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The relationship of tumor microenvironment and clinicopathological parameters in different molecular subtypes of breast cancer

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ABSTRACT

Introduction: Tumor microenvironment plays a significant role in tumor progression. Tumor stroma is one of the strongest modifiers of tumor cell response, cancer behavior, and cancer progression. This study aimed to investigate the correlation of matrix metalloproteinase-9 (MMP-9) expression and tumor-stroma ratio (TSR) with standard clinicopathological parameters in different molecular subtypes of breast cancer.

Methods: Ninety biopsy samples of primary breast cancer diagnosed at the Department of Pathology, School of Medicine, Sarajevo, were selected for this study. The molecular subtype was determined based on the immunohistochemical expression of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, and Ki-67. Stromal and tumoral MMP-9 immunohistochemical expression and the TSR were determined for each tumor.

Results: Tumoral MMP-9 expression correlated positively with the presence of lymphovascular invasion (p= 0.016). TSR showed significant association and correlation with tumor grade (G) (p= 0.031; p= 0.049) and tumor size (pT) (p = 0.049;p= 0.021, respectively). Stromal MMP-9 expression correlated with histologic type, histologic grade of tumor, and lymphocytic inflammatory infiltrate (p= 0.021;p= 0.047, p= 0.038, respectively). A higher percentage of stromal MMP-9 expression correlated with the strongest lymphocytic response (p = 0.007). Significant correlation was observed between molecular subtypes and histologic grade of the tumor (p= 0.032).

Conclusion: Our results, to some extent, confirm the significance of the tumor microenvironment in breast cancer, especially when it is about stromal MMP-9 expression. Although we observed significant association, without linear correlation, we found no significant correlation between molecular subtypes of breast cancer and MMP-9 expression.

Keywords: Breast cancer; molecular subtypes; tumor microenvironment; matrix metalloproteinase-9; tumor-stroma ratio

INTRODUCTION

Due to a high degree of breast cancer heterogeneity, there is a constant need to discover new predictive biomarkers to improve current therapeutic options (1). One of the first steps of this improvement was its classification into different molecular subtypes. Initially, the classification was made based on genetic profiling (2). It was later simplified by surrogate immunohistochemical classification, with four main molecular subtypes of breast cancer – luminal A, luminal B, human epidermal growth factor receptor-2 (HER-2) enriched, and triple-negative (3). Current histopathology reporting relies mainly on evaluating tumor cells with a mandatory interpretation of hormone receptor status and HER-2 expression. More attention was recently focused on breast cancer stroma heterogeneity, together with inflammatory cell response (4).

Malignant phenotype of the tumor is not determined by tumor cells alone, but also by surrounding tumor microenvironment – cancer associated fibroblasts, immune inflammatory cells, endothelial cells, pericytes, stem, and progenitor cells of the tumor stroma (5,6). Tumor cells produce growth factors and proteases that initiate tumor stroma changes (7), leading to remodeling of the extracellular matrix, migration of immune cells, and increased angiogenesis (8). One of the essential enzymes included in the extracellular matrix's remodeling is matrix metalloproteinase – 9 (MMP-9), also known as gelatinase B (9). Due to its specific capability to degrade collagen type IV, MMP-9 promotes the basement membrane's degrading (10), improves tumor invasion and metastases, and

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induces tumor angiogenesis, epithelial-mesenchymal transition, and impacts immunomodulation of tumor microenvironment (11,12). MMPs are produced by tumor cells and stromal cells in the tumor microenvironment due to paracrine stimulation (13). Some immunohistochemistry (IHC)-based studies found MMP-9 to be more consistently expressed by tumor than by stromal cells in breast cancer (14). Those complex interactions between tumor cells and cells in the tumor microenvironment play a significant role in tumor progression (15). One of the valid parameters for the tumor microenvironment is a tumor-stroma ratio (TSR). It has been investigated as a prognostic marker in breast cancer and was significantly associated with poor prognostic factors (16-19). Although tumor stroma has been recognized as a potential modifier of tumor behavior and aggressiveness, cancer cell-stroma interplay still needs to be elucidated (16).

