Prognostic role of matrix metalloproteinase 9 in early breast cancer

KATARINA KALAVSKA[1,2*], ZUZANA CIERNA[3,4*], MARIAN KARABA[5,6], GABRIEL MINARIK[7], JURAJ BENCA[5,8], TATIANA SEDLACKOVA[9], DENISA KOLEKOVA[1], IVANA MRVOVA[3,4], DANIEL PINDAK[5,6], JOZEF MARDIAK[10] and MICHAL MEGO[1,10]

[1]Translational Research Unit, Faculty of Medicine, Comenius University, 833 10 Bratislava; [2]Department of Molecular Oncology, Cancer Research Institute, Biomedical Research Center of the Slovak Academy of Sciences, 945 05 Bratislava; [3]Department of Pathology, Faculty of Medicine, Comenius University, 811 08 Bratislava; [4]Department of Pathology, Faculty Hospital, 917 02 Trnava; [5]Department of Oncosurgery, National Cancer Institute, 833 10 Bratislava; [6]Department of Surgical Oncology, Slovak Medical University, 833 03 Bratislava; [7]Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, 811 08 Bratislava; [8]Department of Medicine, St. Elizabeth University, 810 01 Bratislava; [9]Geneton Ltd., 841 04 Bratislava; [10]Second Department of Oncology, Faculty of Medicine, Comenius University, National Cancer Institute, 833 10 Bratislava, Slovakia

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Abstract. MMP9 is involved in extracellular matrix degradation during various physiological and pathological conditions, including tumorigenesis. The present study aimed to assess the prognostic role of intratumoral MMP9 and to determine its association with circulating tumor cells (CTCs) in patients with early breast cancer. A total of 318 patients with primary breast cancer (PBC) were enrolled into the present study. Specimens were subjected to immunohistochemistry analysis, using the MMP9 monoclonal antibody. MMP9 expression was scored using a weighted histoscore (WH). The results demonstrated that the mean WH ± SEM for MMP9 expression was significantly higher in breast tumor cells compared with tumor associated stromas (132.0±5.2 vs. 50.8±3.7; P<0.00001). Furthermore, a positive association was observed between MMP9 expression, the hormone positive status and proliferation index of analysed breast cancer tumour cells. Notably, the prognostic role of MMP9 was not observed in tumor cells [hazard ratio (HR) =0.96; 95% confidence interval (CI), 0.58-1.59; P=0.864] or tumor associated stroma (HR=1.29; 95% CI, 0.60-2.78; P=0.547). Subgroup analysis demonstrated that patients that were HR negative or triple negative, with low MMP9 expression in tumor cells and stroma had a significantly improved disease-free survival than patients with high MMP9 expression. Taken together, the results of the present study demonstrated that high MMP9 expression in PBC was associated with favorable tumor characteristics. However, the prognostic value of MMP9 was limited to only the HR negative and CTC epithelial-to-mesenchymal transition positive subgroups. Thus, analyzing MMP9 tumor expression may help identify patients with increased risk of disease recurrence in these subgroups.

Correspondence to: Professor Michal Mego, Second Department of Oncology, Faculty of Medicine, Comenius University, National Cancer Institute, Klenova 1, 833 10 Bratislava, Slovakia
E-mail: misomego@gmail.com

*Contributed equally

Abbreviations: MMP9, matrix metalloproteinase 9; CTCs, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; HR, hazard ratio; PBC, primary breast cancer; PR, progesterone receptor; RT-PCR, reverse transcription PCR; NA, non-applicable

Key words: matrix-metalloproteinase 9, early breast cancer, prognostic value, circulating tumor cells

Introduction

Breast cancer is the most common malignancy in women worldwide, whereby 2,088,849 new cases of invasive breast cancer and 626,679 mortalities were reported in 2018 (1,2). Tumor invasion and metastasis affect >90% of patients with breast cancer, and thus notably contribute to the high mortality rate (3-5). This metastatic disease remains incurable, and effective treatment for end-stage metastatic breast cancer are yet to be determined (6-8). The aggressiveness of a tumor is closely associated with its ability to evade natural barriers, to invade adjacent tissues and metastasize distant sites (9). The metastatic cascade is a multistep process where cancer cells escape from the primary tumor site to distant locations, where they can potentially establish new cancer colonies (10,11). Under optimal conditions, epithelial cancer cells detach from the primary tumor site, penetrate and migrate via peripheral circulation, and invade secondary sites, where they ultimately undergo extravasation and populate distant organs (11,12).

Proteolytic degradation of the basement membrane and extracellular matrix (ECM) is considered a crucial aspect of metastatic growth, which enables low anchorage of neoplastic cells (13-17). Several cell-secreted proteolytic enzymes, including matrix metalloproteinases (MMPs) are implicated in the cleavage of ECM (13,18,19). Matrix metalloproteinase 9 (MMP9) is a member of the gelatinase subfamily of MMPs and is secreted by a variety of cell types in an inactive form that undergoes activation upon cleavage by different types of extracellular proteases (18,20). MMP9 activity is modulated via different biochemical molecules, including growth factors and cytokines (19,21). Notably, MMP9 is actively involved in the degradation of type IV collagen, which is a crucial component of the basement membrane (19,22). In addition, MMP9 facilitates the dissemination machinery, and is particularly involved in tumour invasion, tumour-induced angiogenesis, and immunomodulation of the tumour microenvironment, where it is implicated in the formation of so-called premetastatic niches (23,24). Previous studies have focused on the association between high MMP9 expression and the number of distant metastases in patients with breast cancer (25-27), as well as poor prognosis (28,29). It has been speculated that circulating tumor cells (CTCs), which are responsible for distant metastasis formation, use MMPs to form new metastatic sites (19,30). In addition, a previous study demonstrated that elevated MMP1 expression is significantly associated with the presence of CTC_ epithelial-to-mesenchymal transition (EMT) cells in the peripheral blood of patients with primary breast cancer (PBC), as well as with poor prognostic features of their primary tumors (31). The present study aimed to assess MMP9 expression in tumor cells as well as tumor associated stroma of patients with PBC, and determine its association with the presence of CTCs in the peripheral blood of these patients and other clinicopathological characteristics. The prognostic value of MMP9 in patients with PBC was also assessed.

