Potential for Predicting Lymph-node Metastasis in Invasive Breast Carcinoma of No Special Type Using MT1-MMP Immunohistochemistry Staining

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Abstract

BACKGROUND: Lymph-node metastasis (LNM) is the most frequent complication of invasive breast carcinoma (IBC).

AIM: Using immunohistochemistry (IHC), this study aims to determine the role of membrane-type 1-matrix metalloproteinase (MT1-MMP) expression as a biomarker for LNM in IBC of no special type (IBC-NST).

MATERIALS AND METHODS: Primary tumors from individuals with IBC-NST were preserved in paraffin and then categorized as having LNM or not. Tumor size, lymphovascular invasion (LVI), tumor grade, MT1-MMP expression, and other factors were evaluated across a range of ages. MT1-MMP expression was assessed by IHC, with supplemental data acquired from archives. Collecting and analyzing the data required the use of both bivariate and multivariate techniques.

RESULTS: The odds ratio (OR) for LNM was 5.003 (95% CI: 1.68–20.61) for MT1-MMP expression, while the OR for LVI was 4.71 (95% CI: 1.57–18.8). These associations were found using the Firth penalized likelihood Logit analysis method. At an H-score cutoff of 202.22 (70.8% sensitivity and 95.8% specificity), an area under the receiver operating characteristic of 0.9130.038 (95% CI: 0.838–0.989) was found for MT1-MMP expression in diagnosing LNM.

CONCLUSION: In conjunction with LVI, MT1-MMP expression may serve as a predictor of LNM. To further assist data separation in future research, the MT1-MMP expression H-score cutoff of 202.22 could be used.

Introduction

Breast cancer has the highest incidence rate of all female cancers, accounting for 11.7% of all cancer diagnoses and 6.9% of cancer-related deaths [1], [2], which may be either invasive or non-invasive. Eighty-one percentages of breast cancers are invasive and infiltrative, meaning that the cancer cells invade neighboring tissues rather than being contained inside the glands or ducts [3]. There are several subtypes of carcinoma, but around 70% of cases are of the invasive breast cancer that has no known classification invasive breast carcinoma (IBC-NST) [3].

The death rate is greater for invasive cancers. More than 90% of deaths with IBC-NST are caused by distant metastases. Lymph node metastasis (LNM) is the most prevalent kind of metastasis in IBC-NST with 5-year survival 40% [3]. Therefore, accurately diagnosing individuals at high risk and selecting the most efficient therapy for each individual requires accurate prediction of LNM.

Matrix metalloproteinase (MMP) levels are one of numerous biomarkers in IBC-NST used to predict the LNM. MMP is a class of zinc-containing proteolytic enzymes that deteriorate the extracellular matrix. MMPs are proteins that have been linked to tumor invasion and metastasis in breast cancer [4]. MMPs have differed into four main categories named collagenase, gelatinase, stromelysin, and membrane-bound MMPs.

Membrane-type 1-MMP (MT1-MMP) belongs to a family of MMPs that are significantly expressed in many cancers. The role of this protein in breast cancer results in membrane remodeling, tumor invasion, metastases, and angiogenesis [4]. Several studies have shown that MT1-MMP has the potential to predict the prognosis of breast cancer patients [5], [6]. However, the detection methods used are quite advanced, such as immunofluorescence or fluorescence *in situ*

hybridization (FISH), which can only be done in a fully equipped laboratory. The practicality and simplicity of the detection method are very important components of the efficacy of MT1-MMP detection in the clinical setting. Therefore, in this study, we used immunohistochemistry (IHC) to assess MT1-MMP expression for predicting LNM in IBC-NST patients. H-score will be used to quantify MT1-MMP expression as determined by IHC. For future studies, we are also investigating the optimal H-score cutoff for MT1-MMP.

