Matrix Metalloproteinases in Breast Carcinoma

Immunohistology and Prognosis

1

Irena Ranogajec

Abstract

Since breast carcinoma is a group of heterogeneous tumors, the prognosis for each of this is determined in terms of a whole range of clinicopathological factors which can be divided into traditional (tumor size, lymph node status, histological grade, hormone receptors, proliferation index) and new factors, most of which are still being researched. This chapter shows the immunohistology and the prognostic value of the analysed gelatinases (MMP-2 and MMP-9) in breast carcinoma patients. Their expression in breast carcinoma patients is an unfavorable prognostic indicator of the disease, and an indicator of the need for more aggressive treatment in patients with negative lymph nodes. In the future the inhibition of these proteins could play a role in preventing breast carcinoma and in stopping the development of metastases in already existing breast carcinoma. Therefore, there is need for the incorporation of new prognostic factors into future studies and clinical trials that will provide new approach for the breast carcinoma patients.

Keywords

Matrix metalloproteinases · Breast carcinoma · Prognosis · Immunohistology · MMP-2 · MMP-9

I. Ranogajec (✉)
Department of Clinical Cytology, Polyclinic Sunce Zagreb, Trnjanska cesta 108, 10000 Zagreb, Croatia
e-mail: irena.ranoga9@yahoo.com

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1 Introduction

Breast carcinoma is the most frequent malignant tumor in women and it accounts for 27% of all malignant diseases affecting women, whereas it is rare in men. The highest incidence of breast carcinoma has been reported in developed countries, Northern America and Western Europe, and the lowest in Africa and Asia. However, growth of the number of newly detected breast carcinoma patients has recently been observed insofar lower risk countries (China, India, Japan) [1–3]. According to the WHO International Agency for Research on Carcinoma, breast carcinoma is the most frequent carcinoma in women in developed and less developed countries, and its incidence increases at the rate of 10%. In Europe the breast is the most frequent carcinoma site in women; breast carcinoma accounts for 25.5% of all deaths due to newly detected carcinoma sites in women, and for 17.5% of all deaths due to carcinoma in women. The carcinoma risk is 60% higher in Western than in Eastern Europe [4].

Breast carcinoma is also an important health problem for Croatia's female population. It accounts for 22% of new cancer cases, and in terms of incidence and mortality it is the most frequent form of carcinoma in women. According to the records of the National Carcinoma Register, breast carcinoma is diagnosed in about 2,300 women a year, and about 800–900 women die. The highest percentage of deaths due to breast carcinoma and the cancer in general has been reported in the 40–59-year group. Both the incidence and the mortality due to breast carcinoma in Croatia are on the increase, breast carcinoma being the most frequent single cause of death in women aged 35–59 years [5, 6].

In most cases surgical treatment (tumorectomy, mastectomy, dissection of axillary lymph nodes) is just the beginning of therapy, followed by adjuvant chemotherapy, postoperative radiation and, in some cases, adjuvant hormone therapy. Patients with an expression of the receptor of epidermal growth factor 2 (HER-2/neu) in tumor cells are also treated by an antibody blocking the receptor for this factor. According to the currently generally accepted opinion, the majority of women with negative lymph nodes and tumors up to 1–2 cm in size are healed by local therapy only in more than 70% of cases and do not benefit from chemotherapy. Metastases will develop in 20–30% of women in this group, and recent research is focused on detecting patient subgroups that would benefit most from adjuvant therapy [7]. Since breast carcinoma is a group of heterogeneous tumors, the prognosis for each is determined in terms of a whole range of clinical and pathological factors which can be divided into traditional and new factors, most of which are still being researched. The probability of recurrence and the length of survival depend on the stage of the disease (tumor size and histological grade, lymph node involvement, total positive node count), hormone receptor status, proliferation of malignant cell activity, oncogene expression or amplification (HER-2) and general condition of the patient. Since recurrence and repeated surgery can be expected in 25–30% of patients with breast carcinoma and negative lymph nodes, increasing attention is being focused on the discovery of new prognostic

markers which might help this group of patients by providing an additional future therapeutic approach [1, 2]. Matrix metalloproteinases have recently been studied intensively as possible prognostic factors in breast cancer patients. The numerous recent studies suggest their role in carcinogenesis and especially in the dissemination of breast carcinoma [8–15].

