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ORIGINAL ARTICLE

Clinical significance of plasma MMP-2 and MMP-9 levels as biomarkers for tumor expression in breast cancer patients in Egypt

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Abstract

Matrix metallopeptidase 2 (MMP-2) and matrix metallopeptidase 9 (MMP-9) are involved in the breakdown of extracellular matrix in normal physiological processes as well as in disease processes, such as cancer metastasis. We conducted this work to study the role of MMP-2 and MMP-9 in breast cancer by measuring their plasma concentrations before and after surgery. Also, to examine if their levels can reflect the stage of disease and prognosis. Forty-eight breast cancer patients and 13 patients with benign breast diseases were included in the study. MMP-2 and MMP-9 levels were measured by ELISA and semi-quantitative real-time PCR. MMP-2 and MMP-9 levels in plasma were determined by ELISA immediately before surgery and during 6 to 12 months after curative surgery. We observed a significant increase in the level of MMP-9 mRNA expression in breast cancer patients in comparison to their normal breast tissues and to tissues of benign breast disease. In all TNM tumor stages, the plasma levels of MMP-2 and MMP-9 were increased significantly before curative surgery in the studied patients with breast carcinoma and decreased significantly after surgery. Both MMP-2 and MMP-9 may be used as a possible marker for follow-up or as a marker that reflects the response of the disease to treatment.

Keywords MMP-2 · MMP-9 · Breast cancer · TNM staging

Introduction

In Egypt and worldwide, the most common cancer in females is breast. The majority of patients in developed countries came with locally advanced or metastatic disease [1].

In malignant tumors, distant metastasis to vital organs and sites is the main cause of death. The female breast is very rich in lymphatic and venous drainage leading to early and distant spread of breast cancer with their associated high rates of death [2]. The malignant tumors invade the basement membrane to reach the lymphatic and blood vessels after crossing the extracellular matrix (ECM) [3]. Cancer cell invasion is prevented by ECM which maintains tissue architecture and polarity [4].

The vacuolar-H^+-ATPase (V-ATPase) is one of the main regulators of pH in the tumor cells and its positivity leads to invasion and metastasis of malignant cells [5]. Hydrolysis of ATP releases energy that pumps the protons into the extracellular environment and enhances V-ATPase activation. Matrix metalloproteinases (MMPs), which remodel the ECM by enhancement of proteolytic enzyme, is activated by the low PH of the malignant tumor microenvironment. This activation leads to proteolysis of the basement membrane and spread of malignant cells into the circulation and distant metastases [6]. Matrix metalloproteinase, MMP-2 and MMP-9 promotes malignant tumor spread and metastases [7]. In many types of malignant tumors, an association

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between poor tumor differentiation, advanced tumor stage, bad prognosis, and the increased levels of Matrix metalloproteinase, MMP-2 and MMP-9 [8–11].

The present study was conducted to assess the role of MMP-9 and MMP-2 in breast cancer patients. Their role was studied by quantification of plasma levels by ELISA, tumor and adjacent normal tissues mRNA level by real time PCR. Changes in expression levels of studied metalloproteinases before and after tumor resection were also studied. Also, to examine if their levels can reflect the stage of disease and prognosis.

Patients and methods

Characteristics of patients

The study was carried out between May 2015 and June 2017 at Breast Clinic, Clinical Oncology Department, Zagazig University Hospitals. Forty-eight patients with breast carcinoma who underwent a radical or breast conservation resection were included in the study (aged 27–65 years; mean, 46.0 years), 13 patients with benign breast diseases (fibroadenosis and fibroadenomas) (aged 22–53 years; mean, 42.0 years) who underwent surgical excision. Patient's distribution according to TNM staging system [12] is shown in Table 1. Twenty-five healthy subjects of patients' relatives were selected for control blood samples.

All participants give informed consent prior to enrolment to the trial. Ethical approval was obtained from Zagazig University ethical committee.

Table 1 Patient's distribution according to TNM staging system

Patient characteristics	Number
Age (years)	46 ± 18 (27–65)
< 50 years	20 patients
> 50 years	28 patients
Stage	
Stage I	7
Stage II	16
Stage III	25
TNM	
T1	7
T2	2
T3	24
T4	13
N0	23
N1	9
N2	7
N3	7

Specimen collection

During the surgical procedure, tissue samples were taken from breast cancer and other biopsies from surrounding tissues not involved by the tumor as a control. Meanwhile, a tissue sample was taken from the lesion in the 13 patients with benign breast conditions either by excision biopsy or by core biopsy.