To investigate the tumor microenvironment's significance, we evaluated tumor and stromal MMP-9 protein expression regarding standard prognostic factors and TSR in different molecular subtypes of breast carcinoma.

METHODS

Case selection

A cross-sectional retrospective study of 90 paraffin-embedded samples of invasive BC diagnosed at the Department of Pathology, School of Medicine, Sarajevo, in the year 2014-2017, was performed. Approval from the ethical review committee (02-3-4-1210/19) was taken antecedent to conduct this study. All relevant data (age, tumor size, histologic type, grade, lymph node status, and hormonal status) were retrieved from data records. Histologic types were classified as ductal, lobular, and others, comprising papillary, micropapillary, and medullary breast carcinoma (20). Patients without known axillary lymph node status, patients who received neoadjuvant chemotherapy or radiotherapy or had distant metastases at the time of diagnosis, were excluded from the study.

All clinicopathological data are summarized in Table 1.

TABLE 1. Clinical, histopathological, and immunohistochemical data of 90 patients with breast cancer

Parameter	n	%
Age		
Mean±SD	57.74±11.28	
Median (range)	56.00 (33-78)	
Histologic type		
Ductal	77	85.56
Lobular	4	4.44
Others	9	10.00
Tumor grade (G)		
G1	18	20.00
G2	45	50.00
G3	27	30.00
Tumor size (pT)		
pT_1	33	36.67
pT_2	42	46.67
pT_3	7	7.77
pT_4	8	8.89
Lymph node status (pN)		
pN_0	29	32.22
pN_1	21	23.33
pN_2	27	30.00
pN_3	13	14.45
Lvi		
Yes	45	50.00
No	45	50.00
Molecular subtype		
Luminal A	36	40.00
Luminal B	38	42.22
HER2 enriched	4	4.45
Triple-negative	12	13.33
MMP-9 Tumoral		
Positive	76	84.44
Negative	14	15.56
MMP-9 Stromal		
Positive	36	40.00
Negative	54	60.00
TSR		
Stroma low	62	68.89
Stroma high	28	31.11
Total	90	100

LVI: Lymphovascular invasion; TSR: Tumor-stroma ratio; MMP-9: Matrix metalloproteinase-9

Evaluation of TSR

TSR was scored on conventional H&E slides of the primary tumor, using the scoring method introduced by Mesker et al. (21). Two independent observers (MD, NČ) identified the most stroma-abundant area, using a 4× objective. In this area, fields with tumor cells at all borders of the field were used to determine the amount of stroma, using 10× objective. The tumor percentage was scored ten-fold. As mentioned above, the area with the highest stroma ratio, which met all the criteria, was considered decisive. Tumors with a stroma percentage ≤50% were categorized as stroma-low, while tumors with stroma percentage >50% were classified as stroma-high (21).

Immunohistochemical procedures

Protein expression of MMP-9 (Polyclonal rabbit antihuman MMP-9 antibody, code A0150, DakoCytomation, Glostrup Denmark), estrogen receptor (ER) (clone 1D5 FLEX DakoCytomation, Glostrup Denmark), progesterone receptor (PR) (clone 636 FLEX DakoCytomation, Glostrup, Denmark), HER-2, and Ki-67 (Clone MIB-1, FLEX Dako, Glostrup, Denmark) was determined by IHC. All procedures for ER, PR, HER-2, Ki-76, and MMP-9 were conducted using the same manufacturer protocols as in our previous study (22).

Immunohistochemical evaluation

MMP-9 immunoreactivity was determined in tumor and stromal cells and defined as positive when cytoplasmic staining was present. The distinction between stromal and tumor cells was made based on morphology. Stromal cells comprised fibroblasts and mononuclear inflammatory cells. A scoring system based on intensity and percentage of positive cells was used for tumoral and stromal MMP-9 protein expression. The staining intensity was defined as negative, weak, moderate, and strong and scored as 0, 1, 2, or 3, respectively. Percentage of positively stained cells was defined as 0%, 1–25%, 26–50%, 51–75%, and 76–100% and scored as 0, 1, 2, 3, and 4, respectively. Tumors with

a final MMP-9 score >2 (in tumor or stromal cells) were considered positive (23).