Matrix metalloproteinases (matrixins) belong to the family of zinc-dependent structural endoproteinases. In physiological conditions their role is essential in the degradation of the extracellular matrix during development, in the angiogenesis process, ovulation, involution of glandular breast tissue, and wound healing [16]. In 1962 Gross and Lapiere showed that skin cells from metamorphosing tadpole tails, when cultivated on collagen gel, released an enzyme which can degrade the triple collagen helix at neutral pH and 27 °C. The same study described a similar activity in cultures of embryonic skin, post-partial uterus and bone cells [17]. After that first discovery, collagenases were demonstrated in human tissue (MMP-1) and neutrophils (MMP-8). Over time other MMPs were discovered, starting with gelatinase A (MMP-2) and stromelysin (MMP-3), all the way to the identification of enamalysin (MMP-20) [16]. So far more than 20 different human metalloproteinases are known, and they are divided into four basic groups. The first group are collagenases which are involved in the remodeling and degradation of collagen; the second group are gelatinases (or type IV collagenases) which degrade gel and collagen type IV. The third group are stromelysins and matrilisins which degrade various extracellular matrix substances, including proteoglycanes and the non-collagen protein substance. The fourth group are membrane-type metalloproteinases which catalyze various ECM substrates and act as fibrinolytic enzymoplasmin. Proteinase inhibitors stimulating cellular proliferation and inhibiting cellular apoptosis have also been reported [8, 9]. All the metalloproteinases have some common features: each degrades at least one component of the basement membrane; they are active at physiological pH status; they need two zinc molecules for their activity; they are inhibited by metal chelases or tissue metalloproteinase inhibitors. Different physiological substances can inhibit MMPs; such as α2-macroglobulin molecules are larger molecules serum proteins which have an inhibitory effect but cannot cross into tissues. TIMP are smaller molecules displaying expression in different tissues and fluids; TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are known so far. The amino-terminal domain present in all TIMPs is responsible for inhibitory activity. TIMPs form non-covalent complexes with all active MMPs in a 1:1 ratio. The balance between proteases and inhibitors is the key factor determining proteolytic activity. Moreover, MMPs are synthesized in the inactive zymogen zone and require additional extracellular activation. These proenzymes remain inactive owing to interaction between cysteine in the proregion and zinc ions in the active site; the interaction blocks transition into the active form, and this interruption leads to enzyme activation. Trypsin 2, cathepsin, elastases, and plasmin/the plasminogenic system are factors which can lead to transition into the active form and once activated, MMPs can activate the others; MMP-3 can activate MMP-2, MMP-9, and MMP-7. Gelatinases, MMP-2 and MMP-9 can activate one another. The recently discovered MT-MMP subgroup can activate proMMP-2

through the transmembrane domain which is the most important in the process of MMP-2 activation [8, 10]. All latent MMPs comprise at least three domains: 1. the hydrophobic prepeptide domain required for signal secretion; 2. the amino-terminal propeptide domain removed by activation; 3. the catalytic domain containing zinc. This basic structure is present in all matrix metalloproteinases [8, 10].

2 Matrix Metalloproteinases in Breast Carcinoma

The role of metalloproteinases in the carcinogenesis of breast cancer cells is related to tumor initiation and growth, primarily by promotion of angiogenesis in the tumor, and activation of stimulating growth factors. The activation of growth factors and their receptors, and degradation of their inhibitors, is another significant mechanism of their activity [9, 10, 12]. Tumor cell metalloproteinases are probably responsible for invasive tumor growth, while stromal elements mainly influence the remodeling process and the desmoplastic reaction of the tumor [18]. Angiogenesis is stimulated by degradation of the barrier which permits endothelial invasion, and by the release of factors promoting the angiogenic phenotype. Neoangiogenesis is a key moment in the stimulation of tumor growth and development of metastases. Angiogenesis develops in several steps, including the release of angiogenic factors, release of proteolytic enzymes, migration within the extracellular matrix, EC proliferation, and formation and differentiation of microvascular spaces. Proteases are required for the invasion of extracellular space of malignant cells; metastatic cells use proteases to cross the basement membranes, connective tissue and, after that, the basement membranes of small blood vessels and lymph vessels. Type IV collagen is the main structural protein of the basement membrane and extracellular matrix. Studies have shown a correlation between the enhanced expression of MMP-2 and MMP-9 and the occurrence of metastases. It has also been demonstrated that the enhanced expression of MMP in the tumor is correlated with its higher aggressiveness and metastasising capacity [10]. MMP-2, but not MMP-9, releases the ectodomain of the FCG receptor. Since the hydrolysed ectodomain retains the capacity to bind FCG, it can modulate FCG mitogenic and angiogenic activities. Stromelsyn-3 can affect the promotion of MCF-7 cell growth by releasing the extracellular matrix growth factor [13]. The field of carcinoma cell invasion and metastasizing and inhibition by MMP inhibitors has also been studied so far in carcinomas of the pancreas, head and neck, glioma and other CNS tumors, and gastric carcinoma and melanoma. Pathological processes related to MMP activity, the subject of continuous research, include tissue destruction in fibrotic diseases and the weakening of the extracellular matrix in arthritis, oral pathology and periodontal diseases, liver and kidney fibrosis, endometriosis, and aortal aneurysm and heart failure due to the weakening of the extracellular matrix [16]. In situ hybridization techniques have shown metalloproteinases to display expression in tumor cells, and in stromal and inflammatory cells stimulated by factors released by tumor cells [18].

