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Original Article

Quantification of MMP-2 and TIMP-1 expressions in breast cancer

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ABSTRACT

Background and Aims: *MMP-2* and *TIMP-1* are vital molecules in the remodeling of the extracellular matrix, and they have a critical role in the metastatic process of breast cancer. This study aimed to detect expression levels of *MMP-2* and *TIMP-1* genes by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and identify their potential roles in breast cancer prognosis.
Methods: *MMP-2* and *TIMP-1* gene expression levels in 17 breast cancer tumor tissues and normal breast tissue were examined. The expression levels of *MMP-2* and *TIMP-1* were analyzed by qRT-PCR. The association between the expression levels of *MMP-2* and *TIMP-1* and clinicopathological manifestations of breast cancer was examined.
Results: Lower gene expression levels of *MMP-2* and *TIMP-1* were detected in tumors compared to the controls. A statistical correlation was not observed between the expression level of *MMP-2*, *TIMP-1*, and clinicopathological parameters (tumor grade, lymph node involvement, hormone receptor status).

Conclusion: Our findings have been suggesting that expression profiles of *MMP-2* and *TIMP-1* might be independent prognostic and predictive biomarkers for breast cancer. These biomarkers are candidate molecules for personalized therapy. *MMP-2* and *TIMP-1* expression patterns will also aid in the identification of more precise and targeted subgroups of breast cancer.

Keywords: Breast cancer, *MMP2* metalloproteinase, *TIMP-1*

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INTRODUCTION

Breast cancer (BC) is the most frequent cancer adversely affecting women. According to GLOBOCAN, It accounts for 24.9% of cancer cases and is the first cause of cancer-related deaths among women (Ferlay et al., 2019).

The main clinical features for predicting the BC prognosis are age, primary tumor size, histological grade of tumor, distant metastasis, lymph node involvement, ER, and PR status (Ünçel et al., 2015). Despite the development of early detection techniques and advancements in therapy, metastasis from BC continues to be a major cause of mortality and morbidity (Ünçel et al., 2015). One of the key molecules in metastasis is extracellular matrix (ECM) elements which act as a primary set to block the accumulation of tumor cells. Degradation of the basement membrane enhances the metastatic process. For tumor cells to invade and metastasize, this barrier should be digested via MMPs (Matrix metalloproteinases) (Öncel, 2012).

MMP-2 is a member of the MMP family, and it can degrade type IV collagen, a component of basement membranes (Yadav et al., 2014). *MMP-2* facilitates tumor invasion and metastasis by digesting the basement membrane, which separates tumors from surrounding tissue. (Guo, Wu, Hathaway & Hartley, 2012). *MMP-2* has been associated with a variety of cancers, including breast, colon, skin, and lung cancers. Its expression has also been linked to tumor invasion, lymph node metastasis, and survival rates. One of the most effective BC prognostic indicators is *MMP-2* (Jezierska & Motyl, 2009).

Inactive MMPs are activated by proteolytic cleavage and are inhibited specifically by metalloproteinases (TIMP) tissue inhibitors. Up-to-date, four different TIMPs are described: TIMPs 1, 2, 3, and 4 (Arpino, Brck &, Jill, 2015). *TIMP-1* is a natural inhibitor of the *MMP-9*, which also plays an essential role in both normal physiological processes and carcinogenesis (Würtz, Schrohl, Mouridsen&Brünner, 2008) it, has a cancer-promoting effect via stimulating growth and inhibiting apoptosis. High *TIMP-1* expression has been associated with a poor prognosis in several cancers. *TIMP-1* has been suggested as a prognostic and predictive biomarker for BC (Würtz, Schrohl, Mouridsen&Brünner, 2008).

The association between MMP2 and BC's tumor growth, invasion, and metastasis has been corroborated (Guo, Wu, Hathaway & Hartley, 2012). *TIMP-1*, the inhibitor of *MMP-9*, has been extensively studied as a potential biomarker in BC. Overexpression of *TIMP-1* is associated with aggressive tumor behavior in many cancer types and breast cancer (Mahmood, Fakhoury, Yaseen & Moustafa, 2015).

