-apoptotic role of TIMP-1 in breast cancer [51]. In our study, we did not find a correlation between bcl-2 expression and TIMP-1 level.

Our general conclusion is that tumors with high expression of ER presented the highest activity of aMMP-2 form and the highest expression of bcl-2. In line with our results, another group also observed a significant correlation between the expressions of bcl-2 and estrogen receptors in fresh breast cancer specimens [52]. Therefore, we suggest that the level of hormone receptors might modulate the expression of bcl-2, MMPs and TIMPs in breast cancer cells.

Taken together, our results are showing that gelatinases and their tissue inhibitors could be considered as valuable markers for diagnosis and prognosis of breast cancers. It is our hope that, in line with other studies performed by different authors, these markers could be also included in one of the prognostic factors group from the College of American Pathologist Consensus Statement [53].

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