The Ki-67 proliferative index was quantified according to the IHC positive tumor cell nuclei percentage by counting at least 500 tumor cells. It was defined as low in the case of <14% of tumor cells showed positive nuclear expression or high in the case of ≥14% of positive tumor cells.

Estrogen and progesterone positivity was determined according to recommendations of the American society of clinical oncology/college of American pathologists. ER and PR positivity was defined as any positive nuclear staining in ≥1% of tumor cells (24).

HER-2 immunolabeling was measured according to the Hercep Test scoring system (Dako Cytomation) (25), as it follows:

0- no staining or faint incomplete membranous staining in <10% cells;

1-Faint incomplete membranous staining in >10% cells,

2-Weak to moderate complete staining in >10% cells and

3-Strong complete staining in >10% cells.

Cases scored as 2+ were considered equivocal and retested using chromogen *in situ* hybridization.

IHC-based molecular classification of breast cancer

According to St' Gallen IHC-based surrogate molecular classification of breast cancer, we used ER, PR, HER-2, and Ki-67 proliferation index to classify breast cancer into luminal A (ER+, PR+, HER-2 negative, and low Ki-67); luminal B (ER, PR, HER-2 positive, any Ki-67 or ER-positive, PR positive, HER-2 negative, and high Ki-67); HER-2 enriched (ER and PR negative, HER-2 positive, and any Ki-67), and triple-negative subtype (ER, PR and HER-2 negative, any Ki-67) (26).

Statistical analysis

Results are presented as a number of cases, percentages, and median with interquartile range. Testing for differences and correlation was performed using the Chi-square test, the Mann–Whitney test, and the Spearman rank correlation coefficient test. For continuous data, non-parametric Kruskal–Wallis and ANOVA were used. The results were considered statistically significant at the 95% confidence level or $p < 0.05$. The analysis was performed using IBM Statistics SPSS version 23.0.

RESULTS

The mean age of the 90 patients included in the study was 57.74 ± 11.3, range from 33 to 78 years. The majority of patients had ductal breast carcinoma (85.56%), moderately differentiated (50%), and size between 2 and 5 cm (46.67%). The most prevalent molecular subtype was luminal B (42.22%), followed by luminal A with 40% cases (Table 1).

A significant difference was observed between molecular subtypes and histologic grade of the tumor. HER-2 enriched and triple-negative tumors were more likely to be poorly differentiated (G3) in contrast to luminal A and B, which were more frequently well (G1) and moderately (G2) differentiated tumors ($p = 0.032$). We found no significant difference between molecular subtypes of breast cancer regarding other clinicopathological factors (age, tumor size, histologic type, lymph node status, and lymphovascular invasion) ($p > 0.05$) (Table 2).

MMP-9 was more frequently expressed in tumor cells (84.44%) than in stromal cells (40.0%), regardless of molecular subtypes of breast carcinoma (Table 1). The incidence of positive stromal MMP-9 expression was significantly higher in papillary, micropapillary, and medullary carcinomas than in ductal and lobular carcinomas ($p = 0.021$). There was a significant correlation between histologic grade and stromal MMP-9 expression. Poorly differentiated tumors (G3) showed the highest percentage of MMP-9 stromal positivity ($p = 0.047$) (Table 3). We also investigated the correlation of the MMP-9 staining intensity and the percentage of its positivity regarding standard clinicopathological factors. We found that strong intensity of tumoral MMP-9 positivity correlates with the presence of lymphovascular invasion ($p = 0.016$) (Table 4).

Stromal MMP-9 expression correlated positively with lymphocyte response in breast cancer specimens. Tumors with positive MMP-9 stromal expression are more likely to have abundant lymphocytic inflammatory infiltrate ($p = 0.038$). The increase of the percentage of MMP-9 expression increases lymphocytic response, that is, tumors with the highest percentage of positive stromal cells had the strongest lymphocytic response ($p = 0.007$) (Table 5). No significant correlation was found between molecular subtypes of breast cancer and MMP-9 expression ($p > 0.05$). We observed a significant association, but without linear correlation, between the percentage of MMP-9 stromal positivity and molecular subtypes ($p = 0.049$). Triple-negative tumors showed the highest rate (33.3%) of stromal MMP-9 positivity (percentage of positivity >51% – scores 3 and 4) among four molecular subtypes (data not shown in tables). TSR showed significant association and correlation with tumor grade (G) and tumor size (pT). Moderately differentiated tumors comprised the highest percentage of stroma-rich tumors ($p = 0.049$; $p = 0.021$, respectively). Significant association and negative correlation were observed between TSR and tumor size. Compared to smaller tumors, larger tumors were less likely to be stroma-rich ($p = 0.031$; $p = 0.049$) (Table 6).