Patients and methods

Study patients. The present study (Protocol TRU-SK 002; Chair: Michal Mego) enrolled 318 patients with stages I-III PBC who underwent definitive surgery. The samples were collected from the National Cancer Institute (Bratislava, Slovakia) between March 2012 and February 2015. The paraphing embedded tumor tissue and CTCs status in peripheral blood were available for all patients included in the present study. Complete diagnostic evaluation was performed in all patients to exclude the presence of distant metastasis. Patients with concurrent malignancy in the last 5 years, other than non-melanoma skin cancer, were excluded from the present study. The clinicopathological data including age, tumor stage, histology, regional lymph node involvement, hormone receptor status and HER2 status were retrieved and tabulated from the patients' records after obtaining all the relevant ethical approvals.Breast cancer subtypes were identified by immunohistochemical staining (see below) and classified according to the ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up for early breast cancer (32).

The present study was reviewed and approved by the Institutional Review Board of the National Cancer Institute of Slovakia, Bratislava, Slovakia (TRUSK002, 20.6.2011). Written informed consent was provided by all patients prior to the study start.

Tumor pathology. Pathological review was performed at the Department of Patholoχgy, Faculty of Medicine, Comenius University, (Bratislava, Slovakia) by an experienced pathologist (ZC).

Tumor samples and tissue microarray construction. Tumor specimens used in the present study were classified according to the 2019 World Health Organization classification (33). According to the tumor histology results, one or two representative areas containing the most representative part of the hematoxylin and eosin (H&E) stained tumor tissues were observed under a light microscope, original magnification×400. The identified sections were matched to their corresponding wax blocks (donor blocks). The 3-mm diameter cores of the tumors were removed from the donor blocks using the multipurpose sampling tool HarrisUni-Core (Sigma-Aldrich; Merck KGaA) and inserted into the recipient master block. The recipient block was cut into 5-μm-thick sections, which were transferred onto coated slides.

Immunohistochemical (IHC) staining. Deparaffinized slides were rehydrated in phosphate buffered saline solution (10 mM, pH 7.2). Tissue epitopes were demasked using the automated water bath heating process in Dako PT Link (Dako; Agilent Technologies, Inc.) and the slides were incubated in pH 6.0 citrate retrieval buffer at 98˚C for 20 min. The slides were subsequently incubated for 1 h at room temperature with primary mouse monoclonal antibody against MMP9 (Abcam; MMP9 (SB15c); cat. no. ab51203) diluted 1:200 in Dako REAL antibody diluent (Dako; Agilent Technologies, Inc.) and immunostained with anti-mouse/anti-rabbit immuno-peroxidase polymer (EnVision FLEX/HRP, Dako; Agilent Technologies, Inc.) for 30 min at room temperature, according to the manufacturer's instructions. The reaction was visualized using diaminobenzidine substrate-chromogen solution (Dako; Agilent Technologies, Inc.) for 5 min, and the slides were counterstained with hematoxylin. The human clone tissue served as the positive control, and colon tissue subjected to the same procedure omitting the primary antibody was used as the negative control.

IHC evaluation. Tumor scores were blindly assessed by a pathologist (ZC). The results of the IHC analyzes were scored using a weighted histoscore (WH), assessing both the percentage of positive cells (PP) and the staining intensity (SI) of the cytoplasm as follows: The proportion of cells with nuclear staining was multiplied by the intensity of staining to provide a histoscore ranging from 0-300. The histoscore was calculated as follows: Score=(0x percentage not stained) + (1x percentage weakly stained) + (2x percentage moderately stained) + (3x percentage strongly stained) (34). The mid-point of WH histoscore was used as the cut-off criterion similary as previosly (35,36). MMP9 expression was stratified as low vs. high, according to the cut-off value of WH histoscore (150).

Detecting CTCs in peripheral blood. CTCs were identified via reverse transcription-quantitative (RT-q)PCR analysis.

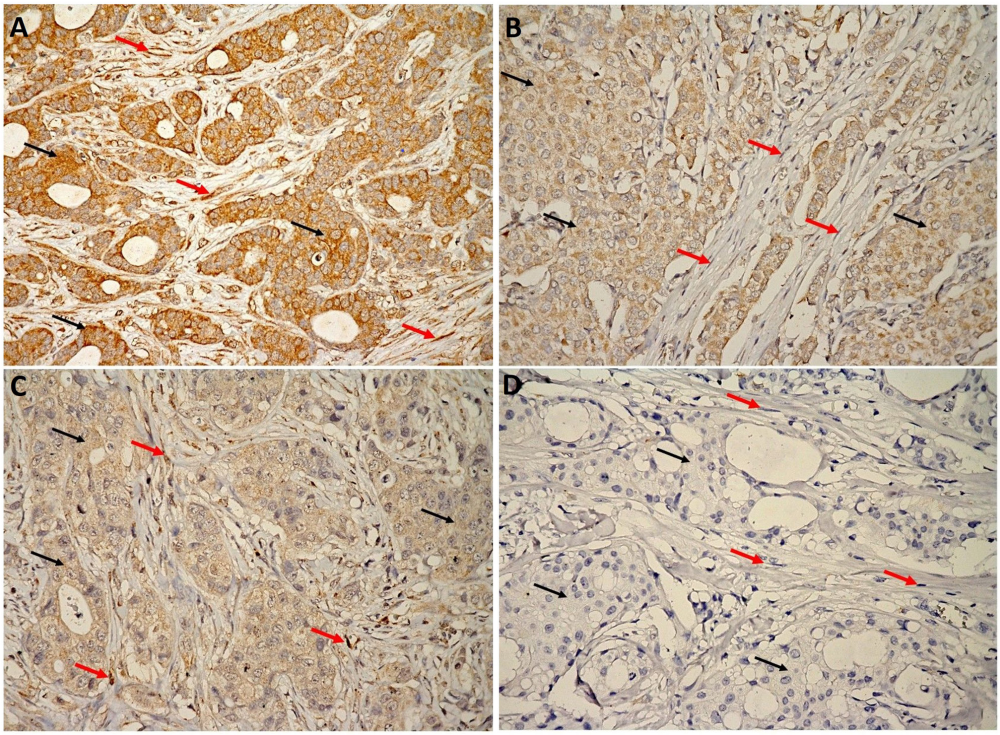

Figure 1. MMP9 expression in primary breast tumors. Immunohistochemical analysis was performed using anti-MMP9 monoclonal antibodies (magnification, x400; visualisation of positive reaction with 3,3'-diaminobenzidine; samples labelled in the same manner). MMP9 expression was evaluated in tumor cells and in stromal cells. (A) Strong expression in tumor cells (black arrows) and stromal cells (red arrows). (B) Moderate positivity in tumor cells (black arrows) and negativity of stromal cells (red arrows). (C) Moderate positivity in tumor cells (black arrows) and strong positivity of stromal cells (red arrows). (D) Negativity in tumor (black arrows) and stromal cells (red arrows).