Different techniques can be used to explore MMPs and their inhibitors at the transcriptional and protein levels. Gelatin zymography, ELISA, immunohistochemistry, in situ hybridization, and qRT-PCR is the most frequently used techniques in research (Hadler-Olsen, Winberg, &Uhlin-Hansen, 2013).

MMP-2 and *TIMP-1* were found to be upregulated according to the pooled microarray data (unpublished) from another study of our group by Cine et al. (Cine et al, 2014)

In this study, we analyzed the expression levels of *MMP-2* and *TIMP-1* by qRT-PCR in the same primary BC and adjacent non-tumor samples individually. We aimed to observe the potential biomarker role of *MMP-2* and *TIMP-1* for the prognosis of BC.

MATERIALS AND METHODS

Tissue collection

Seventeen samples from malign tumors and normal breast tissues were collected from patients who underwent surgery during diagnosis for BC at the Department of General Surgery, Kocaeli University, 2009-2010. This study was approved by the Kocaeli University Ethics Committee. (Approval no: 2008/76 IAEK 11/9.). Clinicopathological features of patients were retrieved from medical reports.

Total RNA isolation

Frozen tissue samples were divided for RNA isolation. Total RNA was extracted from tissue samples using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A260/280 ratios were measured to detect the purity of samples. The quality of RNA was confirmed by RNA LabChip (Agilent Technologies, Waldbronn, Germany) and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA integrity value of ≥6.10 was considered acceptable.

cDNA synthesis and qRT-PCR

Complementary DNA (cDNA) synthesis was performed with a commercial cDNA synthesis kit (Transcriptor First Strand cDNA synthesis kit, Mannheim, Germany, Roche). qRT-PCR (Syber Green, Mannheim, Germany, and Roche) experiment was applied as described previously to measure *MMP-2* and *TIMP-1* gene expression (Savlı, Aalto, Nagy, Knuutila & Pakkala, 2002; Savlı et al., 2003). Beta-actin was used as a housekeeping gene for normalizing gene expression values. Sequences of *MMP-2*, *TIMP-1*, and Beta-actin primers (Integrated DNA Technologies, Illinois, and USA) are shown in Table 1. Gene expression ratios were compared in tumor and non-tumor tissue relative expression software tools (REST ©, 2009, Qiagen, Hilden, Germany).

Statistical analysis

All data analysis was performed using GraphPad Prism version 7.3(GraphPad Software Inc., San Diego, CA). Statistical differences of at least $p<0.05$ were accepted as statistically significant.

Table 1. The sequences of primers used for RT-PCR.

GENE	PRIMER SEQUENCE
TIMP-1	
	(F) 5′ – CTT CTG GCA TCC TGT TGT TC- 3′
	(R) 5′ –AGA AGG CGG TCT GTG GGT – 3′
MMP-2	
	(F) 5′- CGC TCA GAT CCG TGG TGA G- 3′
	(R) 5′- TGT CAC GTG GCG TCA CAG T- 3′
BETA-ACTIN	
	(F) 5′-TGA CTT TGT CAC AGC CCA AGA- 3′
	(R) 5′-AAT CCA AAT GCG GCA TCT TC- 3′

The association between the expression of *MMP-2*, *TIMP-1*, and clinicopathological features was analyzed with the One-Way ANOVAs variance test.

RESULTS

Seventeen patients were aged from 38 to 73 years, with a mean of 52 years old. Of the 17 BC, 9 (52%) were classified as infiltrative ductal carcinoma, 5 (29%) were invasive ductal carcinoma, 1 (%5.8) was invasive micropapillary carcinoma, and 1 (%5.8) was ductal carcinoma in situ. One tumor sample remained unclassified. The clinical and pathological features of the patient group are listed in Table 2.

MMP-2 expression levels and clinical significance

We observed decreased *MMP-2* expression levels more frequently in tumor tissues than in non-tumor tissues (10/17). Relative expression values of all samples are shown in Figure 1. The relative expression levels of *MMP-2* with clinicopathologic parameters were compared and calculated p values were greater than 0, 05 for all parameters and are shown in Figure 2. Our study showed no statistical correlation between tumor grade, lymph node involvement, and hormone receptor status.