Materials and Methods

Design of study

From February 2020 to December 2020, cross-sectional research was carried out at the Universitas Indonesia laboratory. In June of 2020, the University of Indonesia Ethics Committee accepted the protocol (20-09-1169). Every participant gave informed, written agreement before the study began, and the researchers ensured that their work complied with the Declaration of Helsinki [7]. To complete our analysis, we needed to access data from the department's archives, in which we did for the years 2019 and 2020. Patient age, tumor type, age, tumor size, LNM, tumor grade, and lymphovascular invasion (LVI) were all obtained. Further, information on MT1-MMP expression was gathered by quantifying the stained findings of IHC.

Samples

Histopathologically newly diagnosed IBC-NST with or without LNM patients from Asia having a mastectomy for breast cancer provided primary tumor paraffin blocks. Patients with other forms of cancer, those with unreliable paraffin blocks, and those with systemic comorbidities were restricted from being able to participate.

Each sample was classified based on whether or not LNM was detected. With an alpha of 5%, a 95% confidence interval, and 80% power, a total of 23 samples were collected across all categories. This research used 24 LNM samples and 24 non-LNM samples. To avoid any potential for bias, just one researcher got access to the final groupings. Before the analysis is done, no one except the author knows to which category each study belongs.

IHC staining

Xylol was used to deparaffinized the breast cancer specimen's paraffin section before it was rehydrated in a succession of 96% and 70% absolute alcohol and distilled water for 5 min. Tris EDTA at pH 9.0 was used for 20-min antigen retrieval in a high-heat decloaking chamber. After antigen retrieval and a peroxidase block for 15 min, the sections were washed in phosphate-buffered saline with a pH of 7.4. A primary antibody against MT1-MMP (Merck, Jakarta, Indonesia) was used to incubate the slice for 1 h, and this was followed by further incubation with a secondary antibody and Novolink polymer. Tissue slices were stained with the brown chromogen diaminobenzidine, counterstained with hematoxylin from the same company, and finally bluing with 5% lithium carbonate for microscopic examination.

MT1-MMP expression quantification

The IHC staining was examined and interpreted by two histopathology specialists. Leica LAZ EZ software and a white balance camera were used to view each preparation under a ×400 light microscope. Minimum of 500 tumor cells were evaluated for MT1-MMP expression across five randomly selected visual fields (×400), with each tumor area has 100 cells. Tumor cell membranes and cytoplasm were stained brown, indicating MT1-MMP expression [8]. Using Image J's cell counter, staining was classified based on its color intensity [9]. The H-score is used to quantify the MT1-MMP expression [10]. Until all of the computations in the sample have been evaluated, one researcher compiles the findings to eliminate any potential for bias. The H-score will be calculated as the mean of the two raters' scores.

Statistical analysis

Microsoft Excel was used to insert collected data into the primary table before analysis. Tabulated data will be analyzed in SPSS 20 and viewed in GraphPad Prism 8 for visualization. We estimated the intraclass correlation coefficient (ICC) to evaluate data quality by comparing H-score variation among observers, which use the two-way mixed-average absolute agreement. Poor, moderate, good, and exceptional dependability are indicated by ICC values of <0.5, between 0.75 and 0.9, and higher than 0.90, respectively [11]. The average of the two observers' H-scores was used to classify patients into high- or low-risk categories [12]. The levels of MT1-MMP expression in each sample are described by these categories.

For bivariate analysis, we looked at the correlation between LNM and the following factors: Patient age (50 y.o vs. 50 y.o), tumor grade (Grade III [high] vs. Grade I-II [low]), tumor size (5 cm vs. >5 cm), LVI (yes/no), and MT1-MMP expression (high/low). For the variable with p-value, multivariate analysis will follow <0.2. Firth penalized likelihood Logit analysis

for reducing bias in small sample analyses [13]. Area under the receiver operating characteristic (AUROC) curve was used to evaluate the model's discriminatory power. If p < 0.05, then the results are reliable. The ROC curve of the MT1-MMP H-score will be evaluated to determine the best possible cutoff H-score for the MT1-MMP. The H-score continuous data on MT1-MMP expression was utilized for this study. 0.5 indicates no differentiation, 0.7–0.8 is fair, 0.8–0.9 is excellent, and more than 0.9 is exceptional for the AUROC [14]. From the ROC curve, the Youden index cutoff value for MT1-MMP expression was determined [15] and also the K-Index [16].