2.1 Matrix metalloproteinase-2 (MMP-2) belongs to the gelatinase subgroup (type IV collagenases) and is also called gelatinase A, 72-kD gelatinase; type IV collagenase is involved in the degradation of the basement membrane, elastase, and denatured type I, II, and III collagen [8, 16, 19]. The MMP-2 gene is located in chromosome 16q13, it is 17 kb long with 13 exons 110–901 bp in size and 12 introns 175–4.350 bp in size. 1–4 and 8–12 introns of type IV collagenase coincide with intron localizations in genes for MMP-1 and MMP-3, indicating also the close structural correlation of these genes with metalloproteinases. Unlike the stromelysin genes, there is no TATA box in the promotor region, but there are two GC boxes. There is no CAAT box, but the potential binding site to the transcription factor AP-2 is on the first exon [20]. In 1972 Harris and Kane studied gelatinase activity in rheumatoid sinovial tissue, probably related to MMP-2 activity. In 1978 Sellers et al. separated gelatinase activity from the action of collagenase 1 and stromelysin 1 in a hare cell culture. Collier described the chemical structure of gelatinase which consists of a triple repeat domain of type II fibronectin type introduced into the catalytic domain [16]. MMP-2 is secerned in the latent, proenzyme form, and activated through proteolytic modification by the activator MMP-2, i.e., the membrane type 1 (MT1-MMP), the enhanced activity of which in malignant breast tumors has so far been discussed in several studies [21–23]. ProMMP-2 activation does not depend on uPA/plasmin activity, but it can be activated by thrombin, plasmin, MMP-1 and MMP-7. Nevertheless, activation under the influence of MT-MMP on the surface of the cell appears to be physiologically the most acceptable [16]. ProMMP-2 forms a complex with TIMP-2, and this complex binds on the surface of the cell with MT1-MMP and, by releasing TIMP-2, MT1-MMP then activates proMMP-2. The activation of proMMP-2 through MT2-MMPa is direct and does not depend in TIMP-2. MMP-2 is suppressed by inhibitor TIMP-2 [24]. This inhibitor is related to a reduced metastatic potential and invasion; it is a nonglycosyling 21 kDa protein [10]. Although MMP-2 is referred to as gelatinase, this enzyme can attack the native form of collagen type I, and elastin and other proteins [16].

2.2 Matrix metalloproteinase-9 (MMP-9) is also known as gelatinase B, and is part of the gelatinase group. Like MMP-2 it degrades gel (denatured collagen), elastin, laminin, collagen type IV, V, XI and XVI, but not interstitial collagen. It also activates growth factors such as TGFb and pro TNF [8, 25–27]. Sopata et al. isolated it from human neutrophil granulocytes. Gelatinase B is the largest metalloproteinase and weighs 92 kDa. It is made up of three fibronectin domains and a similar collagen V domain. TIMP-1 binds to proMMP-9 creating a complex which regulates activation into MMP-9. Latent MMP-9 activators can include tripsin, as the most efficient, plasmin, cathepsin G, chimotripsin, tissue kallikrein, elastase and proteinase. ProMMP-9 can be activated by a cascade reaction through an intermediate link by the influence of other metalloproteinases: MMP-1, MMP-2, MMP-3, MMP-7, MMP-10 and MMP-13 [8]. So far MMP-9 expression in tumor cells and in the stroma of breast carcinoma has been studied by immunohistochemical methods or in situ hybridisation techniques [28–31]. It has also been

demonstrated that an MMP-9 promoter with a T allele displays a significantly high transcription activity as compared with the C allele. Accordingly, genetic polymorphism is also important for gene expression and, thereby, MMP-9 activity, ultimately enhancing the risk of tumor invasion and disease progression in patients with such a CT/TT genotype of the MMP-9 gene [32]. It has been demonstrated that fibroblasts, i.e., the stromal cells of breast carcinoma, produce MMP-9, with the involvement of TNF-alpha and TGF-beta inductors produced by breast carcinoma cells [33]. Fibroblasts can also be active in regulating the invasion of breast carcinoma cells through the production of thrombospondin-1 in fibroblasts which in turn induces MMP-9 production [34]. FGF also induces MMP-9 expression, as demonstrated by cell lines in vitro; this is related to the enhanced activity of NF-kappa B and AP-1 [35]. Because of the degradation of type IV collagen, which is particularly abundant in the basement membrane, MMP-2 and MMP-9 are most frequently and most strongly involved in tumor initiation, tumor growth and metastasizing, especially in breast carcinoma. In this regard another important is the promotion of angiogenesis by MMP-2 and MMP-9 which includes the degradation of the basement membrane of vascular, interstitial spaces, and the release of VEGF which is an angiogenic factor [22].

3 Immunohistology and Prognosis

Gelatinases and their tissue inhibitors can be valuable diagnostic and prognostic markers in breast carcinoma patients. On the basis of current and future studies by various authors they could eventually be incorporated into a standard prognostic group for breast carcinoma patients. In view of the foregoing, quite a few studies focused on the influence and application of metalloproteinase inhibitors and the future therapeutic potential of these substances. Numerous studies have shown a correlation of metalloproteinases with the initiation and progression of tumor growth [10, 12, 24].

The expression of MMP-2 in different tissues has been analysed immunohistochemically and by in situ hybridisation. MMP-2 is an enzyme present in normal tissue, most strongly in stromal elements. In breast carcinoma the very tumor cells modulate the level of MMP-2 by MT1-MMPa and TIMPa action. Since both tumor cells and the surrounding stroma in tumorous tissue display MMP-2 expression, a possible explanation is that tumor cell MMP influences invasive growth, while stromal elements influence the remodeling process and the desmoplastic reactions around the tumor cells [10]. In vivo studies have also confirmed the claim that stromal cells, i.e., fibroblasts, in most carcinomas play an important role in MMP-2 production. Along with carcinoma cells, fibroblasts are also probably stimulated to produce the higher MMP-2 levels observed in malignant tumors [12]. So far numerous studies have demonstrated the positive correlation between enhanced MMP-2 activity and tumor invasiveness and metastatic capacity [10, 18, 24, 25, 28, 29, 36–38]. In breast carcinoma cells enhanced MMP-2 expression was observed

compared with expression in benign breast lesions, and in vitro studies have also shown that active MMP-2 expression is linked with the more aggressive malignant cell potential in breast carcinoma cell lines [24]. The level of pro-MMP2/MMP-2 affects the determination of cell invasive and metastatic capacity, and the higher MT-MMPa level influences MMP-2 activation in carcinoma of the breast, cervix, and lungs by promoting invasiveness and metastatic capacity [10].