MMP-2 levels were compared within molecular subtypes, and Figure 4 shows the relative *MMP-2* levels of each molecular grade.

TIMP-1 expression levels and clinical significance

We observed decreased *TIMP-1* expression levels more frequently in tumor tissues than in non-tumor tissues (12/17). Relative expression values for all samples are shown in Figure 1. The relative expression levels of *TIMP-1* with clinicopathologic parameters were compared. Calculated p values were greater than 0, 05 for all parameters and are shown in Figure 3.

There was no statistically significant correlation found between the main clinical parameters and *TIMP-1* levels. *TIMP-1* levels were compared within molecular subtypes, and Figure 4 shows the relative *TIMP-1* levels of each molecular grade.

DISCUSSION

This study focused on detecting expression levels of *MMP-2* and *TIMP-1* in BC patients individually using qRT-PCR in breast

Table 2. *MMP-2* and *TIMP-1* expression levels and patients' clinicopathological data.

Pathological Data	Number of Patients	*MMP-2* Expression		*TIMP-1* Expression	
		Low	High	Low	High
Age					
≤50	9	4 (%44)	5 (%56)	5 (%56)	4 (%44)
≥50	8	5 (%62,5)	3 (37,5)	6 (%75)	2 (%25)
Histological Grade					
Grade 1	3	3 (%100)	-	2 (%66)	1 (%33)
Grade 2	3	1 (%33)	3 (%66)	2 (%66)	1 (%33)
Grade 3	7	4 (%57)	3 (%43)	5 (%71)	2 (%29)
No Information	4	1 (%25)	3 (%75)	4 (%100)	-
Lymph Node Metastasis					
Negative	7	5 (%71)	2 (%29)	4 (%57)	3 (%43)
Positive	10	4 (%40)	6 (%60)	8 (%80)	2 (%20)
ER Status					
Negative	7	3 (%43)	4 (%57)	6 (%84)	1 (%16)
Positive	10	6 (%60)	4 (%40)	6 (%60)	4 (%40)
PR Status					
Negative	11	5 (%45)	6 (%55)	8 (%72)	3 (%28)
Positive	6	4 (%66)	2 (%34)	2 (%34)	4 (%66)
CerbB2 Status					
Negative	8	4 (%50)	4 (%50)	4 (%50)	4 (%50)
Positive	9	5 (%56)	4 (%44)	8 (%87,5)	1 (%12,5)
Molecular Subtypes					
Luminal A	5	3 (%60)	2 (%40)	2 (%40)	3 (%60)
Luminal B	4	1 (%33)	2 (%66)	3 (%100)	-
Triple Negative	3	2 (%66)	1 (%33)	2 (%66)	1 (%33)
Her2/Neu	4	1 (%25)	3 (%75)	3 (%75)	1 (%25)

cancer and aimed to see if *MMP-2* and *TIMP-1* are prognostic biomarkers of BC.

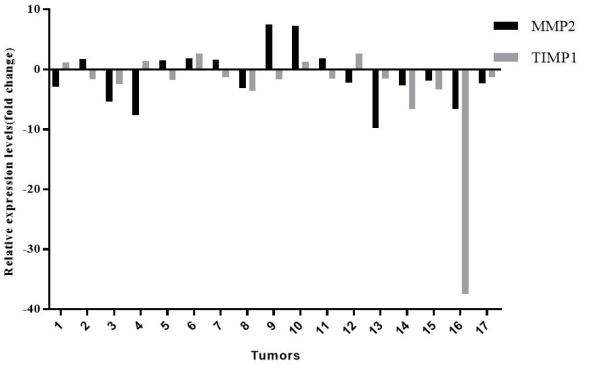

Figure 1. Column graphs demonstrate relative expression trends of *MMP-2* and TIMP-1 of each tumor.

In a systematic meta-analysis study by Chen et al., a considerable number of studies reported high expression levels of *MMP-2* in breast cancer tumors (Chen, Wang, Chen, Dong & Zhang, 2015). A study of qRT-PCR expression analyses revealed that *MMP-2* levels were significantly higher in breast cancer stages II-III than in benign breast tumor tissues (Mahmood et al., 2015). Figuera et al. observed high levels of *MMP-2* in tumor samples according to adjacent non-tumor tissue (Figueira et al., 2009). However, in our study, the mean expression levels of tumors were lower than controls.