Results

The expression of MT1-MMP was examined by IHC staining in all 48 samples. The typical IHC staining findings are shown in Figure 1a-d. Each picture shows varying staining: (a) No tumor cell staining, (b) mild, (c) moderate, and (d) strong. The images are all sections of the same slide. This exemplifies the ability to distinguish several cells of varying brightness within a single visual field and over multiple slides. In the cytoplasm, MT1-MMP expression can be analyzed.
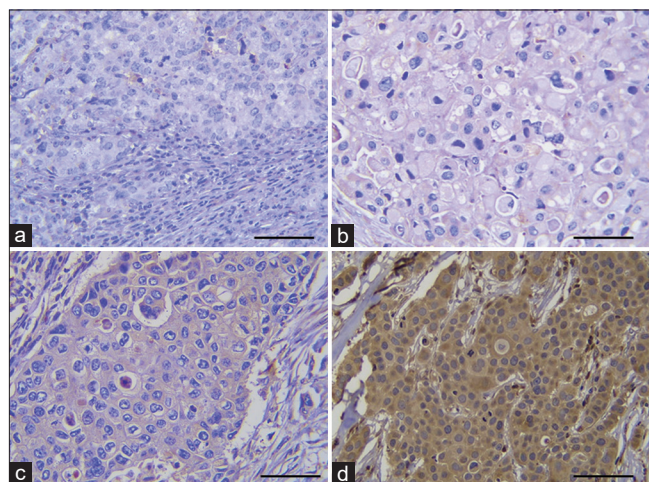

Figure 1: MT1-MMP expression in the cytoplasm of IBC-NST tumor cells, as shown by IHC staining (×400). (a) Negative, (b) low positive, (c) positive, and (d) high positive. Each picture has a scale bar of 50 µm

All 48 samples were evaluated separately by two researchers. Figure 2 shows the H-score distribution among the various samples. Both measures were determined to have a moderate degree of dependability. With a 95% CI ranging from 0.579 to 0.867, the average ICC for all measures was 0.763 (F (47.47) = 4.218, p = 0.001).

Table 1 shows each sample's clinicopathologic features. These confounding variables (or "covariates") have been connected to LNM incidence rates. Thus, we also conducted a bivariate test to determine the link between each of these factors and LNM. Several factors were subjected to bivariate analysis to see whether or not they were correlated with LNM. Table 2 displays the results of a series of tests conducted on the independent variables of LVI, tumor size, tumor grade, and MT1-MMP expression level.

Table 1: The clinicopathological characteristics of samples

Clinicopathological characteristic	n or value	%
Age (years)		
≥50	27	56.25
<50	21	43.75
Mean (SD)	50.94 (12.29)	
Median (Min-Max)	50 (29–75)	
Tumor grade		
Grade I	5	10.4
Grade II	16	33.3
Grade III	27	56.3
Tumor size (cm)		
<2	2	4.2
2–5	28	58.3
>5	18	37.5
Lymphovascular invasion		
Yes	27	56.25
No	21	43.75

MT1-MMP expression was shown to have a statistically significant correlation with LNM (p = 0.018) in a bivariate study. In addition, LNM was substantially linked to LVI (p = 0.02). LNM was unrelated to patient age, tumor grade, tumor size, or tumor volume.

Table 2: Bivariate analysis of LNM variables

Variables	Category	Lymph node metastasis			p-value	OR	95% CI	
		Yes (%)	No (%)	Total			Min	Max
Age	≥50 y.o	14 (51.9)	13 (48.1)	27	1	1.19	0.38	3.71
	<50 y.o	10 (47.6)	11 (52.4)	21				
Tumor grade	High	15 (55.6)	12 (44.4)	27	0.56	1.67	0.53	5.265
	Low	9 (42.9)	12 (57.1)	21				
Tumor size	>5 cm	7 (38.9)	11 (61.1)	18	0.37	0.49	0.148	1.602
	≤5 cm	17 (56.7)	13 (43.3)	30				
Lymphovascular invasion	Yes	18 (66.7)	9 (33.3)	27	0.02*	5	1.45	17.27
	No	6 (28.6)	15 (71.4)	21				
MT1-MMP expression	High	14 (58.3)	10 (41.7)	24	0.018*	5.32	1.49	19.06
	Low	5 (20.8)	19 (79.2)	24				

LNM: Lymph node metastasis, MT1-MMP: Membrane-type 1-matrix metalloproteinase, OR: Odds ratio.