Tissues taken were immediately frozen by liquid nitrogen and preserved at − 70 °C till the time of the study. A 3 ml blood specimen was also taken at the time of operation from each patient. A 3 ml blood specimen was taken also from 25 healthy subjects of patients' relatives as controls.

Blood was collected in heparinized tubes and centrifuged. The supernatant fluid was taken and kept in a refrigerator at − 20 °C till the time of analysis.

Tissue homogenate: the homogenate is prepared by rinsing the tissue sample with phosphate buffer saline (PBS) to remove excess blood and then the tissue was weighed and minced into small pieces and homogenized in 5–10 ml PBS and extraction buffer in the homogenizer. The sample was placed on the shaker at 4°C for 1 h then centrifuge the sample at approximately 10,000×*g* for 5 min the supernatant was removed and assayed immediately or aliquots were kept at − 80 °C until analysis.

Extraction of RNA from tissue samples

Total RNA was extracted from frozen specimens using the isolation kit provided by Promega according to their protocol (Promega Corporation, Madison, WI, USA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The reaction was done using GoScriptTM Reverse Transcription System supplied by Promega (Promega Corporation, Madison, WI, USA). The cDNA synthesis was carried out briefly as follow: 3 µg of total RNA was mixed with Oligo dT15 primers and Nuclease-Free Water at 65 °C for 5 min then the tube was placed in iced water at least for 10 min and centrifuged. After centrifugation, the following mixture (GoScript™ 5 × Reaction Buffer, 0.5 mM of PCR Nucleotide Mix, 2–5 mM of $MgCl_2$, RNasin® Ribonuclease Inhibitor, GoScript™ Reverse Transcriptase, and Nuclease-Free Water) was added, and the tube was placed in a thermal cycler for 5 min at 25 °C, followed by 60 min at 42 °C. The reaction was then terminated by heating for 5 min at 70 °C. The cDNA was stored at − 20 °C until the qRT-PCR analysis.

The synthesized cDNA may be added directly to PCR amplification, In each PCR reaction the following PCR mix was used: 10× Buffer 5 µl, $MgCl_2$ 25 mM 4 µl, dNTPs* 4 µl, Primer 1 50 µM 1 µl, Primer 2 50 µM 1 µl, Taq 5 µ/µl 0.4 µl, cDNA 1 µl and H_2O to 50 µl (dNTPs* composed of: 2 mM each dATP, dCTP, dGTP; 1.98 mM dTTP; 0.02 mM DIG-dUTP).

The following primers were used for MMP-2 and MMP-9 genes

MMP-2: Forward: 5′-CTAGACTGCTACCATCCGTC-3′ Reverse: GTATACCGCATCAATCTTTTCCG-3′

MMP-9: Forward: 5′-CACCTTCACTCGCGTGTAC-3′ Reverse: 5′-CATCTGCGTTTCCAAACCGAG-3′

PCRs were carried out under the following conditions: the PCR mixture was denaturized for the 60 s at 90 °C followed by another 60 s, 40 cycles of denaturation under 95 °C for 40 s, the 40th cycle was set for 120 s under 72 °C. A final 41st cycle was similar except the extension step extended for 10 min.

The outcome of PCR reaction was separated on agarose gel electrophoresis and standardized with β-actin [13].

ELISA detection of MMP-2 and MMP-9

We measured MMP-2 and MMP-9 in the study participants by a commercial ELISA kit purchased from Chemicon International; Temecula, CA, (Catalog No. CC073). The procedure was done according to the manufacturer's instructions.

The serum levels of MMP-2 and MMP-9 were estimated in all patients with the carcinoma of the breast before surgery and 6–12 months thereafter.

Statistical analysis

Statistical analyses were done by SPSS version 22. Data were expressed as mean ± standard error (mean ± SE) and presented by Box-plots. Comparison of expression values between the groups was performed by the non-parametric tests (two-tailed Mann–Whitney-U-test or Kraskal–Wallis test and p-values < 0.05 were considered as statistically significant.