Enrichment of CTC from peripheral blood by depleting $CD45^+$ cells was performed using the Rossette Sep™ kit (15162; Stemcell Technologies, Inc.), as previously described (37,38). Briefly, RNA isolated from CD45-depleted peripheral blood samples were transcribed into cDNA, which was subjected to RT-qPCR analysis to assess the expression levels of epithelial-to-mesenchymal transition (EMT-TF) genes, including TWIST1, SNAIL1, SLUG and ZEB1. Compared with healthy donors, patient samples with higher EMT-TF gene transcript levels were classified as CTC EMT positive, based on the preclinical study and human sample testing. The highest expression values in healthy donors were used as a cut-off value to determine CTC positivity (39).

Statistical analysis. Patient characteristics were summarized using the median (range) values for continuous variables and frequency (percentage) for categorical variables. The distribution of MMP9 histoscore was significantly different from the normal distribution (Shapiro-Wilk test), thus non-parametric tests were used for analyses. Mann-Whitney U test was used to compare the differences in distributions of MMP9 expression between two groups of patients with PBC, whereby MMP9 expression was categorized as absent or present. Fisher's exact test or the χ^2 test were used where appropriate.

The median follow-up period was estimated as a median observation time among all patients and among those still alive at the time of their last follow-up. Disease-free survival (DFS) was calculated from the date of CTCs measurement to the date of disease recurrence (locoregional or distant), secondary cancer, death or last follow-up. DFS was estimated using the Kaplan-Meier product limit method and log-rank test. Two-sided $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using NCSS 11 statistical software (2016; NCSS, LLC.; ncss.com/software/ncss).

Results

Patient characteristics. The present study enrolled 318 patients with PBC. The median age of the assessed cohort was 60 years (age range, 25-83 years). The majority of patients had node negative (60.1%) and hormone positive (83.6%) tumors; 48/318 patients (15.1%) had a HER-2/neu amplified status. Patient characteristics are summarized in Table I.

CTCs detection. To establish overexpression of the EMT-inducing TF gene transcripts in patients with PBC, the expression levels were compared between patient samples and healthy donors, as previously described (39). Among the patient samples, CTCs were detected in 83 patients (26.1%). CTCs with only epithelial markers were detected in the peripheral blood of 34 patients (10.7%), while CTCs with an EMT phenotype were present in 56 patients (17.6%).

Association between MMP9 expression, and patients/tumor characteristic and CTCs. MMP9 protein expression in tumor cells was assessed in all patients (n=318) (Fig. 1). However, pathologists were unable to detect stromal cells in 9/318 tumor tissues due to the small sample size, which only constituted tumor cells. Thus, MMP9 expression in stroma was only assessed in 309 patients. MMP9 expression intensity at least 1+

