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Comparison of MMP-9 Density Between Triple Negative and HER2 Enriched Breast Carcinoma Subtypes

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ABSTRACT

Background
Matrix metalloproteinase-9 (MMP-9) is a protease that plays an important role in various biological processes of breast cancer. This marker may be useful in determining the prognosis and become a promising therapy, especially in breast carcinoma with an aggressive subtype including HER2 enriched and triple negative. Both subtypes have high MMP-9 expression, but it is necessary to differentiate them because they have different biological and molecular aspects. This study aims to compare the density of MMP-9 between triple negative and HER2 enriched breast carcinoma subtypes and their relationship with clinicopathological characteristics.

Methods
This is a cross sectional study using 32 samples of paraffin blocks and slides of breast carcinoma patients with triple negative and HER2 Enriched subtypes at the Department of Anatomical Pathology Faculty of Medicine Sriwijaya University/Dr. Mohammad Hoesin Hospital Palembang for the period January 1, 2016 to December 31, 2019. Each sample was stained with MMP-9 antibodies immunohistochemistry (polyclonal, Abcam). Comparative analysis of MMP-9 density between the two subtypes was carried out using the median value based on the Mann-Whitney analysis. MMP-9 density was categorized as high and low based on the ROC curve and analyzed by using chi-square test to assess its association with clinicopathological characteristics.

Results
Mann-Whitney test showed that the MMP-9 density was higher in the HER2 enriched subtype (median 12.0) than triple negative (median 8.8) with a p value of 0.632. The relationship distribution of high MMP-9 density was found in the category of age group >40 years old (60.0%), positive lymphovascular invasion (86.7%) and histological grade III (80.0%) with p values of 0.798, 0.402 and 0.691.

Conclusion
MMP-9 density in HER2 enriched subtype was higher than triple negative subtype, but statistically not significant.

Keywords: MMP-9 expression density, HER2 enriched breast carcinoma, triple negative

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INTRODUCTION

Breast cancer is the most common malignancy in women in the world and is the fifth leading cause of death from cancer in women in the world. Based on data from the 2020 Global Burden of Cancer Study (GLOBOCAN), the incidence of breast cancer in women is 11.7% of all cancer cases.[1] The Incidence of breast cancer varies by age for each country.[1] Several studies have shown that young women are more suffer from breast cancer with HER2 enriched and triple negative molecular subtypes which have a poor prognosis and cause of death at ≤40 years old.[2]

The importance of tumor micro-environment, one of which is the extracellular matrix, is widely known. The extracellular matrix is a complex and highly dynamic molecular network, and serves to provide biochemical signals in the process of development, invasion and metastasis of breast cancer.[3,4] Changes in breast cancer extracellular matrix are not only limited to the composition of the extracellular matrix, but also the remodeling enzymes, one of which is the matrix metalloproteinase (MMP) enzyme.[3]

Matrix Metalloproteinase-9 (MMP-9) is one of the most frequently studied MMPs and is a protease enzyme that plays an important role in extracellular matrix remodeling by degrading the extracellular matrix, which increased breast tumor growth, invasion, metastasis, angiogenesis, and proliferation. MMP-9 expression can be performed by using MMP-9 antibody immunohistochemistry which stained positively in the cell cytoplasm. In another study, MMP-9 expression was carried out semi-quantitatively on tumor cells and stromal cells for each subtype of breast carcinoma because they have different levels of MMP-9 expression.[5–7]

MMP-9 expression has the potential to be a useful biomarker in determining prognosis and has become a promising therapy, especially in breast carcinoma with aggressive subtypes including HER2 enriched and triple negative. In addition, both subtypes have different aggressiveness, metastatic tendency in certain organs and different molecular aspects, so the expression of MMP-9 will be different.[6,8,9] Therefore, we are interested in comparing the MMP-9 density between triple negative and HER-2 enriched breast carcinoma subtypes at the Department of Anatomical Pathology Faculty of Medicine Sriwijaya University/Dr. Mohammad Hoesin Hospital Palembang and also analyze the relationship of MMP-9 density with its clinicopathological characteristics.

METHODS

This study was an analytical observational study with a cross-sectional design in cases of triple negative and HER2 enriched breast carcinoma subtype which had been diagnosed histopathologically and immunohistochemically at the Department of Anatomical Pathology Faculty of Medicine Sriwijaya University/Dr. Mohammad Hoesin Hospital Palembang for the period 1 January 2016 to 31 December 2019.