TSR showed no significant association with molecular subtypes of breast cancer nor with MMP-9 expression ($p > 0.05$) (data not shown in tables).

DISCUSSION

We used surrogate IHC-based classification (26) and divided breast tumors according to ER, PR, HER-2, and Ki-67 expression, into luminal A, luminal B, HER-2 enriched, and triple-negative subtype. To avoid unnecessary subgrouping, due to relatively small sample size, luminal B HER-2 positive, and luminal B HER-2 negative tumors were all grouped as luminal B. The previous studies shown that triple-negative tumors are more aggressive with higher histologic grade, shorter disease-free, and relative survival period, and regarding luminal A and luminal B tumors (27). To some extent, our results confirmed findings of molecular

TABLE 2. Clinicopathologic characteristics of molecular subtypes of breast cancer

Variable	Molecular subtypes				*p*-value*
	Luminal A n (%)	Luminal B n (%)	HER 2 enriched n (%)	Triple-negative n (%)	
Age mean±SD	59.3±12.1	55.7±11.4	63.3±10.3	57.7±7.8	0.426 0.632
pT					
pT_1	16 (44.4)	12 (31.6)	2 (50.0)	3 (25.0)	0.597
pT_2	15 (41.7)	17 (44.7)	2 (50.0)	8 (66.7)	0.989
pT_3	1 (2.8)	5 (13.2)	0 (0.0)	1 (8.3)	
pT_4	4 (11.1)	4 (10.5)	0 (0.0)	0 (0.0)	
pN					
pN_0	12 (33.3)	11 (28.9)	1 (25.0)	5 (41.7)	0.268
pN_1	8 (22,2)	10 (26.3)	1 (25.0)	2 (16.7)	0.701
pN_2	13 (36.1)	7 (18.4)	2 (50.0)	5 (41.7)	
pN_3	3 (8.3)	10 (26.3)	0 (0.0)	0 (0.0)	
Tumor grade					
G1	7 (19.4)	10 (26.3)	0 (0.0)	1 (8.3)	0.009
G2	23 (63.9)	15 (39.5)	4 (100.0)	3 (25.0)	0.032
G3	6 (16.7)	13 (34.2)	0 (0.0)	8 (66.7)	
Histologic type					
Ductal	30 (83.3)	32 (84.2)	4 (100.0)	11 (91.7)	0.773
Lobular	1 (2.8)	3 (7.9)	0 (0.0)	0 (0.0)	0.416
Others	5 (13.9)	3 (7.9)	0 (0.0)	1 (8.3)	
LVI					
No	20 (55.6)	18 (47.4)	2 (50.0)	5 (41.7)	0.830
Yes	16 (44.4)	20 (52.6)	2 (50.0)	7 (58.3)	0.373

**p*-value obtained for ANOVA (mean age), Chi-square test, and Spearman correlation test. pT: Tumor size, pN: Regional lymph node status, LVI: Lymphovascular invasion

TABLE 3. Correlation of MMP-9 expression and standard clinicopathological parameters of breast cancer