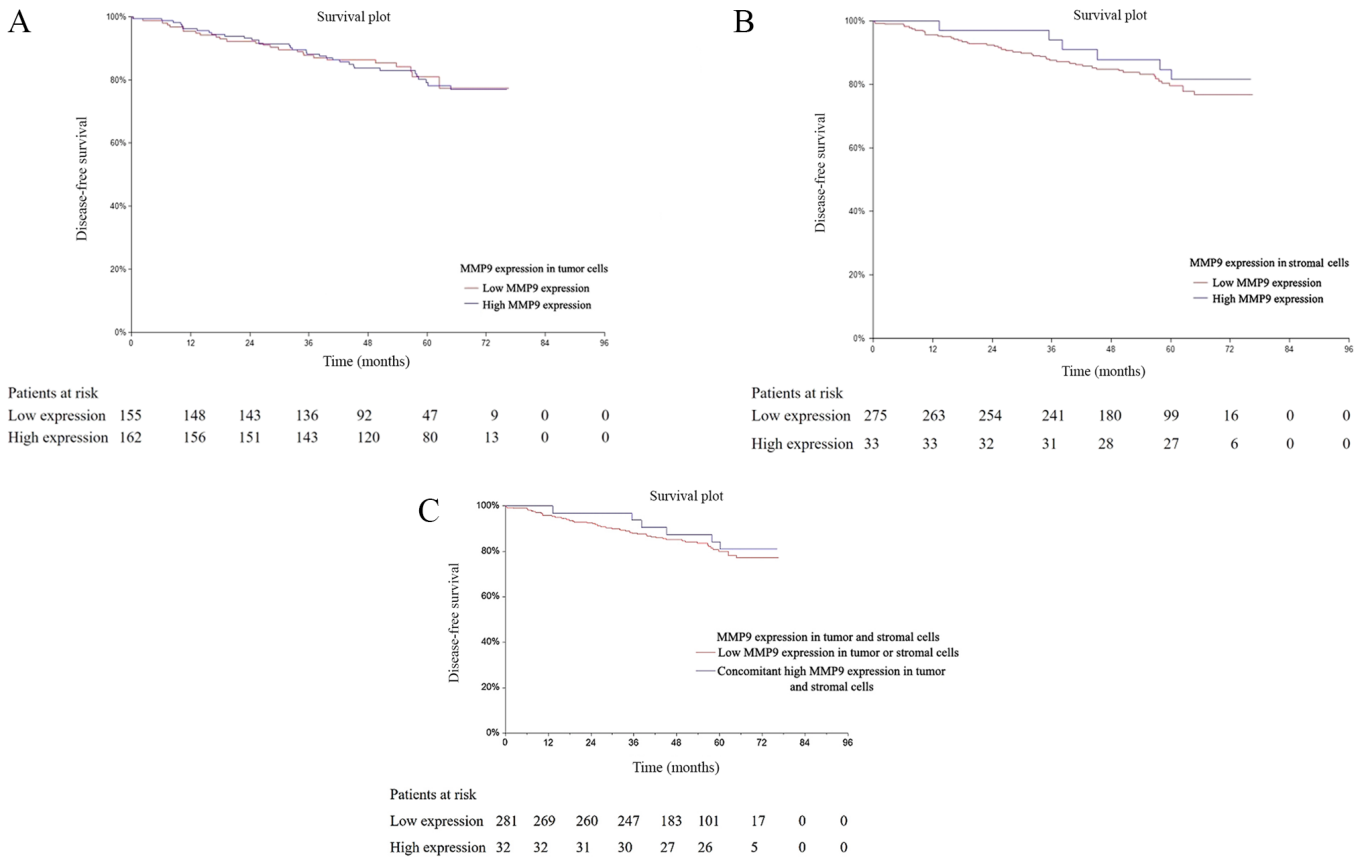

Figure 2. Disease-free survival in all patient groups according to MMP9 expression. (A) Tumor cells (P=0.864) and (B) tumor associated stroma (P=0.547) results are presented. (C) Patients with concomitant high MMP9 expression in tumor and stromal cells (P=0.573) results are presented.

and higher was detected in 255 samples (80.2%) in breast tumor cells and in 307 samples (99.4%) of tumor associated stroma (P<0.00001). The mean WH ± standard error of the mean (SEM) for MMP9 expression was significantly higher in breast tumor cells compared with tumor associated stroma (132.0±5.2 vs. 50.8±3.7; P<0.00001). The association between MMP9 expression in tumor cells and clinicopathological characteristics, as well as its association with CTCs are presented in Table II. The results demonstrated that elevated MMP9 expression was significantly associated with EP/PR positive breast cancer cells (mean WH ± SEM=137.6±5.6 vs. 103.4 ±12.8, P=0.011) and low proliferating tumors (Ki67 <20%) (mean WH ± SEM=141.1±6.7 vs. 117.9±8.1, P=0.018), while elevated MMP9 expression in tumor associated stroma was associated with hormone receptor (EP/PR) status (mean WH ± SEM=54.6±4.0 vs. 30.7±9.1, P=0.021) (Table III). In our analysis, there was found any association between MMP9 expression in breast cancer cells, or in tumor associated stroma and CTCs.

Prognostic value of MMP9 in PBC. The median follow-up time was 54.9 months (range, 0.2-76.6 months). In the assessed cohort, 61 patients (19.2%) experienced a disease progression during follow-up. Among the subgroup of patients where MMP9 expression in tumor associated stroma was assessed (n=309), the median follow-up time was 55.3 months (range, 0.2-76.6), and 59 patients (19.1%) experienced a DFS event. Due to insufficiency of overall survival data, only DFS data are presented in the present study.

Univariate analysis was performed to determine the prognostic value of MMP9 in PBC cells [hazard ratio (HR)=0.96; 95% confidence interval (CI), 0.58-1.59; P=0.864; Fig. 2A], as well as in tumor associated stroma (HR=1.29; 95% CI, 0.60-2.78; P=0.547; Fig. 2B). Exploratory subgroup analysis was performed to determine a potential subgroup-related prognostic value of MMP9 (Tables IV and V). In addition, also the univariate analysis in group of patients with concomitant high MMP9 expression in tumor and stromal cells was carried out. However, no prognostic value was found using this analysis (HR=1.27, 95% CI 0.59-2.75, P=0.573) (Fig. 2C). The results demonstrated that low MMP9 expression in tumor cells was associated with better DFS in hormone receptor (ER/PR) negative and triple negative patients with PBC (HR=0.33; 95% CI, 0.12-0.93; P=0.025; Fig. 3A) and (HR=0.17; 95% CI, 0.05-0.57; P=0.003; Fig. 3B), respectively. Notably, the prognostic value of MMP9 in tumor cells was also observed in the CTC_EMT-positive subgroup of patients (HR=0.40; 95% CI, 0.16-0.95; P=0.047; Fig. 3C). Among the subgroup of patients where MMP9 expression in tumor associated stroma was assessed, the prognostic value of MMP9 was observed in the hormone receptor (ER/PR) negative subgroup of patients (HR=0.14; 95% CI, 0.00-4.81; P=0.002; Fig. 4A), triple negative (HR=0.12; 95% CI, 0.00-4.89; P=0.001; Fig. 4B). In addition, among the subgroup of CTC_EMT positive patients