Since MT1-MMP expression and LVI both yielded statistically significant findings, a multivariate analysis was conducted to determine the correlation between the two variables by incorporating LVI as a covariate. Table 3 displays the results of a Firth penalized likelihood Logit analysis performed to lessen the impact of sample size on the study.

Table 3: Firth penalized likelihood Logit analysis results

Predictor	β	SE β	Wald's χ^2	df	p-value	e^β (odds ratio)
MT1-MMP expression	1.61	0.694	6.259	1	0.012	5.003
Lymphovascular invasion	1.55	0.678	6.058	1	0.014	4.71
Constant	−1.50	0.61	8.16	1	0.004	
Test						
Overall model evaluation			12.86	2	0.0016	

MT1-MMP: Membrane-type 1-matrix metalloproteinase.

Table 3 demonstrates that MT1-MMP expression is related with LNM with an odds ratio (OR) of 5.003 (95% CI: 1.68–20.61) and LVI with 4.71 (95% CI: 1.57–18.8). Five times as much metastasis was seen in cases where MT1-MMP expression was high as in cases where it was low. Furthermore, the likelihood of metastases in samples with LVI was 4.71 times higher than in samples without LVI. The predictors together provided a robust distinction between LNM and non-LNM, as shown by the fact that the entire model performed better than a constant-only model (p = 0.0016).

Figure 3 displays the ROC curve used to evaluate MT1-MMP expression for its capacity to
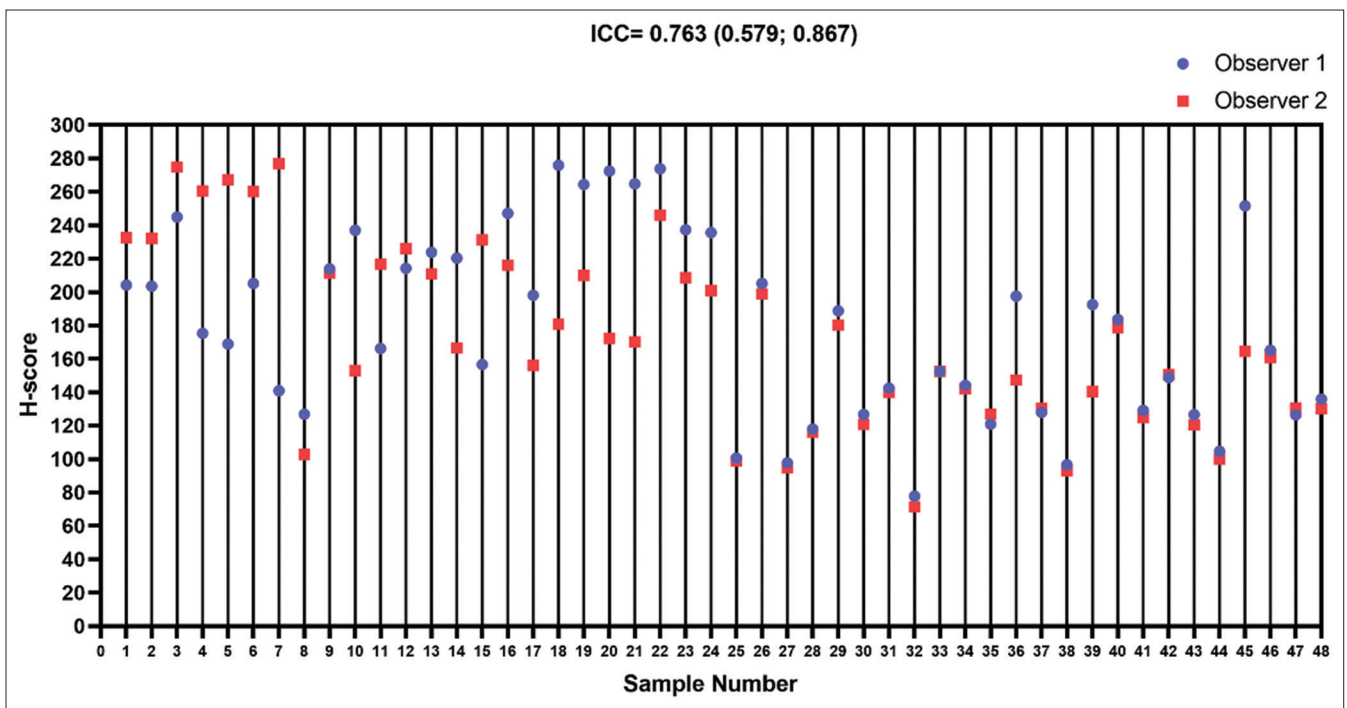
Figure 2: The H-score between two observers, shown as a dot plot for the whole sample