Clinicopathological parameter	MMP-9					
	Stromal		*p*-value*	Tumoral		*p*-value*
	Negative	Positive		Negative	Positive	
Age mean±SD	57.2±12.6	58.6±8.9	0.568 0.365	53.5±13.2	58.5±10.8	0.126 0.167
Histologic type						
Ductal	50 (92.6)	27 (75.0)	0.044 0.021	12 (85.7)	65 (85.5)	0.816
Lobular	1 (1.9)	3 (8.3)		1 (7.1)	3 (3.9)	0.950
Others	3 (5.6)	6 (16.7)		1 (7.1)	8 (10.5)	
Tumor grade						
G1	13 (24.1)	5 (13.9)	0.022 0.047	1 (7.1)	17 (22.4)	0.357
G2	29 (53.7)	16 (44.4)		9 (64.3)	36 (47.4)	0.551
G3	12 (22.2)	15 (41.7)		4 (28.6)	23 (30.3)	
pT						
pT_1	19 (35.2)	14 (38.9)	0.074 0.469	6 (42.9)	27 (35.5)	0.962
pT_2	30 (55.6)	12 (33.3)		6 (42.9)	36 (47.4)	0.610
pT_3	2 (3.7)	5 (13.9)		1 (7.1)	6 (7.9)	
pT_4	3 (5.6)	5 (13.9)		1 (7.1)	7 (9.2)	
pN						
PN_0	18 (33.3)	11 (30.6)	0.543 0.380	2 (14.3)	27 (35.5)	0.459
PN_1	15 (27.8)	6 (16.7)		4 (28.6)	17 (22.4)	0.153
PN_2	14 (25.9)	13 (36.1)		5 (35.7)	22 (28.9)	
PN_3	7 (13.0)	6 (16.7)		3 (21.4)	10 (13.2)	
LVI						
No	30 (55.6)	15 (41.7)	0.141 0.201	9 (64.3)	36 (47.4)	0.192
Yes	24 (44.4)	21 (57.3)		5 (35.7)	40 (52.6)	0.250

**p*-value obtained for ANOVA (mean age), Chi-square test, and Spearman correlation test. pT: Tumor size, pN: Regional lymph node status, LVI: Lymphovascular invasion; MMP-9: Matrix metalloproteinase-9

subtypes, in order that HER-2 enriched and triple-negative tumors were more likely to be poorly differentiated in contrast to luminal A and B, which were more frequently well and moderately differentiated tumors ($p < 0.05$).

TABLE 4. Correlation of tumoral MMP-9 intensity and standard clinicopathologic parameters in breast cancer

Clinicopathological parameter	Tumoral MMP-9				*p*-value*
	Negative	Weak	Moderate	Strong	
Age mean±SD	52.3±15.3	58.2±10.1	57.5±12.0	60.4±9.7	0.394 0.196
Histologic type					
Ductal	9 (100.0)	29 (85.3)	26 (83.9)	13 (81.3)	0.885
Lobular	0 (0.0)	2 (5.9)	1 (3.2)	1 (6.3)	0.305
Others	0 (0.0)	3 (8.8)	4 (12.9)	2 (12.5)	
Tumor grade					
G1	0 (0.0)	6 (17.6)	9 (29.0)	3 (18.8)	0.289
G2	7 (77.8)	15 (44.1)	16 (51.6)	7 (43.8)	0.411
G3	7 (77.8)	15 (44.1)	16 (51.6)	7 (43.8)	
pT					
pT_1	4 (44.4)	11 (32.4)	13 (41.9)	5 (31.3)	0.395
pT_2	5 (55.6)	14 (41.2)	14 (45.2)	9 (56.3)	0.793
pT_3	0 (0.0)	6 (17.6)	1 (3.2)	0 (0.0)	
pT_4	0 (0.0)	3 (8.8)	3 (9.7)	2 (12.5)	
pN					
pN_0	1 (11.1)	9 (26.5)	16 (51.6)	3 (18.8)	0.121
pN_1	3 (33.3)	8 (23.5)	6 (19.4)	4 (25.0)	0.301
pN_2	4 (44.4)	9 (26.5)	6 (19.4)	8 (50.0)	
pN_3	1 (11.1)	8 (23.5)	3 (9.7)	1 (6.3)	
LVI					
No	7 (77.8)	14 (41.2)	20 (64.5)	4 (25.0)	0.015
Yes	2 (22.2)	20 (58.8)	11 (35.5)	12 (75.0)	0.016

**p*-value obtained for ANOVA (mean age), Chi-square test, and Spearman correlation test. pT: Tumor size; pN: Regional lymph node status; LVI: Lymphovascular invasion; MMP-9: Matrix metalloproteinase-9