The expression of MMP-9 is significantly higher in malignant breast carcinoma cells than in benign lesions. As observed, MMP-9 expression is the highest in the most aggressive invasive ductal carcinoma (NOS) of the breast, while the value of the TIMP-1 inhibitor is the lowest. These results show that the MMP-9/TIMP-1 imbalance can influence the development and growth of ductal carcinoma (NOS), and indicated the correlation between MMP-9/TIMP-1 and tumor size [22]. It has been demonstrated that the high expression of MMP-9, especially in stromal breast carcinoma cells, is related to the less differentiated ductal type tumors with a poorer prognosis of survival and with the shorter recurrence time in breast carcinoma patients [25]. Recent studies have also demonstrated the presence of higher MMP-9 expression in cells of lobular carcinoma in situ, both immunohistochemically and by the RT-PCR method; this makes MMP-9 an interesting therapeutic and chemoprotective agent in the future treatment of lobular neoplasias [39].

In view of the foregoing considerations quite a few studies have considered the experimental models of cell lines in examining the influence and application of metalloproteinase inhibitors and the future therapeutic potential of these substances. Numerous studies have demonstrated the correlation between metalloproteinases and tumor growth initiation and progression [10, 12, 24]. Thus, the integration of their inhibitors is linked with the prevention of carcinogenic growth and the inhibition of invasion and metastasizing [10].

3.1 Our study of matrix metalloproteinases in breast carcinoma hypothesized that the enhanced expression of gelatinases (MMP-2, MMP-9) in tumor and stromal cells in invasive breast carcinoma patients ought to be an unfavorable prognostic factor, and suggested the need for more aggressive treatment in patients with negative lymph nodes and HER-2 protein expression [40, 41]. The median age of breast carcinoma patients in this study was 56 years and the majority of them was postmenopausal, had a tumor less than 2 cm, ductal histological type, estrogen positive, intermediate histological grade, with a low proliferative index. Lymph node metastases were found in 36.9% of patients and HER-2 expression in 15.9% of our study of matrix metalloproteinases. In 48.1% of patients more than 100 tumor newly formed vessels/mm^2 were found (Table 1) [40, 41].

3.2 The expression of MMP-2 and MMP-9 in tumor cells, in our study, was evaluated by the semiquantitative method as cytoplasmic and membrane staining. The expression of MMP-2 and MMP-9 in tumor and stromal cells was compared with standard clinicopathological prognostic factors and patient survival. We showed MMP-2 and MMP-9 expression in cytoplasm and membrane of the tumor cells and also positivity of stromal cells of the tumor in breast carcinoma patients (Fig. 1 and 2) [40, 41].

Table 1 Overview of studied clinicopathological factors in 138 breast carcinoma patients [41]

Factor	Number of patients	%
Age		
<50 years	49	35.5
>50 years	89	64.5
Tumor size		
<2 cm	74	53.6
>2 cm <5 cm	57	41.3
>5 cm	7	5.1
Histological tumor type		
Ductal	81	58.7
Lobular	30	21.7
Other	27	19.6
Tumor grade		
1	21	15.2
2	73	52.8
3	44	32.0
Vascular invasion		
Negative	134	97.1
Positive	4	2.9
New blood vessels/square mm		
<50	19	14.5
>50 < 100	49	37.4
>100	63	48.1
Ki-67		
<10%	81	60.9
>10%	52	39.1
Lymph nodes		
Negative	60	43.5
Positive	51	37.0
Unknown	27	19.5
Estrogen receptors		
Negative	43	42.6
Positive	58	57.4
Progesteron receptors		
Negative	56	55.4
Positive	45	44.6
HER-2		
Negative	116	84.1
Positive	22	15.9

In 2004 Pellikainen et al. also demonstrated MMP-2 and MMP-9 expression in the cytoplasm of tumor and stromal cells. Positive MMP-2 stromal cells (46%) were associated with strong HER-2 expression in the group of patients with negative

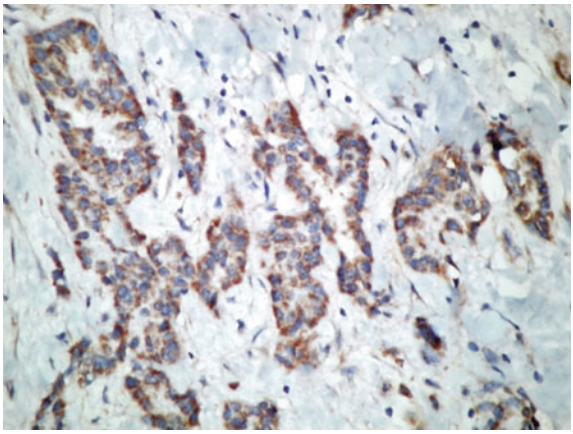

Fig. 1 Membrane and cytoplasmic MMP-2 positivity of tumor cells. (MMP-2 × 40) [41]