differentiate against LNM. The 95% CI for the AUROC was 0.913 ± 0.038. The ROC curve analysis revealed that an H-score of 202.22 was the most sensitive cutoff for detecting MT1-MMP expression, with a correspondingly high Youden index (0.667) and low K-index (0.295). With a sensitivity of 70.8% and specificity of 95.8%, this cutoff is very effective.
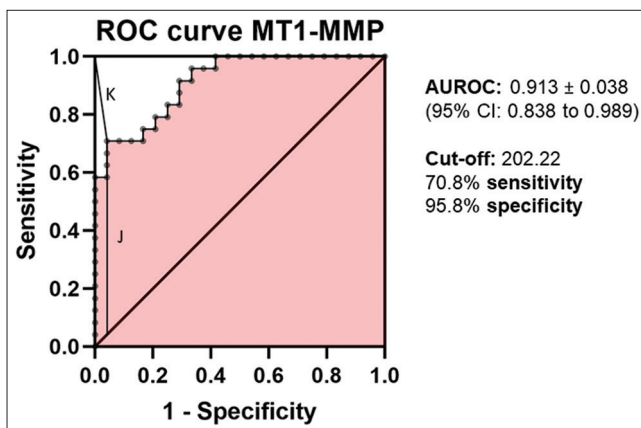

Figure 3: ROC curve of MT1-MMP expression. J: Youden index; K: K-index

Discussion

MT1-MMP is a protein that can act as a biomarker. Proteolytic enzyme MT1-MMP has a function in the development of breast cancer [6]. Breast cancer cells may proliferate, invade, and advance when the extracellular matrix is degraded by MT1-MMP [17]. It is

clear that MT1-MMP activity testing may provide details regarding breast cancer development. This cannot be separated from MT1-MMP's function as a biomarker. FISH and polymerase chain reaction (PCR) are only two of the many molecular approaches available for detecting MT1-MMP.

IHC staining, a straightforward method, was employed in our research. In contrast to IHC, the increasingly popular PCR and FISH have limited use in the clinic. Using an appropriate antibody, IHC may detect MT1-MMP both at the cell membrane and in the cytoplasm. As shown in Figure 1, the chromogen 3,3'-diaminobenzidine is utilized to create the brown hue. Browns with deeper tones indicate higher MT1-MMP expression levels in the cells. The brown tint is concentrated mostly in the cell membranes and cytoplasm, where MT1-MMP is present. In a similar vein, Li *et al.* found that breast carcinoma cells had overexpressed staining for MT1-MMP in their cell membranes and cytoplasms [18].