Results

mRNA of MMP-2 in breast tumors

Analysis of MMP-2 mRNA levels showed no significant difference between normal breast tissues and breast cancer tissues groups ($p > 0.05$). No difference in MMP-2 mRNA level between those with lymphatic metastasis and those without metastasis. ($p > 0.05$) (Fig. 1a).

MMP-2 tissue expression by ELISA

MMP-2 levels were significantly elevated in breast cancer tissue (mean ± SE = 182.4 ± 98.3 ng/mg total protein) compared to tissues of benign breast diseases (mean ± SE = 52.7 ± 16.6) and normal breast Tissue (mean ± SE = 4.3 ± 0.5) ($p = 0.01$). The elevated levels of MMP-2 were noted in all stages of breast cancer with lymph node metastases (N1-3) and without (N0) lymph metastases (Fig. 1b).

Plasma level of MMP-2 by ELISA

The levels of MMP-2 were elevated in the plasma of patients with breast carcinoma (mean ± SE, 572.9 ± 44.8 ng/ml) compared to the healthy control group (mean ± SE, 76.4 ± 5.7 ng/ml) ($p < 0.0001$) and to those with benign breast diseases (mean ± SE, 216.4 ± 9.0 ng/ml) ($p < 0.001$). This elevation was also correlated with TNM tumor stage and with nodal metastasis. The plasma levels were significantly different with tumor stage ($p < 0.0001$) (Fig. 1c) and with axillary nodal metastasis (N1-3) (mean ± SE = 590 ± 45.2) and (N0) (500 ± 30.4) (($p < 0.01$) (Fig. 1d).

mRNA of MMP-9 in breast tumours

MMP-9 levels were elevated in both malignant breast tissues and benign breast conditions in comparison to normal breast tissues ($p < 0.03$). Breast cancer patients with nodal metastases had significantly elevated mRNA-MMP-9 in comparison to those without nodal metastasis (N0) ($p < 0.04$) (Fig. 2a).

Breast tissues levels of MMP-9 by ELISA

The levels of MMP-9 were elevated in the malignant tissues of patients with breast carcinoma (mean ± SE, 498.4 ± 71.9 ng/mg total protein) compared with those with benign breast diseases (BBD) (mean ± SE, 83.6 ± 32.4) and with normal breast tissues (mean ± SE, 6.5 ± 3.5) ($p < 0.01$) (Fig. 2b). When considering nodal metastasis in the analysis of MMP-9 levels, there was no correlation between patients with and those without lymph nodes involvement.

Plasma level of MMP-9 by ELISA

The levels of MMP-9 were elevated in the plasma of patients with breast carcinoma (mean ± SE, 58.7 ± 7.9 ng/