This study used 32 samples of hematoxylin eosin archive slides and paraffin block/formalin fixed paraffin embedded (FFPE) breast carcinoma patients which had been stained with immunohistochemical markers ER, PR, HER2 and Ki-67. The study sample was taken retrospectively from a population with total sampling technique that met the criteria for exclusion and inclusion. Breast carcinoma with HER2 enriched subtype is characterized by expression of negative ER, negative PR and positive HER2. The triple negative subtype is characterized by ER, PR and HER2 negative expression.

Immunohistochemistry on this study is by using the MMP-9 polyclonal antibody (ab38898, Abcam), was carried out to detect the active form of the MMP-9 enzyme. The positive criteria is characterized by brown staining (weak-strong intensity) in the cytoplasm of tumor cells as well as the cells in the tumor microenvironment (Figures 1 and 2). In this study, the intensity of the staining was negligible in determining the positivity of MMP-9. This is because the cold ischemia time may vary for each specimen sent and the age of stored paraffin block varies.[10–13] Positive control in this study is by using colorectal carcinoma specimens.

The interpretation was carried out by one person, the researcher (dr. Eka Putra Pratama), initially with a weak magnification (10 times) to select 5 focus of high density cells that were positive with MMP-9. Then, each of the focus was examined with a strong magnification (40 times), photographed using a DP 21 camera with Olympus brand BX51 binocular light microscope and calculated the number of positive cells using Image J, summed and divided by 5 to determine the average of each sample. The average of all samples was analyzed according to the ROC

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curve to determine the cut-off point values and categorized into high and low MMP-9 density.

Bivariate analysis was performed to see the difference and comparison of MMP-9 density between triple negative and HER2 enriched breast carcinoma using the Mann-Whitney U test. This analysis was also used to see a relationship between MMP-9 density and clinicopathological characteristics such as age, lymphovascular invasion and histopathological grading by using Chi-Square on qualitative data of MMP-9 density that had been categorized based on the ROC curve. The p value is considered significant if p< 0.05 with a confidence interval of 95%. Data analysis using the Statistical Program for Social Sciences (SPSS) tool version 26.0. This research has received proper ethical approval at the Dr. Mohammad Hoesin Hospital Palembang, according to seven standards of WHO 2011 with ethic number 70/kepkrsmh/2022.

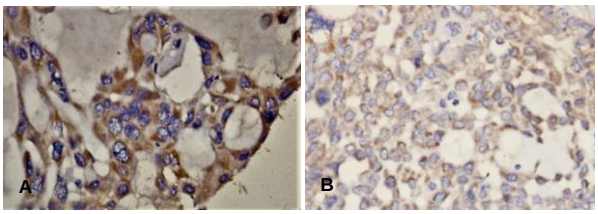

Figure 1. High MMP-9 density. A) High MMP-9 density with strong intensity in HER2 enriched subtype (magnification 40 times). B) High MMP-9 density with low to moderate intensity in triple negative subtype (magnification 40 times).

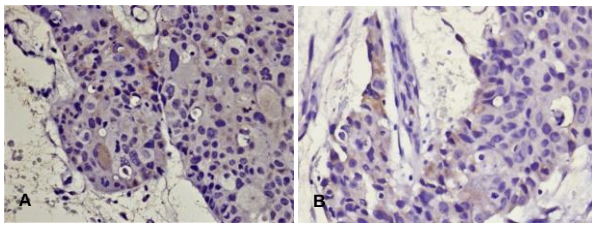

Figure 2. Low MMP-9 density. A) Low MMP-9 density in triple negative subtype (magnification 40 times). B) Low MMP-9 density in HER2 enriched subtype (magnification 40 times).

RESULTS

Clinicopathologic Characteristics in Breast Carcinoma Subtypes

Descriptive analysis of clinicopathological characteristics is shown in Table 1. The total sample consists of 32 samples with 25 samples of triple negative subtype and 7 samples of HER2 enriched subtype. The most common age group in this study was >40 years old consists of 21 samples (65.6%), with the youngest age 26 years old and the oldest age 74 years old. Positive lymphovascular invasion was found in 25 samples (78.1%). The most common histopathological grading is grade III, which is 24 samples (75.0%) and grade II 8 samples (25.0%). In this study, there were no samples with grade I.