TABLE 5. MMP-9 expression and lymphocyte response

Parameter	Lymphocyte response			*p*-value*
Tumoral MMP-9	Weak	Moderate	Strong	
Negative	8 (57.1)	3 (21.4)	3 (21.4)	0.883
Positive	39 (51.3)	16 (21.1)	21 (27.6)	0.631
Tumoral MMP-9 intensity				
Negative	5 (55.6)	2 (22.2)	2 (22.2)	0.742
Weak	14 (41.2)	8 (23.5)	12 (35.3)	0.323
Moderate	19 (61.3)	5 (16.1)	7 (22.6)	
Strong	9 (56.3)	4 (25.0)	3 (18.8)	
Tumoral MMP-9 %				
0	5 (55.6)	2 (22.2)	2 (22.2)	0.996
1	3 (60.0)	1 (20.0)	1 (20.0)	0.679
2	4 (44.4)	3 (33.3)	2 (22.2)	
3	8 (53.3)	3 (20.0)	4 (26.7)	
4	27 (51.9)	10 (19.2)	15 (28.8)	
Stromal MMP-9				
Negative	33 (61.1)	10 (18.5)	11 (20.4)	0.036
Positive	14 (38.9)	9 (25.0)	13 (36.1)	0.038
Stromal MMP-9 intensity				
Negative	28 (63.6)	7 (15.9)	9 (20.5)	0.045
Weak	7 (38.9)	5 (27.8)	6 (33.3)	0.045
Moderate	11 (50.0)	5 (22.7)	6 (27.3)	
Strong	1 (16.7)	2 (33.3)	3 (50.0)	
Stromal MMP-9 %				
0	31 (62.0)	9 (18.0)	10 (20.0)	0.048
1	4 (57.1)	2 (28.6)	1 (14.3)	0.007
2	3 (30.0)	5 (50.0)	2 (20.0)	
3	7 (53.8)	1 (7.7)	5 (38.5)	
4	2 (20.0)	2 (20.0)	6 (60.0)	

**p*-value obtained for Chi-square test and Spearman correlation test

TABLE 6. Correlation between TSR and clinicopathological parameters

Parameter	T/S ratio		*p*-value*
	Low	High	
Age mean±SD	58.3±11.3	5.6±11.3	0.511 0.491
Histologic type			
Ductal	52 (83.9)	25 (89.3)	0.792
Lobular	3 (4.8)	1 (3.6)	0.504
Others	7 (11.3)	1 (3.6)	
Tumor grade			
G1	13 (21.0)	5 (17.9)	0.049
G2	26 (41.9)	19 (67.9)	0.021
G3	23 (37.1)	4 (14.3)	
pT			
pT_1	28 (45.2)	5 (17.9)	0.031
pT_2	24 (38.7)	18 (64.3)	0.049
pT_3	6 (9.7)	1 (3.6)	
pT_4	4 (6.5)	4 (14.3)	
pN			
PN_0	18 (29.0)	11 (39.3)	0.548
PN_1	17 (27.4)	4 (14.3)	0.757
PN_2	18 (29.0)	9 (32.1)	
PN_3	9 (14.5)	4 (14.3)	
ER			
Negative	12 (19.4)	5 (17,9)	0.558
Positive	50 (80.6)	23 (82.1)	0.868
PR			
Negative	17 (27.4)	7 (25.0)	0.513
Positive	45 (72.6)	21 (75.0)	0.813
HER-2			
Negative	55 (88.7)	23 (82.1)	0.296
Positive	7 (11.3)	5 (17.9)	0.402
Ki-67			
Low	31 (50.0)	16 (57.1)	0.345
High	31 (50.0)	12 (42.9)	0.535