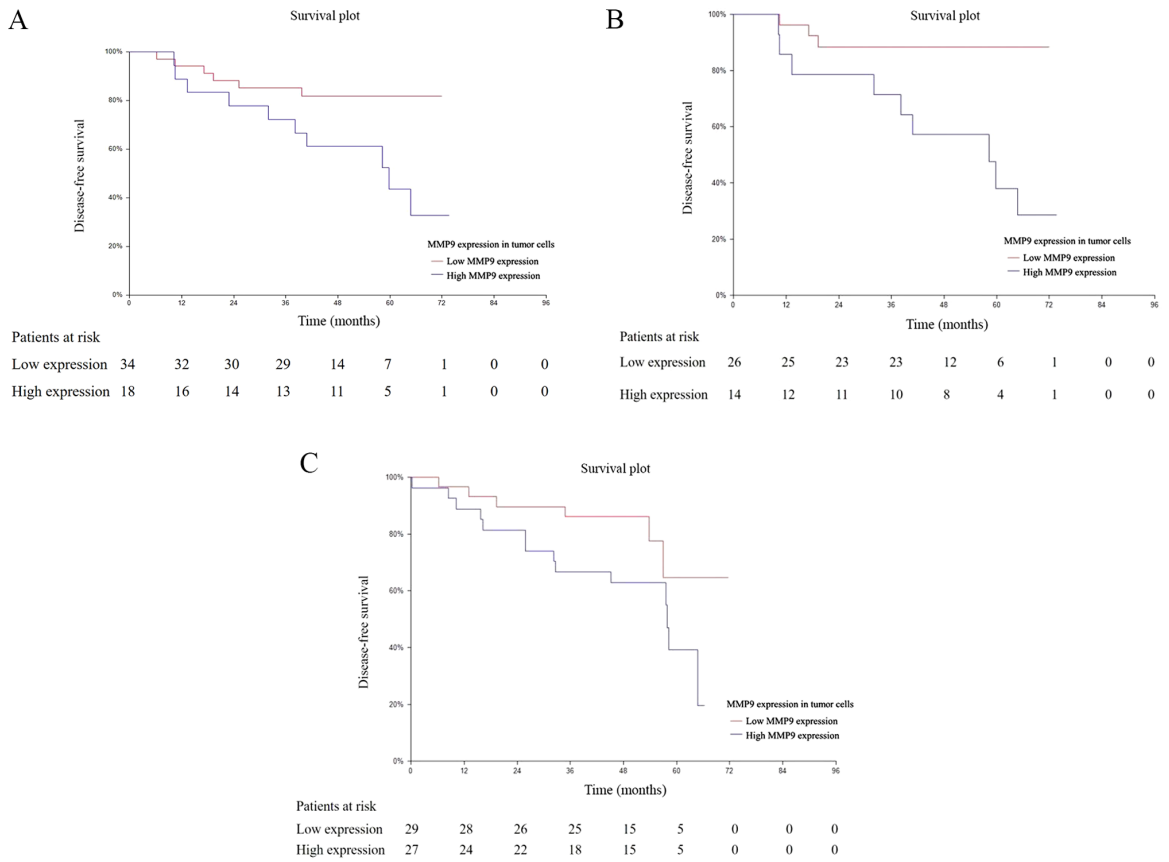

Figure 3. Kaplan-Meier disease-free survival analysis. Disease-free survival according to MMP9 expression in tumor cells of (A) hormone-negative (P=0.025), (B) triple-negative (P=0.003), (C) CTC EMT-positive (P=0.047) and (D) CTC EP-positive (P=0.675) patients is presented. CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial-positive.

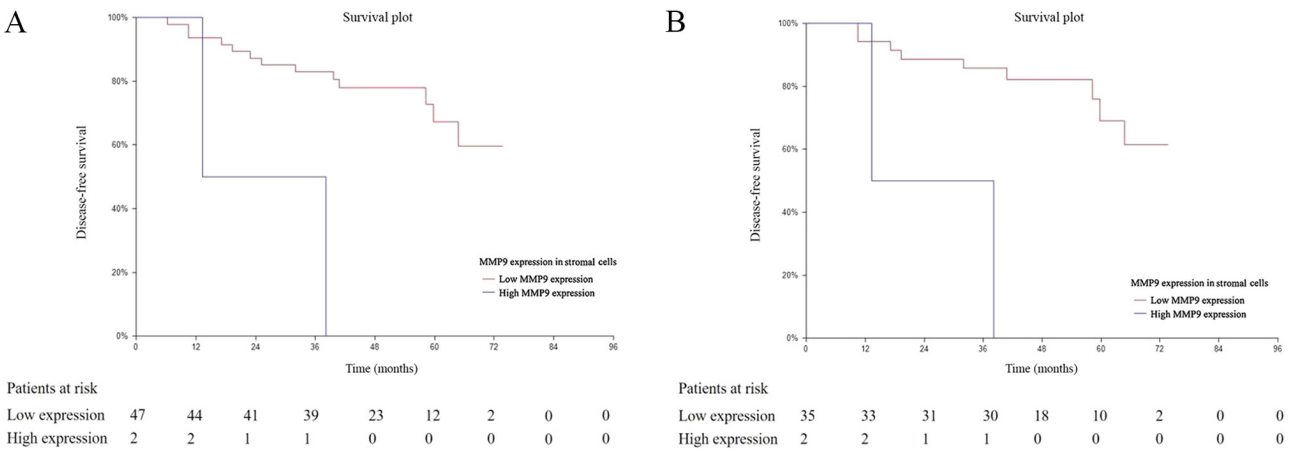

Figure 4. Kaplan-Meier disease-free survival analysis. Disease-free survival according to MMP9 expression in stromal cells of (A) hormone-negative (P=0.002), (B) triple-negative (P=0.001). CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition

was progression of the disease documented in 1 of 2 patients with high MMP9 expression in stromal cells compared to 22 of 51 patients with low MMP9 expression within 4-years follow up. In subgroup of the CTC_EP positive patients 2 of 4 patient with high MMP9 expression in stromal cells experienced progression of disease compared to 6 of 22 patient with low MMP9 expression after 4-years follow up.

Notably, combinatorial survival analysis of CTC_EMT and MMP9 expression in tumor cells demonstrated that CTC_EMT positive patients with high MMP9 expression had a significantly shorter DFS compared with CTC_EMT negative patients (P<0.00001; Fig. 5).

Discussion

MMPs represent a large family of proteolytic enzymes of the extracellular matrix that are involved in extracellular matrix degradation, tumor cell invasion, metastasis and

Table I. Patient characteristics.

Characteristic	n (%)
All patients	318 (100.0)
Histology	
Invasive ductal carcinoma	272 (85.5)
Invasive lobular carcinoma	32 (10.1)
Other histological subtypes	14 (4.4)
Grade	
Low and intermediate	200 (62.9)
High grade	110 (34.6)
Unknown	8 (2.5)
T stage	
T1	218 (68.6)
T2 and more	100 (31.4)
N stage	
N0	191 (60.1)
N1mi	10 (3.1)
N1	68 (21.4)
N2	27 (8.5)
N3	19 (6.0)
Unknown	3 (0.9)
Hormone receptor status (cut-off 1%)	
Negative for both	52 (16.4)
Positive for either	266 (83.6)
HER2 status	
Negative	270 (84.9)
Positive	48 (15.1)
Ki67 status	
<20%	189 (59.4)
≥ 20%	128 (40.3)
Unknown	1 (0.3)
Molecular subtype	
Luminal A	166 (52.2)
Luminal B	99 (31.1)
$HER2^+$	13 (4.1)
Triple negative	39 (12.3)
Unknown	1 (0.3)
P53 status	
Negative	193 (60.7)
Positive	124 (39.0)
Unknown	1 (0.3)
BCL-2 status	
Negative	92 (28.9)
Positive	225 (70.8)
Unknown	1 (0.3)
CTC EP	
Negative	235 (73.9)
Positive	27 (8.5)
CTC EMT	
Negative	235 (73.9)
Positive	56 (17.6)
CTC any	
Negative	235 (73.9)
Positive	83 (26.1)

CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial-positive.