The value of the ROC curve cut off point for MMP-9 density in this study was 11.1 (Figure 3). Based on this cut off point, MMP-9 density was low if <11.1 and it was high if >11.1 (Figures 1 and 2). The distribution of MMP-9 density can be seen in Table 2.

Table 1. Clinicopathological characteristics distribution in breast carcinoma. Highest triple negative and HER2 enriched breast carcinoma subtype distribution is in age group >40 years old (65.6%), positive lymphovascular invasion (78.1%) and histopathological grade III (75.0%).

Clinicopathological characteristics	Molecular subtypes		Total
	Triple negative	HER2 enriched	
	n (%)	n (%)	n (%)
Age			
≤40 years old	9 (36.0)	2 (28.6)	11 (34.4)
>40 years old	16 (64.0)	5 (71.4)	21 (65.6)
Lymphovascular invasion			
Positive	20 (80.0)	5 (71.4)	25 (78.1)
Negative	5 (20.0)	2 (28.6)	7 (21.9)
Histopathological grade			
I	0 (0.0)	0 (0.0)	0 (0.0)
II	4 (16.0)	4 (57.1)	8 (25.0)
III	21 (84.0)	3 (42.9)	24 (75.0)

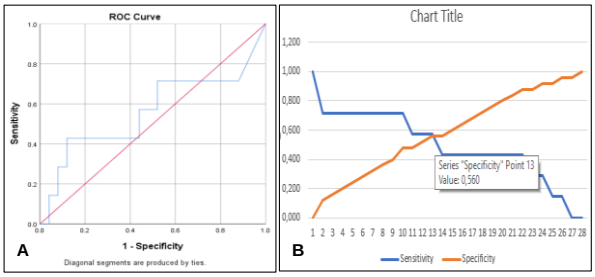

Figure 3. Matrix metalloproteinase (MMP-9) cut-off point based on ROC curve analysis. A) AUC 0.560 (IK95% 0.278 – 0.842). B) cut-off point value was 11,1 with specificity of 56.0% (red line) dan sensitivity of 57.1% (blue line).

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Table 2 Matrix metalloproteinase (MMP-9) density distribution. HER2 enriched breast carcinoma had high MMP-9 density for about 57.1% and triple negative breast carcinoma had high MMP-9 density for about 44.4%.

MMP-9 density	Molecular subtypes		Total
	Triple negative n (%)	HER2 enriched n (%)	n (%)
High	11 (44.0)	4 (57.1)	15 (46.9)
Low	14 (56.0)	3 (42.9)	17 (53.1)

Table 2 showing high MMP-9 density in 15 samples (46.9%) and low MMP-9 density in 17 samples (53.1%). Highest MMP-9 density is in HER2 enriched breast carcinoma subtype for about 57.1% and triple negative molecular subtype for about 42.9%.

Comparison between MMP-9 density in triple negative and HER2 enriched subtype

Shapiro-Wilk normality test showing abnormal distribution for MMP-9 density in both subtypes. Therefore, Mann-Whitney test was used to analyze the comparison between MMP-9 density in triple negative and HER2 enriched subtype. (Table 3).

Table 3. Comparison between MMP-9 density in triple negative and HER2 enriched breast carcinoma subtype. HER2 enriched breast carcinoma subtype had higher MMP-9 density (median 12.0) than triple negative subtype (median 8.8).

Molecular subtype	MMP-9 density			*p
	Median	Minimum	Maximum	
Triple negative	8.800	0.0	125.2	0.632
Her2 enriched	12.000	0.0	105.0	
Total	9.500	0.0	125.2	

*Mann-Whitney test, significant if p< 0.05.

Mann-Whitney test analysis showing MMP-9 density was higher in HER2 enriched than in triple negative breast carcinoma molecular subtypes, but the result was not statistically significant with p value of 0.632.

Relationship between MMP-9 density and clinicopathological characteristics

Chi-square test was used to analyze the association between MMP-9 density and age (Table 4). Table 4 showing that there is no association between MMP-9 density and age (p=0,798). The age group with a high MMP-9 density was more common in the age group >40 years old (60.0%) than in the age group <40% (50.0%).