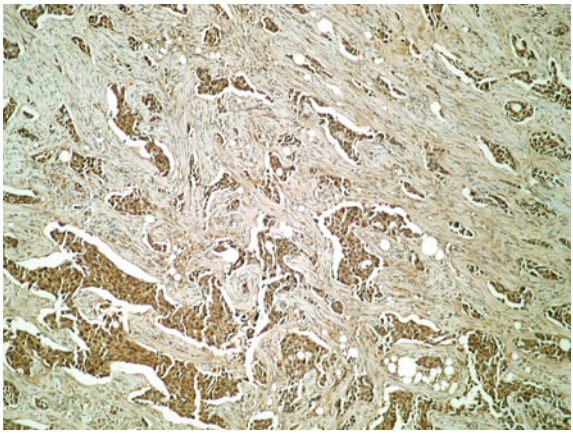

Fig. 2 MMP-9 positivity of tumor and stromal cells. (MMP-9 × 10)

lymph nodes, and this expression is correlated with more aggressive factors. The strong MMP-2 expression in tumor cells was associated with strong stromal expression to a statistically significant degree ($p = 0.009$). Stromal MMP-9 expression was observed in 38% of cases and was correlated, to a statistically significant degree, with HER-2 expression in hormone positive tumors. MMP-2 expression in tumor cells was not correlated with HER-2 expression [25]. In 1999 Jones et al. demonstrated MMP-2 expression in more than 90% of studied breast carcinoma patients, mainly in terms of cytoplasmatic staining; membrane expression was noted in 34% of patients. MMP-9 expression was seen in 68 of patients as tumor cell or stroma staining [29]. In 2002 Singer et al. confirmed the importance of cell–cell interaction and demonstrated, on an in vitro fibroblast and tumor cell culture, enhanced expression and activity of both gelatinases, MMP-2 and MMP-9. They thereby substantiated the claims regarding the importance of stroma in tumor progression through the release of angiogenic substances, cytokines affecting cellular growth, and protein degrading enzymes such as PDGF, EFG, FGF, IL-1,

TNFα, and EMMPRIN [42]. In the opinion of some authors—since both the tumor cells and the surrounding stroma in tumor tissue display MMP-2 expression—the possible explanation might be that tumor cell MMP affects invasive growth, while stromal elements influence the remodeling process and desmoplastic reaction round the tumor cells. In vivo studies also corroborate the claim that, in most carcinoma cases, stromal cells, i.e., fibroblasts, play an important role in MMP-2 production. Along with carcinoma cells, fibroblasts are also probably stimulated to produce the higher MMP-2 levels noted in malignant tumors [12]. In 2006 Tetu et al. demonstrated stromal MMP-2 expression in about 50% of breast carcinoma patients, whereas tumor cells were not positive as shown by the results obtained by in situ hybridisation. The same author has demonstrated that breast cancer cells produce factors (TGF, PDGF, EMMPRIN) inducing stromal cells to produce proteases which in their turn stimulate tumor cells by binding to receptors. These results support the hypothesis that biological behavior of the tumor does not depend only on its characteristics but also on its stroma. Moreover, this finding conceals possible therapeutic potential since reactive stromal cells display greater genetic stability than carcinoma cells and are hence theoretically less subject to mutation and resistance to therapy [24].

3.3 Our study presented the correlation of tumor and stromal MMP-2 and MMP-9 expression with other prognostic factors in breast carcinoma patients (Table 2). The results have shown a correlation between the studied factors and MMP-2 expression in tumor cells, but the correlation is not statistically significant ($p > 0.05$). However, stromal MMP-2 expression showed a statistically significant difference with regard to neoangiogenesis and tumor size, meaning that a stronger MMP-2 stromal expression also denotes stronger neoangiogenesis and greater probability of >5 cm tumors [40, 41].

In 2006 Liu et al. demonstrated the statistically significant correlation between MMP-2 expression and tumor size, tumor grade and metastasis development, concluded that MMP-2 expression can reflect the possible invasiveness of breast cancer and that, therefore, different selective MMP inhibitors could eventually be used as potential anti-metastatic drugs taking tumor size into account [43]. A group of Chinese authors, Peihong et al., showed in 2007 a strong MMP-2 expression in DCIS, and thereby demonstrated MMP-2 correlation with tumor invasion, i.e., more aggressive biological tumor potential, and suggested the possible use of MMP-2 expression as an early prognostic factor of invasiveness [44]. On the other hand, another group of authors, Kim et al., failed in 2006 to demonstrate a statistically significant difference in MMP-2 between DCIS and DIC [30]. In 2007 Ogura et al. demonstrated the statistical significance of MMP-2 expression in patients with T1 N0 grade breast carcinoma and recurrence within 10 years, and thereby suggested the possible use of MMP-2 as a promising predictor of recurrence risk in patients with earlier breast cancer stages [45].