Table II. Association between MMP9 expression in tumour cells, patients, tumour characteristics and circulating tumor cells.

	MMP9 expression in tumor cells				
Characteristic	N	Mean	SEM	Median	P-value
MMP9 expression weighted histoscore	318	132.0	5.2	150	NA
Histology					0.081
Invasive ductal carcinoma	272	136.0	5.6	150	
Other	46	108.6	13.6	100	
Grade					0.242
Low and intermediate	200	135.1	6.5	150	
High grade	110	130.1	8.8	110	
Unknown	8	80.0	32.7	0	
T-stage					0.163
T1	218	136.4	6.3	150	
>T1	100	122.6	9.3	100	
N stage					0.468
N0	201	135.4	6.5	150	
N^+	114	127.1	8.7	150	
Unknown	3	90.0	53.6	100	
Hormone receptor status (cut-off 1%)					0.011
Negative for both	52	103.4	12.8	100	
Positive for either	266	137.6	5.6	150	
HER2 status					0.792
Negative	270	131.4	5.6	150	
Positive	48	135.7	13.4	150	
Ki67 status (cut-off 20%)					0.018
<20%	189	141.0	6.7	170	
≥20%	128	117.9	8.1	110	
Unknown	1	250.0	92.0	250	
Molecular subtype					0.0711
Luminal A	166	138.6	7.2	160	
Luminal B	99	134.8	9.3	150	
$HER2^+$	13	106.2	25.6	100	
Triple negative	39	102.4	14.8	100	
Unknown	1	250.0	92.3	250	
P53 status					0.632
Negative	193	131.1	6.7	150	
Positive	124	134.1	8.3	150	
Unknown	1	50.0	92.9	50	
BCL-2					0.229
Negative	92	126.0	9.7	110	
Positive	225	134.0	6.2	150	
Unknown	1	250.0	92.7	250	
CTC EP					0.851
Negative	235	134.1	6.0	150	
Positive	27	138.1	17.5	150	
CTC EMT					0.300
Negative	235	134.1	6.1	150	
Positive	56	120.4	12.4	135	
CTC any					0.472
Negative	235	134.1	6.1	150	
Positive	83	126.1	10.2	150	

CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; NA, not applicable; EP, epithelial-positive.

Table III. Association between MMP9 expression in stromal cells, patients, tumour characteristics and circulating tumor cells.

	MMP9 expression in stromal cells				
Characteristic	N	Mean	SEM	Median	P-value
MMP9 expression weighted histoscore	309	50.8	3.7	25	NA
Histology					0.434
Invasive ductal carcinoma	266	51.9	4.0	30	
Other	43	44.2	9.8	20	
Grade					0.489
Low and intermediate	194	50.9	4.6	30	
High grade	108	51.7	6.2	20	
Unknown	7	34.3	24.4	0	
T-stage					0.469
T1	213	52.6	4.4	25	
>T1	96	46.8	6.6	25	
N stage					0.536
N0	197	54.9	4.6	30	
N^+	110	43.5	6.1	20	
Unknown	2	50.0	45.6	50	
Hormone receptor status (cut-off 1%)					0.021
Negative for both	49	30.7	9.1	5	
Positive for either	260	54.6	4.0	30	
HER2 status					0.872
Negative	263	50.7	4.0	20	
Positive	46	51.6	9.5	30	
Ki67 status (cut-off 20%)					0.137
<20%	183	56.3	4.8	30	
≥20%	126	42.9	5.7	20	
Molecular subtype					0.094
Luminal A	163	57.0	68.7	30	
Luminal B	97	50.5	62.0	30	
$HER2^+$	12	15	24.3	0	
Triple negative	37	35.8	55.7	5	
P53 status					0.537
Negative	188	48.1	4.7	30	
Positive	120	55.5	5.9	20	
Unknown	1	0.0	64.5	0	
BCL-2					0.995
Negative	88	47.9	6.9	30	
Positive	221	52.0	4.3	20	
CTC EP					0.350
Negative	229	54.4	4.1	30	
Positive	27	41.3	12.8	20	
CTC EMT					0.400
Negative	229	54.4	4.1	30	
Positive	53	40.3	10	10	
CTC any					0.168
Negative	229	54.4	4.1	30	
Positive	80	40.6	7.2	20	

CTC, circulating tumor cells; EP, epithelial; EMT, epithelial-to-mesenchymal transition; NA, not applicable.

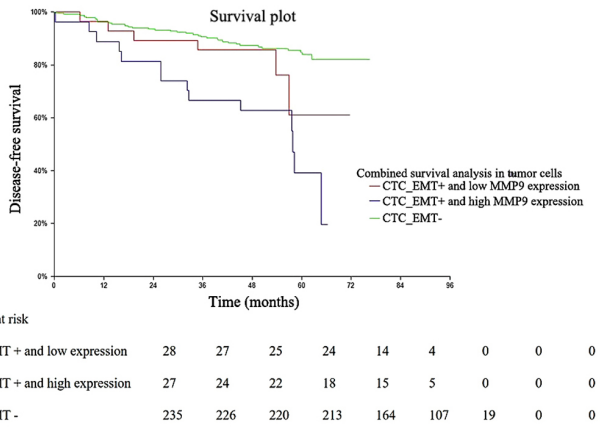

Figure 5. Kaplan-Meier DFS analysis for a combination of CTC EMT and MMP9. CTC EMT positive patients with MMP9 expression had a worse DFS than patients that are CTC EMT negative. P<0.00001. DFS, disease-free survival; CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition.

angiogenesis (19,40-42). The results of the present study demonstrated that elevated MMP9 expression levels in tumor cells and tumor associated stroma were significantly associated with favorable tumor characteristics. Hormone-positive tumors exhibited significantly higher MMP9 expression in tumor cells, as well as in tumor associated stromal cells. In addition, the results demonstrated an association between increased MMP9 expression and low proliferation index of Ki67. Although the role of MMP9 and its association with breast cancer has been extensively studied, data regarding the prognostic value of MMP9 are inconsistent. On one hand, it has been reported that MMP9 expression is associated with a shorter relapse-free survival time in patients with primary breast tumours (26,29,43,44). The association between upregulated MMP9 expression and an increased risk of overall survival and relapse-free survival in breast cancer has also been confirmed via meta-analyses by Song *et al* (45) and Ren *et al* (46). Conversely, some studies have identified MMP-9 as a favourable prognostic marker for breast cancer (9,47).