Most of the samples with high MMP-9 density had lymphovascular invasion, which was 86.7%. Whereas 13.3% did not have lymphovascular invasion. The results of the analysis showed that there was no significant relationship between MMP-9 density and lymphovascular invasion (p=0.402).

The majority of samples with high MMP-9 density were found in histopathological grade III (80.0%) than grade II (20.0%). However, there was no significant relationship between MMP-9 density and breast carcinoma histopathological grade. In this study, there were no grade I breast carcinoma samples.

Table 4. Association between MMP-9 density and clinicopathological characteristics in breast carcinoma.

Clinicopathological characteristics	MMP-9 density				Total		*p
	High		Low				
	n	%	n	%	n	%	
Age							
≤40 years old	6	40.0	5	29.4	11	34.4	0.798
>40 years old	9	60.0	12	70.6	21	65.6	
Lymphovascular invasion							
Positive	13	86.7	12	70.6	25	78.1	0.402
Negative	2	13.3	5	29.4	7	21.9	
Histopathological grade							
Grade I	0	0.0	0	0.0	0	0.0	0.691
Grade II	3	20.0	5	29.4	8	25.0	
Grade III	12	80.0	12	70.6	24	75.0	

*Chi-Square test, significant if p< 0.05

DISCUSSION

Several previous studies have shown that breast cancer with HER2 enriched and triple negative subtypes is more common in the age group <40 years old.[2,14] However, in this study the most common age group was >40 years old, which is not in accordance with the previous study.[2] This may be caused by the large number of breast cancer patients who come for treatment at old age, as well as the existence of an unhealthy lifestyle due to lack of physical activity, consuming

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excess fatty foods, lack of consumption of fruits and vegetables, which have an impact on high levels of obesity which play a role as a risk factor for triple negative breast carcinoma.[15-17] Excess body mass index (BMI) can lead to the production of pro-inflammatory factors in the form of a metabolically activated phenotype consisting of genotoxic carcinogens and toxic metabolites caused but the accumulation of saturated fat, which are released by insulin resistance adipocytes. The metabolic pro-inflammatory factors are associated with BRCA1 and BRCA2 gene mutations which are often found in triple negative breast carcinoma.[17]

Triple negative and HER2 enriched breast carcinoma subtype in this study generally had positive lymphovascular invasion and was in accordance with the previous studies. This variable is a significant poor predictor if breast cancer patients and is associated with increased lymph node metastases, distant metastases, increased mortality and decreased response to therapy.[18,19]

In this study, most breast carcinoma with triple negative subtypes have histopathological grade III. In contrast to the HER2 enriched molecular subtypes which generally have histopathological grade II. These findings are consistent with several other studies which show that breast carcinoma with triple negative and HER2 positive molecular subtypes mostly has a high histopathological grading.[20,21] Mutations of p53, deregulation of Rb and integrin, overexpression of EGFR and decreased expression of PTEN, have important roles in increasing the histopathological grade of breast carcinoma, which is associated with poor prognosis and increases the aggressiveness of breast carcinoma.[22]

Comparison between MMP9 density in triple negative and HER2 enriched breast carcinoma subtype

The results of the analysis showed that the HER2 enriched subtype had a higher MMP-9 density than the triple negative subtype. This is consistent with a study conducted by Yousef et al which showed that the highest MMP-9 expression was found in the HER2 enriched molecular subtype compared to other molecular subtypes, although this was not statistically significant. This is because HER2 enriched and triple negative both have high MMP-9 density.[6]

Tumor cells and tumor microenvironment cells can secrete local inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis alpha (TNF-α) by causing activation of NF-κB, a transcription factor protein that increases MMP-9 production.[6,23] Triple negative breast carcinoma subtype can also increase TGF-β cytokine secretion which is associated which various pro tumorigenic products and affecting MMP-9 expression through interaction with the MMP-9 promoter. In addition, TGF-β secretion in triple negative breast carcinoma can also be caused by MMP-9 expression in the surrounding tumor microenvironment cells, one of which is fibroblast.[24]

Decreasing the amount of TIMP-1 may be a factor in increasing MMP-9 activation in HER2 enriched breast carcinoma, but its relationship with the triple negative breast carcinoma is unknown.[25] Active MMP-9 can be neutralized by protease inhibitors such as TIMP-1.[6]

c-ErbB-2 ligand which played a role in HER2 enriched molecular breast carcinoma subtype can increase MMP-9 expression through the JAK3/ERK pathway and this ligand was not found in triple negative breast carcinoma subtype.[26] SK-BR-3 is a breast cancer cell lineage that overexpresses ErbB-2 and associated with HER2 enriched breast carcinoma subtype.[27] Cho et al stated that SK-BR-3 cells could significantly increase MMP-9 expression.[28]