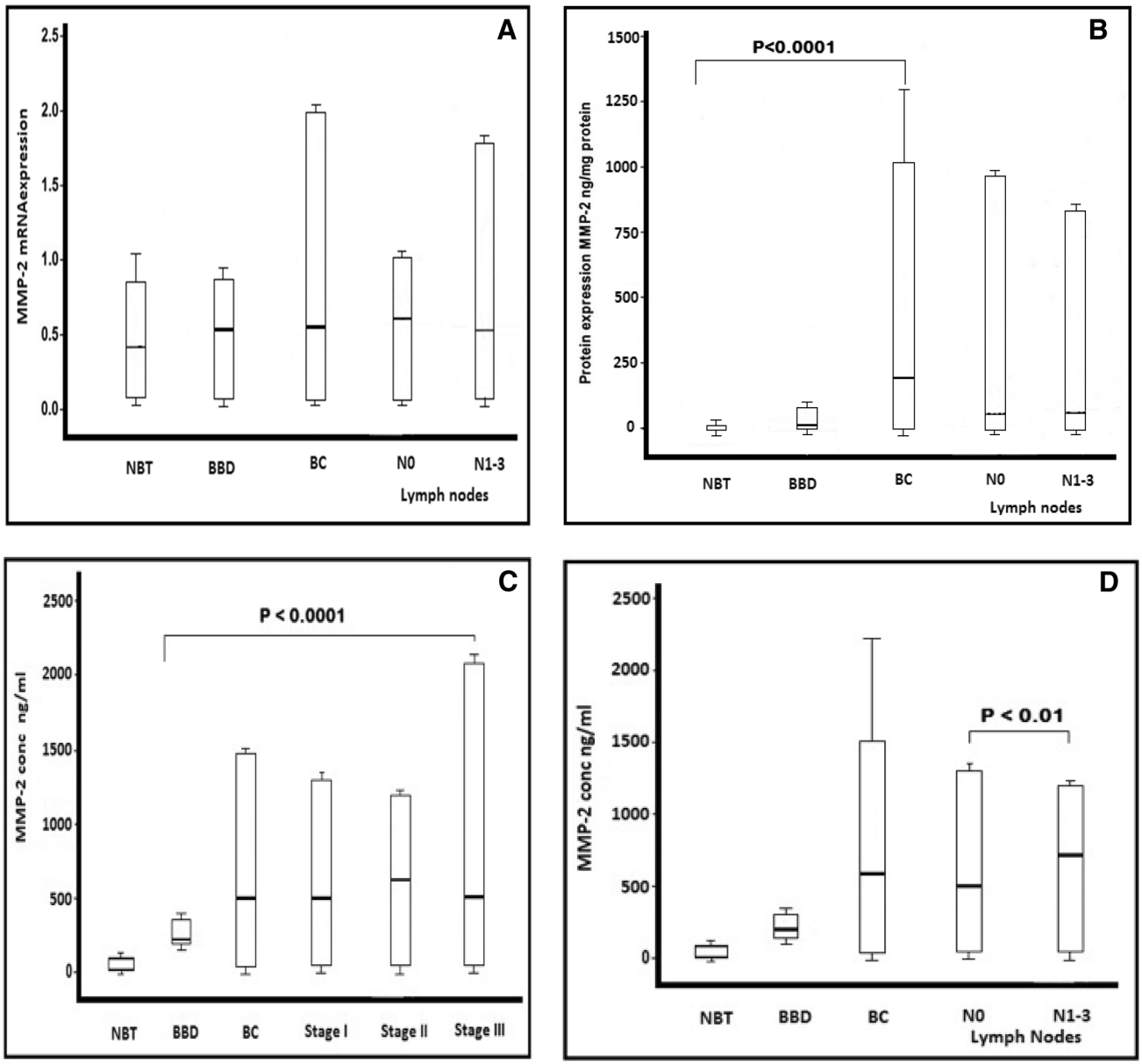

Fig. 1 MMP-2 mRNA and protein expression in breast tissue. **a** MMP-2 mRNA tissue expression values by RT-PCR. No significant difference was demonstrated between normal breast tissue (NBT), benign breast disease (BBD) and breast cancer (BC) samples or between those without (N0) or with (N1-3) nodal metastases. **b** MMP-2 tissue protein expression by ELISA was significantly elevated in breast cancer tissue compared to normal breast tissue (NBT) and benign breast disease (BBD) ($p < 0.01$). Plasma MMP-2 Level by ELISA in all studied group. **c** Plasma MMP-2 level was significantly increased in breast cancer patients compared to the healthy control group ($p < 0.0001$) and to those with benign breast diseases ($p < 0.001$). Plasma MMP-2 was increased in stage III tumor than in stage II and stage I ($p < 0.0001$). **d** MMP-2 levels were significantly higher in patients with lymph node metastases (N1-3) compared to those without metastasis (N0) ($p < 0.01$)

ml) compared to the healthy control group (mean ± SE, 18.7 ± 5.2 ng/ml) ($p = 0.0001$) and to those with benign breast diseases (mean ± SE, 18.9 ± 2.5). This elevation was correlated also with tumor stage ($p < 0.003$) (Fig. 3c) but not with nodal metastasis (Fig. 3d).

Comparison between pre-operative and post-operative MMP-2 and MMP-9

Postoperative plasma samples showed marked decrease in the level of MMP-2 (mean ± SE = 136.3 ± 13.42 ng/ml) than the pre-operative level (mean ± SE, 572.9 ± 44.8 ng/