The results of the present study demonstrated a significant association between high MMP9 expression in tumour cells and poor DFS in hormone receptor negative, triple negative, as well as in the CTC_EMT-positive subgroup of patients with early breast cancer. Analysis of stromal cells exhibited this association in the hormone receptor negative and triple negative subgroups of patients.

These results are in concordance with previous studies, confirming the association between MMP9 expression and a shorter progression time, particularly in patients with basal-like or triple negative breast cancer (48,49). Controversy regarding the association between MMP9 expression and clinical outcomes in different types of malignant tumors, including breast cancer, suggests the presence of active and inactive forms of MMP9. MMPs are secreted in the form of inactive proenzymes, whose activation is mediated via different molecular mechanisms (20,21). Thus, the level of active MMP9 in stromal cells and tumor cells may vary, which will subsequently account for the differences in clinical outcomes (50).

Notably, the results of the present study demonstrated the prognostic value of MMP9 in the CTC_EMT-positive

Table IV. Univariate analysis for disease-free survival according to MMP9 expression in tumor cells.

Characteristic	N	HR	95% Low CI	95% High CI	P-value
Overall					0.864
Low MMP9 expression	156	0.96	0.58	1.59	
High MMP9 expression	162				
Invasive ductal carcinoma					0.550
Low MMP9 expression	126	0.84	0.48	1.47	
High MMP9 expression	146				
Other histology					0.335
Low MMP9 expression	30	2.12	0.55	8.17	
High MMP9 expression	16				
Intermediate/low grade[a]					0.901
Low MMP9 expression	93	0.95	0.44	2.07	
High MMP9 expression	107				
High grade[8 pts NA]					0.518
Low MMP9 expression	58	0.80	0.40	1.58	
High MMP9 expression	52				
T1 stage					0.936
Low MMP9 expression	99	0.97	0.48	1.98	
High MMP9 expression	119				
T2 stage and higher					0.484
Low MMP9 expression	57	0.77	0.37	1.60	
High MMP9 expression	43				
N0 stage[b]					0.844
Low MMP9 expression	99	0.92	0.41	2.09	
High MMP9 expression	102				
N^+ stage[b]					0.909
Low MMP9 expression	55	1.04	0.54	2.00	
High MMP9 expression	59				
ER/PR positive for either					0.539
Low MMP9 expression	122	1.20	0.66	2.18	
High MMP9 expression	144				
ER/PR negative for both					0.025
Low MMP9 expression	34	0.33	0.12	0.93	
High MMP9 expression	18				
HER positive					0.712
Low MMP9 expression	22	1.22	0.42	3.49	
High MMP9 expression	26				
HER negative					0.741
Low MMP9 expression	134	0.91	0.51	1.62	
High MMP9 expression	136				
Ki67 low (<20%)					0.523
Low MMP9 expression	84	1.30	0.57	2.99	
High MMP9 expression	105				
Ki67 high (≥20%)					0.149
Low MMP9 expression	72	0.62	0.33	1.19	
High MMP9 expression	57				
Triple negative[c]					0.003
Low MMP9 expression	26	0.17	0.05	0.57	
High MMP9 expression	13				
P53 negative[c]					0.735
Low MMP9 expression	96	0.90	0.49	1.65	
High MMP9 expression	97				
P53 positive[c]					0.829
Low MMP9 expression	59	1.11	0.43	2.82	
High MMP9 expression	65				
BCL2 negative[c]					0.124
Low MMP9 expression	51	0.53	0.24	1.18	
High MMP9 expression	41				
BCL2 positive[c]					0.445
Low MMP9 expression	105	1.29	0.67	2.49	
High MMP9 expression	120				
CTC EP negative					0.387
Low MMP9 expression	115	1.33	0.69	2.57	
High MMP9 expression	120				
CTC EP positive					0.675
Low MMP9 expression	12	1.52	0.20	11.24	
High MMP9 expression	15				
CTC EMT negative					0.387
Low MMP9 expression	115	1.33	0.69	2.47	
High MMP9 expression	120				
CTC EMT positive					0.047
Low MMP9 expression	29	0.40	0.16	0.95	
High MMP9 expression	27				
CTC any negative					0.387
Low MMP9 expression	115	1.33	0.69	2.57	
High MMP9 expression	120				
CTC any positive					0.113
Low MMP9 expression	41	0.51	0.23	1.14	
High MMP9 expression	42				

[a]Data not available in 8 patients; [b]data not available in 3 patients; [c]data not available in one patient; ER, estrogen receptor; PR, progesterone receptor; CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial; HZ, hazard ratio; CI, confidence interval; NA, not applicable.

subgroup of patients. Generally, ETM is considered a developmental process, facilitating the resistance to apoptosis and increased invasion, and is closely associated with development of a cancer stem cell phenotype (19,51) This machinery can be directly induced by MMPs in the target epithelial cells. Expression of proteases, including MMPs is upregulated during reorganization of ECM in EMT. In addition, the process of MMP-induced EMT has been best characterized in mammary epithelial cells (52,53). According to the results of the present study, there was no significant association between any subpopulations of CTCs and MMP9 expression. Contrary to MMP1, MMP9 does not actively participate in the release of CTCs into the blood stream of patients with PBC (31). However, these changes may result in the resistance to therapy, and development of a cancer stem cell phenotype closely associated with poor DFS. Given the limited treatment options for these subgroups of patients (triple-negative and CTC_EMT-positive PBC), MMP9 may potentially offer a novel therapeutic target. In addition, the results from the combinatorial survival analysis demonstrated that CTC_EMT

Table V. Univariate analysis for disease-free survival according to MMP9 expression in stromal cells.