The tumor cell MMP-9 expression in our study demonstrated a statistically significant correlation with the histological tumor type (ductal and other tumor types) ($p < 0.05$, data not shown), hormone status ($p = 0.02$), and a marginal

Table 2 Correlation of MMP-2 and MMP-9 expression with clinicopathological factors [40]

Prognostic factors	MMP-2 tumor cells *p*	MMP-2 stromal cells *p*	MMP-9 tumor cells *p*	MMP-9 stromal cells *p*	MMP-2/MMP-9 tumor cells *p*	MMP-2/MMP-9 stromal cells *p*
Age	0.28	0.32	0.06	0.53	0.55	0.35
Tumor type	0.63	0.52	0.06	0.08	0.50	0.51
Tumor grade	0.86	0.97	0.25	0.67	0.87	0.73
Vascular invasion	0.64	0.26	0.51	0.45	0.64	0.27
Estrogen receptors	0.25	0.47	**0.02***	0.44	0.46	0.53
Progesteron receptors	0.42	0.57	**0.02***	0.43	0.31	0.37
Lymph node	0.99	0.24	0.99	0.96	0.83	0.41
HER-2	0.47	0.28	0.08	0.41	0.52	0.49
Ki-67	0.40	0.14	0.12	0.12	0.39	0.06
Neoangiogenesis	0.28	**0.04***	0.78	0.74	0.13	0.67
Tumor size	0.31	**0.01***	0.94	0.46	0.39	**0.01***

*Significantly different

significance for HER-2 expression ($p = 0.08$) and patient age ($p = 0.06$). This means that there is a statistically significant correlation between higher MMP-9 expression in tumor cells and positive estrogen and progesterone receptors, ductal and other breast carcinoma types, HER-2 expression, and postmenopausal patient status [40, 41].

In 2006 Jinga et al. compared gelatinase activity and expression with prognostic factors in breast carcinoma patients (tumor stage, histological type and grade, tumor size, nodal status, and NPI). Because of the small number of patients in the studied groups they failed to demonstrate statistically the correlation between MMP and the tumor stage and histological grade: a weak positive correlation was found between tumor size and MMP-9, while correlation between MMP-9 expression and histological tumor type was statistically significant. Thus, in invasive breast carcinoma NOS MMP-9 expression was the strongest, and TIMP-1 value the lowest. Positive correlation was found between MMP-2 expression and lymph node status; enhanced MMP-2 expression and reduced TIMP-2 expression were found in patients with tumor cell invasion into lymph nodes. A positive correlation was found between MMP-2 expression and estrogen receptor status in breast tumors; in tumors with a stronger ER expression a stronger MMP-2 activity was observed. Estradiol is deemed to stimulate, through estrogen receptors, the signal transduction cascade leading to gelatinase (MMP-2 and MMP-9) activation. Gelatinases can be considered to be valuable diagnostic and prognostic markers in breast carcinoma patients, and in the future they can be incorporated into the prognostic factor group [22]. In 2007 Nilsson et al. demonstrated that estradiol and tamoxifen regulate

MMP-2 and MMP-9 in hormone positive breast carcinoma. During tamoxifen therapy MMP-2 and MMP-9 activity is enhanced by MMP modulation through anti-angiogenic fragments [46]. In 2005 Di et al. demonstrated in their in vitro study that gelatinase expression can be stimulated by estrogen in hormone dependent breast carcinomas which have positive estrogen as well as progesterone receptors. Thus, during therapy the blocking of estrogen or aromatase by aromatase inhibitors can reduce tumor growth and the tumor metastatic potential [31].

In our results the stromal expression of MMP-9 was correlated, to a statistically significant extent, with lobular breast carcinoma ($p = 0.02$, data not shown), whereas for ductal carcinoma the statistical significance was marginal ($p = 0.08$) [40, 41]. A similar result was confirmed by Jones et al. by demonstrating a statistically significant correlation between MMP-9 and the histological tumor type; in invasive lobular carcinoma, unlike the invasive ductal type, a more homogeneous cytoplasmatic MMP-9 expression was observed [29]. In 2008 Dengfeng et al. correlated MMP-9 expression with lobular carcinoma in situ. Accordingly, activated MMP-9 is believed to be involved in the formation of the typical *indian file* histological picture of lobular carcinoma; similarly, MMP-9 RNA and protein are believed to act as precursors of (LCIS) stage invasive lobular carcinoma, and factors activating it can trigger the development of the invasive disease. This makes MMP-9 an interesting therapeutic and chemopreventive target for patients with lobular invasion of the infiltrating or non-infiltrating type [39]. Similarly, Pellikainen correlates stromal MMP-9 expression with poor tumor differentiation, hormone-negative tumors, and ductal carcinoma [25]. In 2004 Rahko et al. demonstrated MMP-9 expression in 61.3% of patients with breast cancer. With MMP-9 expression, 5-year DFS amounted to 37%, as compared with 63% of patients with negative MMP-9 in the subgroup of hormone negative tumors. The study did not confirm the correlation of MMP-9 expression with clinical stage, histological prognostic factors, and hormone status [26].

Our study also analysed the tumoral and stromal MMP-2 and MMP-9 coexpression, and the correlation between this coexpression and other prognostic factors (Table 2). The results show a correlation with all factors, but it is statistically significant in stromal MMP-2/MMP-9 coexpression regarding tumor size ($p = 0.01$), i.e., positive tumors were more often larger [40, 41].