To isolate the effect of MT1-MMP expression as a predictor of LNM, it is necessary to account for and adjust for a number of potential confounding variables. Inclusion and exclusion criteria helped to exclude a number of potential sources of bias related to LNM [19]. Despite this, the research also accounted for a number of other factors that may have acted as confounds. However, bivariate and Firth penalized likelihood Logit analyses mitigated their influence on LNM. Only LVI exhibits a meaningful connection to LNM among these potential confounders. After analyzing the correlation between MT1-MMP and LNM expressions using Firth penalized likelihood Logit

analysis, we discovered that the impact of MT1-MMP expression on LNM was not modified by controlling for LVI. Thereby, MT1-MMP expression may serve as a predictor of LNM.

An investigation of the link between MT1-MMP expression and LNM in IBC-NST would be fascinating. The OR for MT1-MMP expression was 5.003 (95% CI: 1.68–20.61) in the logistic regression analysis. These results showed that high MT1-MMP expression on IHC staining was associated with a fivefold greater frequency of LNM in the primary tumor samples than low MT1-MMP expression. The underlying molecular pathways allow for the connection between MT1-MMP expression and LNM [19]. MT1-MMP is produced in an inactive form but can be active in the cell membrane. Degradation of the extracellular matrix, which includes proteins such as laminin and fibronectin, is facilitated by the active MT1-MMP, which, in turn, activates MMP2 and MMP13 [6]. The breakdown of the matrix will facilitate tumor cell invasion and subsequent metastasis. This explains why metastasized primary tumors display significant levels of MT1-MMP in the axillary lymph nodes. Maquoi *et al.* found similar results, demonstrating that apoptosis bypassed by MT1-MMP activity increases tumor development [20]. Jiang *et al.* found that compared to wild-type cells, tumor cells with reduced MT1-MMP expression were less invasive when cultured *in vitro*, providing more evidence for the role of MT1-MMP in tumor progression [6]. High MT1-MMP expression, however, was not necessarily linked to cancer cell metastasis, as discovered by Cepeda *et al.* [5] In his research, Cepeda showed that the overexpression of MT1-MMP was no more than 1000 times that of normal cells. Instead, it was more optimal for causing cancer cell invasion and metastasis. By all means, this cannot be compared with the high H-score in this study. However, this is something that can trigger further research.

However, this research also established a link between LVI and LNM. The OR for LVI in the aforementioned model was 4.71 (95% CI: 1.57–18.8). This shows that the likelihood of lymph node metastases in initial tumor samples with LVI was 4.71 times higher than in those without LVI. Several studies, including Melzer *et al.*, have hypothesized that MMP has a role in initiating LVI in IBC-NST; however, this is still up for debate [21]. The study of Perentes *et al.* even identified the potential for MT1-MMP to induce LVI [22]. Furthermore, LVI is involved in systemic metastases, as shown by Nathanson *et al.* [23]

The potential of MT1-MMP as a predictor of lymph no metastasis certainly needs to be further elaborated. One method is to examine the ROC curve using LNM discrimination analysis. The 95% CI for the AUROC was 0.913 ± 0.038, indicating high precision. The results of this research showed that MT1-MMP expression was highly discriminatory of lymph node metastases and may be utilized as a threshold to facilitate the separation of H-score data. The technique section explains why the research used an H-score of 200 as the threshold for inclusion. The authors acknowledge, however, the value of a marker-specific IHC cutoff test in making therapy response predictions. Therefore, we identified a specific cutoff for the MT1-MMP H-score, 202.22. This cutff can assist the separation of MT1-MMP H-score data in various further studies. Of course, this cutoff still requires validation with a larger data set, but this research is expected to be a pioneer for further studies. The limitation of this research is that this study has a small sample size. However, even with a small sample, this study already presents a promising picture for research with a larger sample.

Conclusion

MT1-MMP expression serves as a good predictor for LNM. Even if it plays a secondary role in prediction, LVI is nevertheless useful. Both contribute to the ability to foretell LNM in IBC-NST. IHC staining is a useful approach for identifying MT1-MMP expression, and an H-score cutoff of 202.22 may be used to categorize samples into those with high or low MT1-MMP expression. Future studies on MT1-MMP expression in IBC-NST may make advantage of this cutoff.

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