*p-value obtained for Chi-square test and Spearman correlation test

More attention was dedicated to investigating the tumor microenvironment, also known as the tumor-associated stroma. Some studies showed that stromal response has similarity to wound healing, affecting the extracellular matrix's remodeling, cell motility, and angiogenesis (28). As one of the primary regulators of ECM remodeling and degradation, MMP-9 expression was extensively investigated in breast cancer, with exceptional attention to its heterogeneity in different molecular subtypes (23,29,30). Since ECM degradation is a crucial step in tumor dissemination, MMP-9 is one of the most investigated potential biomarkers in breast cancer. Some studies reported that high MMP expression in cancer cells correlates with small and early-stage tumors, while stromal MMP expression correlates with more aggressive factors (31). We observed a significant association between positive stromal MMP-9 expression and higher histological grade of the tumor ($p < 0.05$). Furthermore, we observed a significant correlation between histologic type and stromal MMP-9 expression. Papillary, micropapillary, and medullary breast carcinomas showed a higher percentage of stromal MMP-9 expression than ductal and lobular carcinomas ($p < 0.05$). Joseph et al. study revealed that elevated cytoplasmic and stromal MMP-9 expressions were associated with high grade tumors, poor Nottingham Prognostic Index (NPI), and hormone receptor negativity. Stromal MMP-9 expression was associated with the presence of LVI (29). Some previous studies (32,33) found an association between tumoral MMP-9 expression and negative prognostic factors for breast cancer. Investigating plasma levels of MMP-9: TIMP-1 complex, Thorsen et al. found that it has no prognostic information in primary breast cancer (34). In our study, high tumoral MMP-9 protein expression was associated with the presence of lymphovascular invasion ($p < 0.05$), but we found no significant correlation of tumoral MMP-9 expression with other standard clinicopathological features.

Kim et al., investigating the immunohistochemical expression of different MMPs and their tissue inhibitors (TIMPs), found that the incidence of tumoral MMP-9 expression was significantly higher in HER-2 overexpressing tumors than in luminal A tumors (23). Youssef et al. investigated MMP-9 expression *in silico* analysis on DNA microarray and RNA sequencing data, together with immunohistochemical MMP-9 expression, and found that MMP-9 was differentially expressed among molecular subtypes, that is, that overexpression correlates with more aggressive molecular subtypes as HER-2 enriched and triple-negative breast cancer (35). Mehner et al. study revealed that MMP-9 is most highly expressed in basal-like and triple-negative tumors and contributes to metastatic progression (30). Although we found no significant correlation between molecular subtypes and MMP-9 expression, we observed a significant association between the percentage of MMP-9 stromal expression and molecular subtypes. In our study, stromal MMP-9 expression was higher in triple-negative tumors than luminal A and luminal B tumors ($p < 0.05$).

Due to the findings mentioned above, that triple-negative tumors were frequently higher grade and had a higher incidence of stromal MMP-9 positivity, we correlated TSR and molecular types of breast cancer, as well as TSR and MMP-9 expression. Although we expected triple-negative tumors to have a high stroma ratio, we found no significant correlation between TSR and molecular subtypes of breast cancer. Since we noticed higher MMP-9 stromal expression in high-grade tumors, we expected stroma-rich tumors to be more frequently MMP-9 positive or to have a higher percentage of MMP-9 expression. Still, we found no statistical significance between investigated parameters. Gujam et al. found that patients with high TSR were older, frequently had HER-2 positive tumors consistently, low tumor inflammatory infiltrate, and shorter cancer-specific survival (18). Since one of the hallmarks of cancer initiation and progression is inflammation (6), and since MMPs are involved in the local immune regulation at different points (36), we investigated the association between MMP-9 expression and the inflammatory infiltrate. We found that tumors with high MMP-9 stromal expression were associated with strong inflammatory response ($p < 0.05$). Among MMPs, MMP-9 is known as inflammation-related MMP, expressed in stromal lymphocytes, neutrophils, and macrophages (37).

CONCLUSION

Considering the limitations of the presented study, as relatively small sample size and a lack of correlation with clinical

outcomes and survival, our results confirm the significance of tumor microenvironment in breast cancer, emphasizing stromal MMP-9 expression. Although we found no significant correlation between molecular subtypes of breast cancer and MMP-9 expression, we observed a significant association, without linear correlation, between the percentage of MMP-9 positive stromal cell expression and molecular subtypes. Further studies with larger sample size, particularly with a higher proportion of triple-negative tumors, will be necessary to determine the impact of tumor microenvironment in different molecular subtypes of breast cancer in Bosnian women.

COMPETING INTERESTS

The authors declare no conflict of interest.

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