Matrix metalloproteinase-9 (MMP-9) is a gelatinase-B and secreted as an active zymogen. This gelatinase is tightly regulated by several mechanisms, one of which is the transcription factor activator protein 2 (AP-2). This AP-2 protein can be expressed by breast carcinoma cells as well as tumor microenvironment cells.[26] Increased transcription of HER2 protein can occur due to activation of the HER2 promoter by transcription factors, including AP-2.[29] So it can be concluded that increasing AP-2 expression can increase MMP-9 expression in breast carcinoma with the HER2 enriched subtype.

Increased expression of MMP-9 in triple negative breast carcinoma can be caused by increased expression of ADAM9 mRNA which interacts with integrin β1 in regulating MMP-9 synthesis. ADAM9 is an enzyme that can work by binding to integrin receptors to activate PI3K/Akt in NF-κB signal transduction, which is a protein transcription factor that increases MMP-9 production.[30,31] Zhou et al stated that ADAM9

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expression was increased in triple negative breast carcinoma subtype and could be an important target in therapeutic, but no studies stated that ADAM9 could be found in other breast carcinoma subtypes.[30]

Tumor microenvironment can also influence MMP-9 expression, including CD8+ T lymphocytes, CD4+ cells, B cells, macrophages and fibroblasts. However, in cases of aggressive breast carcinoma subtypes, usually MMP-9 is often secreted by intrastromal cells, namely fibroblast. Intrastromal fibroblast cells can produce cytokines in the form of TNF-α and TGF-β which will bind to their receptors, the TNF-α receptors I and II, that can affect MMP-9 expression through activation of MAPK and SMAD signaling pathways. TNF-α also regulates CDKN1A/p21 expression which plays an important role in increasing MMP-9 expression.[32,33]

Relationship between MMP-9 density and clinicopathological characteristics

Increasing age can increase MMP-9 activity which is influenced by TGF-β.[34] Accumulation of senescent cells is a sign of aging effects and the initiation of carcinogenesis. These cells can cause accumulation of free radicals, OH and/or HO_2 in cells, resulting in oxidative stress and disruption of DNA repair. Transforming growth factor- β (TGF-β) is a superfamily of cytokines that control cellular function and contribute to cell proliferation, migration, differentiation and apoptosis in various cell types. Recent reports show that both DNA damage and accumulation of free radicals can increase TGF-β expression.[35] In addition, excessive oxidative stress and DNA damage can also stimulate the release of pro-inflammatory cytokines such as NF-κB and IL-1, which are known to increase MMP-9 expression by inducing AP-1.[36,37] This is consistent with the results, where the age group >40 years old had a higher density of MMP-9, when compared to the age group <40 years old, although it was not statistically significant.

The MMP-9 enzyme has the ability to degrade collagen, including collagen type IV, which plays an important role in basement membrane degradation, increasing migration, invasion and metastasis.[38] MMP-9 is also known to increase angiogenesis around tumor cells through the production of VEGF and increase the migration of endothelial cells which also produce VEGF, so that tumor cells can easily migrate into the blood vessels that form around them and can degrade the basement membrane of blood vessels.[39] This is consistent with the results that high MMP-9 expression in tumor cells is associated with increased lymphovascular invasion, although it was not statistically significant.

Previous studies have shown that there was a significant difference between histopathological grade and MMP-9 expression, where the study also showed that high MMP-9 expression was more often found in histopathological grade III.[40] This is consistent with the results, where a high MMP-9 density has a histopathological grade III of 80%, although it is not statistically significant. The mechanism of MMP-9 in increasing the aggressiveness of breast carcinoma occurs through the activation of TGF-β which increases cell growth and development, as well as cell invasion.[3]

CONCLUSION

MMP-9 density in HER2 enriched was higher than in triple negative breast carcinoma molecular subtypes, but statistically not significant. High MMP-9 density distribution belongs to the following categories: age group >40 years old, positive lymphovascular invasion and histopathological grade III.

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