Characteristic	N	HR	95% Low CI	95% High CI	P-value
Overall					0.547
Low MMP9 expression	276	1.29	0.60	2.78	
High MMP9 expression	33				
Invasive ductal carcinoma					0.458
Low MMP9 expression	237	1.41	0.63	3.18	
High MMP9 expression	29				
Other histology					0.825
Low MMP9 expression	39	0.79	0.08	7.84	
High MMP9 expression	4				
Intermediate/low grade[a]					0.970
Low MMP9 expression	174	1.02	0.31	3.38	
High MMP9 expression	20				
High grade[a]					0.458
Low MMP9 expression	96	1.56	0.57	4.24	
High MMP9 expression	12				
T1 stage					0.974
Low MMP9 expression	189	1.02	0.36	2.90	
High MMP9 expression	24				
T2 stage and higher					0.457
Low MMP9 expression	87	1.71	0.54	5.43	
High MMP9 expression	9				
N0 stage[b]					0.460
Low MMP9 expression	174	1.71	0.53	5.55	
High MMP9 expression	23				
N^+ stage[b]					0.871
Low MMP9 expression	100	0.92	0.31	2.70	
High MMP9 expression	10				
ER/PR positive for either					0.323
Low MMP9 expression	229	1.67	0.71	3.88	
High MMP9 expression	31				
ER/PR negative for both					0.002
Low MMP9 expression	47	0.14	0.00	4.81	
High MMP9 expression	2				
HER positive					0.242
Low MMP9 expression	39	3.12	0.83	11.74	
High MMP9 expression	7				
HER negative					0.900
Low MMP9 expression	237	1.06	0.43	2.64	
High MMP9 expression	26				
Ki67 low (<20%)					0.486
Low MMP9 expression	159	1.67	0.50	5.53	
High MMP9 expression	24				
Ki67 high (≥20%)					0.679
Low MMP9 expression	117	0.80	0.26	2.50	
High MMP9 expression	9				
Triple negative					0.001
Low MMP9 expression	35	0.12	0.00	4.89	
High MMP9 expression	2				
P53 negative[c]					0.901
Low MMP9 expression	171	1.07	0.39	2.92	
High MMP9 expression	17				
P53 positive[c]					0.482
Low MMP9 expression	104	1.68	0.50	5.69	
High MMP9 expression	16				
BCL2 negative					0.078
Low MMP9 expression	83	0.35	0.05	2.29	
High MMP9 expression	5				
BCL2 positive					0.238
Low MMP9 expression	193	2.00	0.81	4.96	
High MMP9 expression	28				
CTC EP negative					0.143
Low MMP9 expression	202	2.76	1.08	7.09	
High MMP9 expression	27				
CTC EP positive					0.053
Low MMP9 expression	23	0.18	0.01	2.75	
High MMP9 expression	4				
CTC EMT negative					0.143
Low MMP9 expression	202	2.76	1.08	7.09	
High MMP9 expression	27				
CTC EMT positive					0.168
Low MMP9 expression	51	0.37	0.04	3.50	
High MMP9 expression	2				
CTC any negative					0.143
Low MMP9 expression	202	2.76	1.08	7.09	
High MMP9 expression	27				
CTC any positive					0.128
Low MMP9 expression	74	0.44	0.10	1.91	
High MMP9 expression	6				

[a]Data not available in 7 patients; [b]data not available in 2 patients; [c]data not available in patient; ER, estrogen receptor; PR, progesterone receptor; CTC, circulating tumor cells; EP, epithelial; EMT, epithelial-to-mesenchymal transition; HZ, hazard ratio; CI, confidence interval; NA, not applicable.

positive patients with MMP9 expression in tumor cells had a significantly lower DFS compared with CTC_EMT negative patients, suggesting that EMT acts as a negative prognostic marker only in subgroups of patients with high MMP9 expression, while the subgroup of CTC_EMT positive patients, with low MMP9 expression exhibited no effects.

The spectrum of synthetized MMP inhibitors (MMPIs) assessed in clinical trials have demonstrated poor effectiveness and serious side effects (54,55). The limited clinical effect of MMPIs may be due to their poor selectivity. Previous studies have focused on a broad spectrum of MMPs, most of which exert tumorigenic activity. However, it is necessary to take into consideration that some MMPs are characterized by antitumorigenic effects. Another reason for MMPIs inefficiency can be due to their administration to unselected groups of patients (44,56).

In conclusion, this prospective translational study demonstrated the protective role of MMP9 in patients with breast cancer, whereby its increased expression was associated

with favourable tumour characteristics. Thus, as it has been proposed by Pozzi *et al* (57), inhibition of MMP9 antitumorigenic and antiangiogenic activities may result in a paradoxical increase of tumor angiogenesis and tumor growth. Conversely, the results of the present study demonstrated the association between high MMP9 expression and poor DFS in selected subgroups of patients with PBC, particularly hormone receptor negative and triple negative tumors, as well as in CTC_EMT positive patients. These results suggest that MMP9 exerts different biological roles in HR positive vs. negative tumors, further supporting the concept of different biology of breast cancer subtypes according to their HR status. Thus, assessing MMP9 tumor expression may help identify individuals with increased risk of disease recurrence within the aforementioned subgroups of patients with PBC. However, there were certain limitations to the present study, such as the retrospective design of the study and semi-quantitative IHC analysis used for investigating of MMP9 expression. In addition, the study population represent a homogenous cohort of patients, treatment-naïve, without metastatic disease, in order to avoid the effect of the metastatic site heterogeneity factor on analysed variables.

Further studies are required to develop selective MMPIs against the specific protumorigenic MMPs or protumorigenic activities of selected MMPs. Another strategy may be anticancer therapy with antitumorigenic MMPs or with their antitumorigenic subparts. An example of this phenomenon involves the MMP8 enzyme, whereby high MMP8 expression supresses metastasis, while MMP8 silencing induces tumour progression and metastasis (58-60).

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Availability of data and materials

All datasets generated and analyzed during the present study are included in this published article.

Authors' contributions

KK, ZC, JM, MM and GM conceived and designed the present study. KK performed statistical analysis. ZC and IM performed immunohistochemical analysis. GM, TS and DK were involved in CTC detection. MK, JB and DP were involved in patient accrual and performed breast surgery. KK and MM drafted the initial manuscript, and all authors reviewed it critically for important intellectual content. All authors participated in the acquisition, analysis and interpretation of data. All authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Review Board of the National Cancer Institute of Slovakia, Bratislava, Slovakia (approval no. TRU-SK 002; Chair: Professor Michal Mego). Written informed consent was provided by all patients prior to study commencement.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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