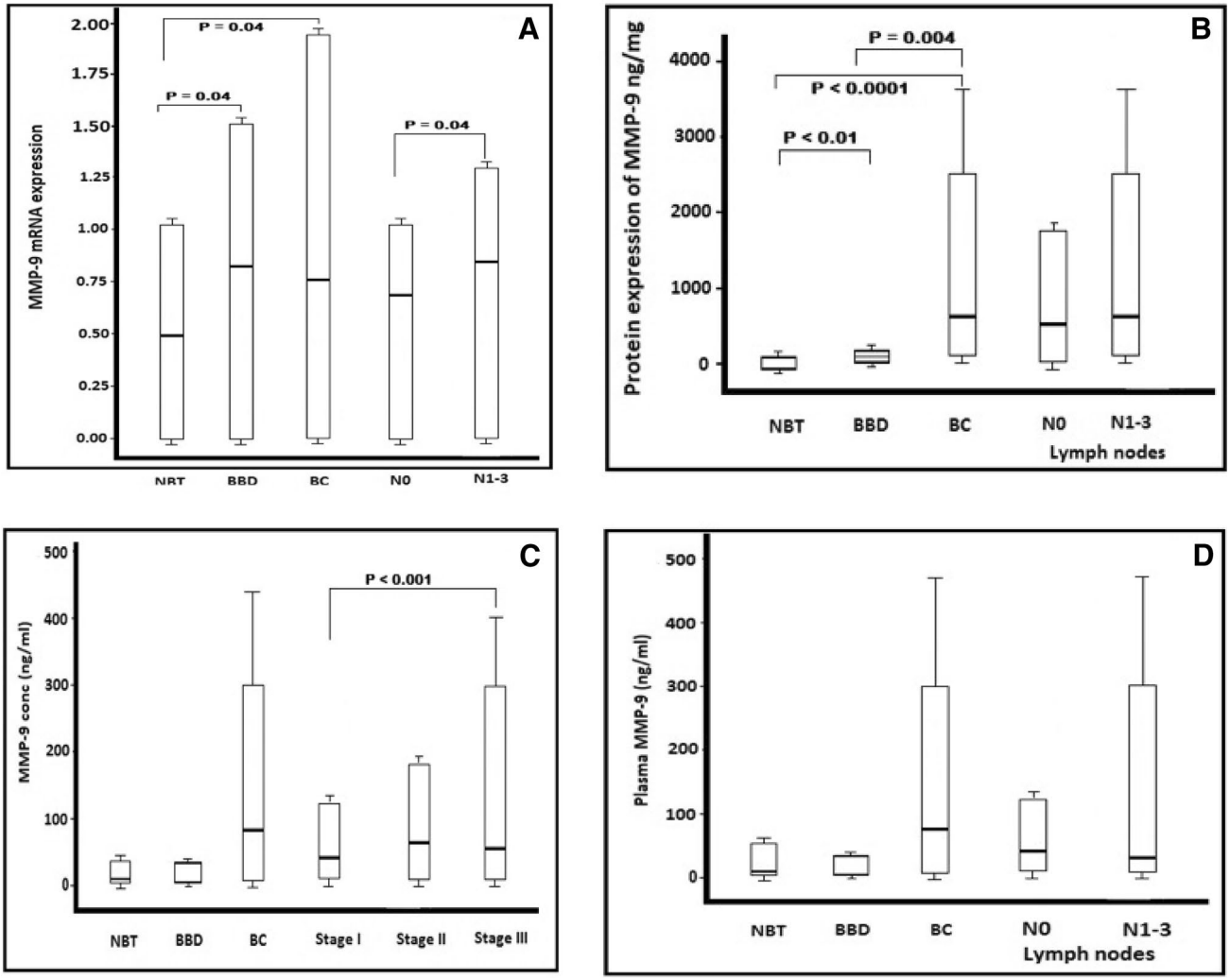

Fig. 2 MMP-9 mRNA and protein expression in breast tissue. **a** MMP-9 mRNA expression by RT-PCR showed significant increase in breast cancer patients and benign breast diseases cases in comparison to its level in normal breast tissue. Patients with nodal (N1-3) metastases showed significant increase in MMP-9 mRNA expression compared to those without lymph node involvement (N0) (p = 0.04 in all). **b** MMP-9 tissue protein expression by ELISA was significantly elevated in breast cancer tissue compared to normal breast tissue (NBT) and benign breast disease (BBD) ($p = 0.01$). Plasma MMP-9 level by ELISA in all studied groups. **c** Plasma levels of MMP-9 was significantly increased in breast cancer patients compared to benign breast diseases and to healthy control females ($p = 0.0001$) and the increase in MMP-9 plasma level was significantly associated with tumor staging (TNM stage) ($p = 0.003$). **d** No significant difference was seen between cancers with lymph node metastases (N1-3) compared to those without lymph node involvement (N0)

ml.) (p< 0.0001) (Fig. 3a). Also, the plasma level of MMP-9 (mean ± SE, 20.1 ± 4.42 ng/ml) was significantly reduced postoperatively than the preoperative level (mean ± SE, 58.7 ± 7.9 ng/ml) (p< 0.0001)(Fig. 3b).