3.4 Our study analysed the correlation between the number of deaths and recurrences, and the MMP-2 and MMP-9 expression in tumor cells and stroma; correlation was demonstrated between tumor cell MMP-2 and the number of deaths ($p = 0.08$, data not shown), but it was marginal in terms of statistical significance; accordingly, the stronger the tumor cell MMP-2 expression the higher is the probable number of deaths for breast carcinoma patients. Survival and recurrence probability curves (Kaplan–Meier) over months showed no statistically significant difference between positive and negative MMP-2 and MMP-9 expression in 138 analysed patients ($p > 0.05$) with the exception of tumor cell MMP-2 expression as related to overall survival, where a statistically significant difference was observed between the curves for the positive and negative group ($p = 0.025$) (Fig. 3). This

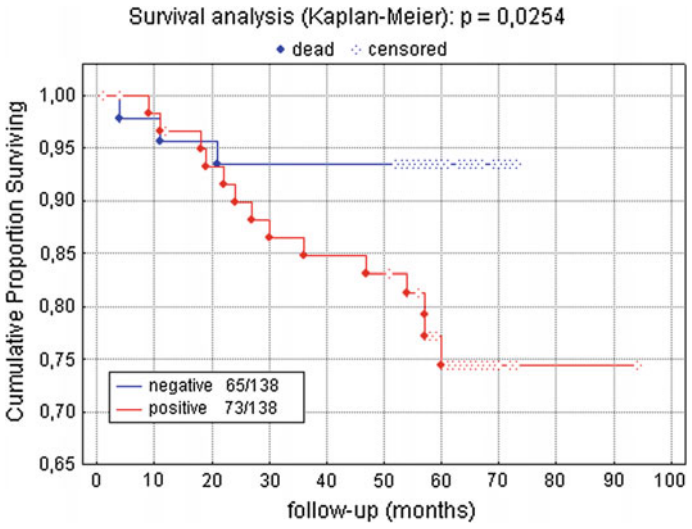

Fig. 3 Overall survival in relation to MMP-2 expression in breast tumor cells [40]

means that breast cancer patients with tumor cell MMP-2 expression die faster during the period of observation as compared with the group with negative MMP-2, and this difference is statistically significant [40, 41].

In 2003 Talvensaari-Matilla et al. demonstrated a statistically significant correlation between MMP-2 expression and survival. In the group of patients with negative progesterone receptors and MMP-2 positive tumors the rate of survival was 58%, and in the group of MMP-2 negative tumors survival observed over 10 years was 95% (p = 0.005). These results showed for the first time that negative MMP-2 in breast carcinoma patients with negative hormone receptors can serve as a marker indicating a much better prognosis [47]. Even earlier, in 1999, the same authors demonstrated a higher recurrence risk in patients with positive lymph nodes and MMP-2 expression in younger than 40 years, but the difference was not statistically significant [48]. In 2006 Tetu et al. showed, when MMP-2, TIMP-2, and MMP-14 were analysed together, that survival was the poorest for patients with strong stromal MMP-2 and MMP-14 and weak stromal TIMP-2 expression (five-year survival rate: 50%); it was the best with weak stromal MMP-2 and MMP-14 expression, and strong stromal TIMP-2 expression (5-year survival = 74%); however, the difference was not statistically significant [24]. In 2003 Wang et al. demonstrated the correlation between MMP-9 and metastases, i.e., positive lymph nodes. The correlation between MMP-9 expression and overall survival was also demonstrated, and the group of patients with survival longer than 3 years had a lower MMP-2 expression unlike the group with survival of less than 3 years [49]. Pellikainen also demonstrated that positive stromal MMP-9 expression is a predictor of a shorter DFS and shorter overall survival in estrogen positive tumors; thus, the rate of 5-year survival in negative stromal MMP-9 tumors was 89% as compared with 70% in positive stromal MMP-9 tumors [25].

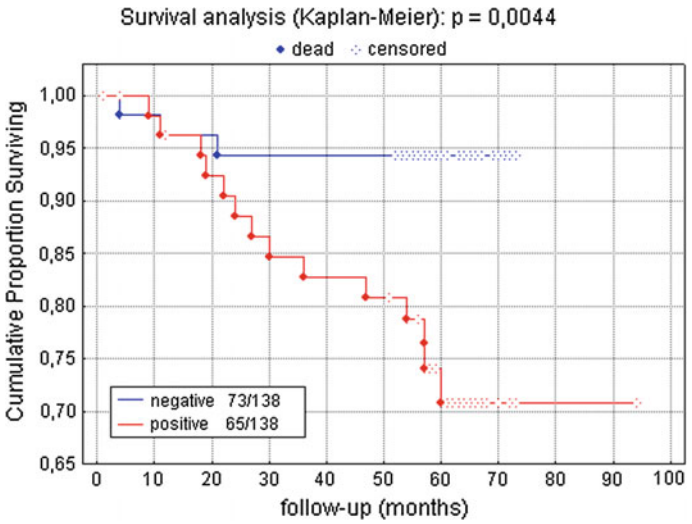

Fig. 4 Overall survival in relation to MMP-2/MMP-9 coexpression in breast tumor cells [40]