Discussion

We carried out the present study to analyze MMP-2 and MMP-9 in early and locally advanced breast carcinoma and to correlate tumor mRNA, protein and pre and postoperative plasma levels.

Wide variety in concentrations of mRNA of MMP-2 was found in many studies. In the current work, there was no meaningful rise in MMP-2mRNA in breast cancer. Our results are different from studies by Pellikainen et al. [14] and Li et al. [15] who announced that MMP-2 levels were elevated in malignant breast tissues and associated with tumor stage. Li et al. [15] detailed additionally locational differences among normal and malignant tissues.

MMP-9 mRNA expression also showed a wide variation in both normal and cancer tissues. In the present study, MMP-9 mRNA was elevated in both benign breast

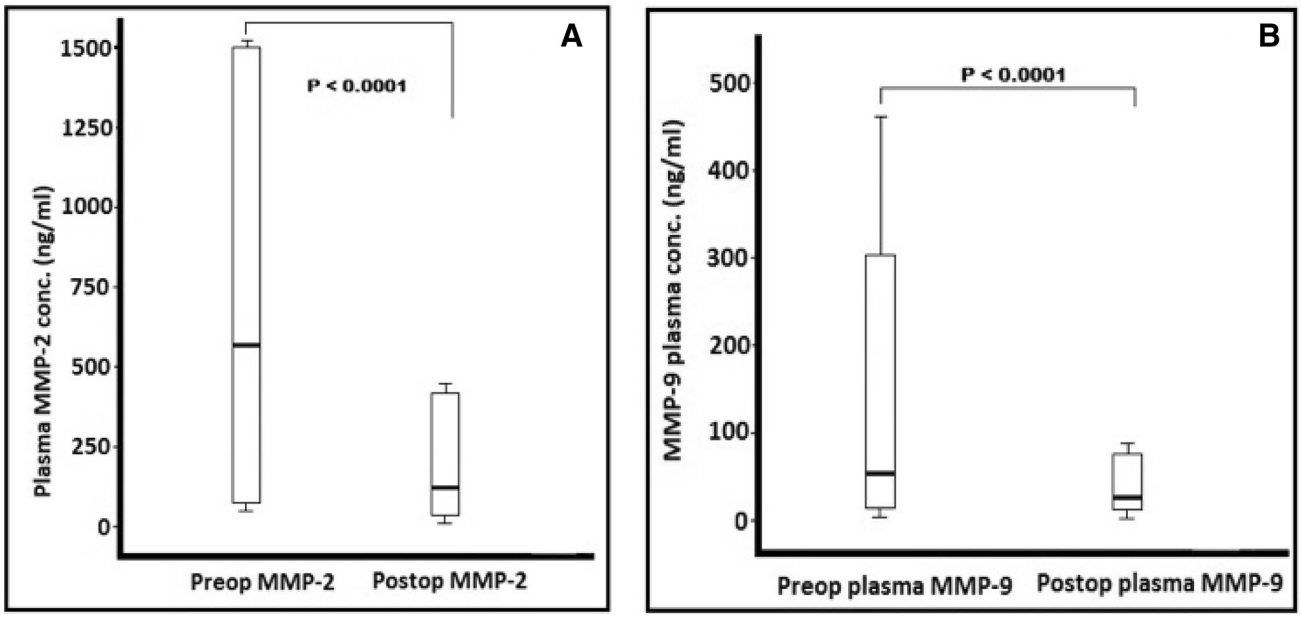

Fig. 3 Comparison of pre and post-operative plasma levels of MMP-2 and MMP-9. **a** Post-operative plasma samples showed significant reduction in plasma level of MMP-2 than the pre-operative level ($p < 0.0001$). **b** Plasma level of MMP-9 was significantly reduced post-operatively than the preoperative level ($p < 0.0001$)

diseases and cancer. Lebeau et al. [16] and Katunina et al. [17] found the same results.

The results of our study discovered that levels of MMP-2 in breast carcinoma agree with the results of Giannelli et al., and Shah et al. indicating elevated levels in malignant tissues in comparison to normal tissues [18, 19]. Moreover, Li et al. [15] noted a correlation between MMP-2 level and TNM clinical stages. Also, we observed higher levels of MMP-2 within tumors with extensive metastases to lymph nodes (N1-3) than in tumors without lymph nodes involvement.

Similar to the mRNA expression, MMP-9 protein expression exhibited increases in both benign breast diseases and cancer. This increased level was seen in different TNM tumor stages and associated with the bad prognosis. Similarly, Yang observed significant increases in the MMP-9 ratio of cancer breast and normal breast tissues [20]. Moreover Radenkovic et al. [21] observed that ProMMP-9 and aMMP-9 activity in tumor tissue showed a positive association with tumor size.

Serum estimations of both MMP-2 and MMP-9 are reported as having limited value for tumor staging and prognosis in other malignancies [22]. In the present study, plasma levels MMP-2 and MMP-9 were examined preoperatively and following curative surgery. We used plasma as the serum levels of MMP-9 can be many times higher than plasma values, may be due to blood clotting resulting in degranulation of neutrophils and release of stored MMP-9 [23].

The predominant plasma species of each MMP-9 and MMP-2 are the latent 92 kDa and 72 kDa forms, as active forms are quickly sequestered and cleared by circulating α2-macroglobulins [23]. In the MMP-2 case, we noted that samples from healthy volunteers were considerably lower than in-patients with breast carcinomas. Plasma levels of MMP-2 were considerably increased in patients with axillary lymph node involvement, indicating that MMP-2 had enhanced this lymph node spread. Li et al., additionally found that MMP-2 and MMP-9 levels in malignant breast specimens were correlative with axillary nodal involvement and clinical stage of the disease in agreement with our results [15]. We found no considerable correlation between plasma levels of MMP-2 and tumor levels. This could be explained by the presence of different types and values of MMP-2 within the tumors and the plasma [23].

Additionally, we found that plasma MMP-9 was considerably increased with respect to TNM stages. As with MMP-2, there was a significant increasing trend in MMP-9 levels with the advancement in tumor stage. This can be in line with the results reportable by Mehner et al. [24]. In contradiction to the previous, Remacle showed that MMP-9 was of limited value for tumor staging and prognosis [25].

Stankovic et al. [26] showed that the activity of proMMP-2 and proMMP-9 significantly increased with each advancing clinical stage of disease and compared to controls.

Following breast carcinoma resections, we noted a marked decrease in mean values of plasma MMP-2 and MMP-9, emphasizing that removal of breast carcinoma

results in normalization of plasma values. This observation draws attention that MMP-2 and MMP-2 levels may be used as markers for complete tumor removal or the presence of micro-metastasis in case of they still high or increasing after tumor removal, but these conditions should be studied in the future in a large series of patients.

Interestingly, the important correlation of MMP-2 and MMP-9 expression with tumor stage were plasma levels. Although mean MMP-9 levels are significantly raised in cancer patients, an elevated preoperative value has a low positive predictive value for use as a screening test as there is a wide range in the normal population.

Furthermore, the levels of MMP-2 and MMP-9 are increased in a number of malignancies of the different human site such as the ovaries [27], liver [28] and cervix [29], therefore, they are not confined for breast malignancies. This character could be useful as markers for follow-up or monitoring response to treatment.

In addition to the previous character, MMP-2 localizes at invasive margins of tumors and is specifically elevated in the plasma of patients with advanced disease. Plasma measurement may, therefore, be useful in patients who are apparent early stage disease based on pathological examination but may be at higher risk of micrometastasis due to high MMP-2 levels and hence may benefit from adjuvant therapy.

Further study with a large number of patients is required to determine the balance between other proteolytic enzymes such as MMP-7, cathepsins and their inhibitors such as TIMP-1 and TIMP-2.

To summarize, the current study showed that plasma levels of MMP-2 and MMP-9 correlate with breast cancer clinical stage, MMP-2 may be used as a measure for tumor burden and lastly, both MMP-2 and MMP-9 levels fall following curative surgical interference of the breast, emphasizing their possible use as markers for persistent or recurrent disease.

Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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