Considering the course of the disease, the correlation between tumoral MMP-2/MMP-9 coexpression, in our study, and the number of deaths and recurrences demonstrated a statistically significant correlation between tumoral MMP-2/MMP-9 coexpression and the number of deaths ($p = 0.001$, data not shown). The classic survival probability curve (Kaplan–Meier) showed a statistically significant difference between positive and negative tumoral MMP-2/MMP-9 coexpression over the followed-up months ($p = 0.004$) (Fig. 4). In other words, after a 60-month follow-up 30% of breast cancer patients with a positive tumoral MMP-2/MMP-9 coexpression died, as compared with only 5% deaths in the group with negative tumoral MMP-2/MMP-9 staining. This confirmed the value of MMP-2/MMP-9 coexpression for the clinical course of breast cancer patients [40, 41]. In 2004 Li et al. studied the prognostic value of immunohistochemical MMP-2/MMP-9 coexpression in breast cancer patients with negative lymph nodes. Positive MMP-2 expression was found in 56.7% of tumors and positive MMP-9 in 59.6%. In this patients group a statistically significant correlation was established between positive MMP-2 and tumor size and histological grade, whereas MMP-2/MMP-9 coexpression was statistically significant with respect to DFS ($p = 0.013$) but not with respect to overall survival ($p = 0.122$). This showed that gelatinases are poor prognostic factors with regard to the shorter survival and demonstrated the statistically significant value of MMP-2/MMP-9 coexpression in patients with negative lymph nodes [28].

In 2003 Fan et al. demonstrated a stronger MMP-2 and MMP-9 expression in breast cancer with positive lymph nodes. Moreover, there was a statistically significant correlation between gelatinases and tumor size and shorter survival. Univariate analysis confirmed MMP-2 and MMP-9 as predictors of an unfavorable

prognosis regarding overall survival ($p < 0.05$) [50]. In 2009 Shah et al. confirmed, in their recent study, that MMP-2 and MMP-9 expression in breast cancer patients and negative lymph nodes can predict the risk of metastasis development in lymph nodes, and that such patients need to be followed-up more closely, with targeted gelatinase inhibitor therapy added in the future [51].

3.5 Multivariate analysis of specific survival, in our study, showed that patients with tumor size larger than 5 cm with positive lymph nodes and coexpression of MMP-2/MMP-9 in tumor cells had an independent prognostic significance (Table 3). Coexpression of MMP-2/MMP-9 in tumor cells was an independent poor prognostic factor in our breast carcinoma patients [40, 41].

Talvensaari-Matilla et al. showed that after a 10-year follow-up 56% of patients with a strongly positive MMP-2 were alive, as compared with 88% of patients with negative MMP-2 regardless of other prognostic factors. In this study MMP-2 was confirmed for the first time, by multivariate analysis, as an independent prognostic factor for survival in breast cancer patients, increasing the death risk 3.6 times over a 10-year follow-up [47].

Our study has demonstrated the value of MMP-2 and MMP-9 determination in breast cancer patients since the expression of these proteins in breast cancer, along with the already existing traditional prognostic factors, represents an additional piece of information on poor prognosis in breast cancer patients. The expression of MMP-2 and MMP-9 (tumor cells and stroma) and their coexpression in breast cancer patients is an unfavorable prognostic indicator of the disease, and an indicator of the need for more aggressive treatment in patients with negative lymph nodes [40, 41].

The latest studies and their results provide information on so far statistically insignificant results in terms of the effect of MMP-2 and MMP-9 expression as independent poor prognostic factors of survival in breast cancer patients, but in combination with other prognostic factors they can provide valuable information, especially in the group with negative lymph nodes and negative hormone receptors. However, considering the still inconsistent results and the still unexplained true nature of behavior of these markers, many authors refer to the necessary additional study of these markers and of the entire metalloproteinase group since this field continues to yield more and more information on the importance of cell–cell interaction and on the influence of the extracellular matrix in carcinogenesis and in the evaluation of the biological tumor behavior.

Table 3 Multivariate model of breast carcinoma specific survival [40]

Risk factors	P	SE	OD	95% CI
Tumor size >5 cm	**0.0044**	1.0241	19.4076	2.560–147.09
Lymph node positivity	**0.0068**	0.6105	5.3544	1.600–17.910
Tumor cell MMP-2/MMP-9 coexpression	**0.0022**	0.8460	13.961	2.619–74.409

OD Odds ratio *CI* Confidence intervals

Where breast carcinoma is involved, in the future the implementation of additional methods will slowly shift from classis prognostic parameters to new markers which can help in selecting therapy and be factors predicting its use. In molecular diagnostics, the basis of additional research in breast cancer continues to be the determination of hormone receptors and the HER-2 status. However, the incorporation of new prognostic factors into the increasing number of studies and clinical trials will provide new contributions to the coming era of personalized medicine and help in the choice of the best individual therapeutic option for each patient.

Theoretically speaking, in the future we can expect that the main contribution of the study of all these compounds will provide for the appearance of a new approach in adjuvant or neoadjuvant therapy of breast cancer patients. Analogously, since metalloproteinases, as demonstrated by recent research, are involved in the initiation of carcinogenesis, in the future their inhibitors can be taken into account in the evaluation of the quest of appropriate chemopreventive substances.

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