Studies focused on the overexpression of *MMP-2* correlation with prognostic factors reported different association statuses with clinicopathological parameters. Chen et al. concluded in a systematic meta-analysis study that *MMP-2* overexpression was associated with lymph node metastasis and poor survival (Chen et al., 2015). Mahmood et al. showed that *MMP-2* expression levels were correlated with tumor grade, tumor stage,

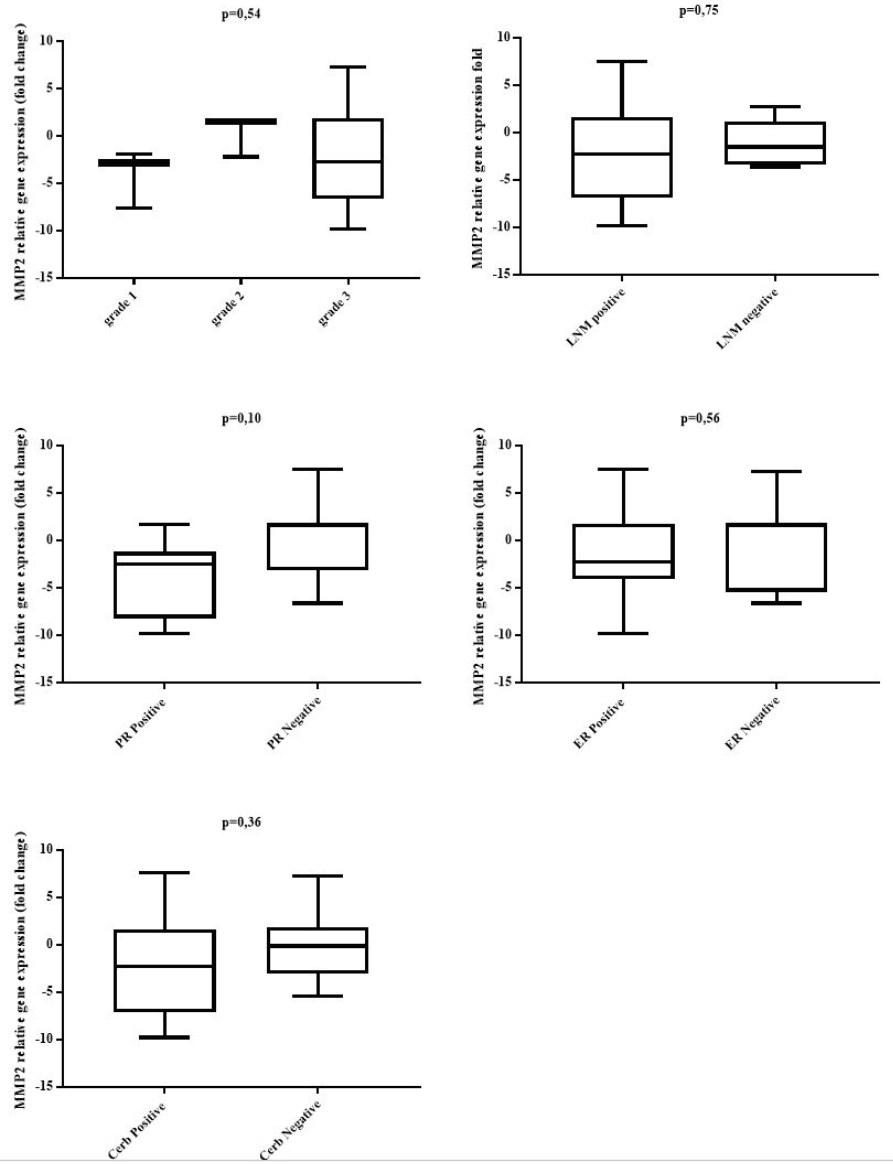

Figure 2. Box plots showing relative *MMP-2* expression trends and the clinicopathological parameters.

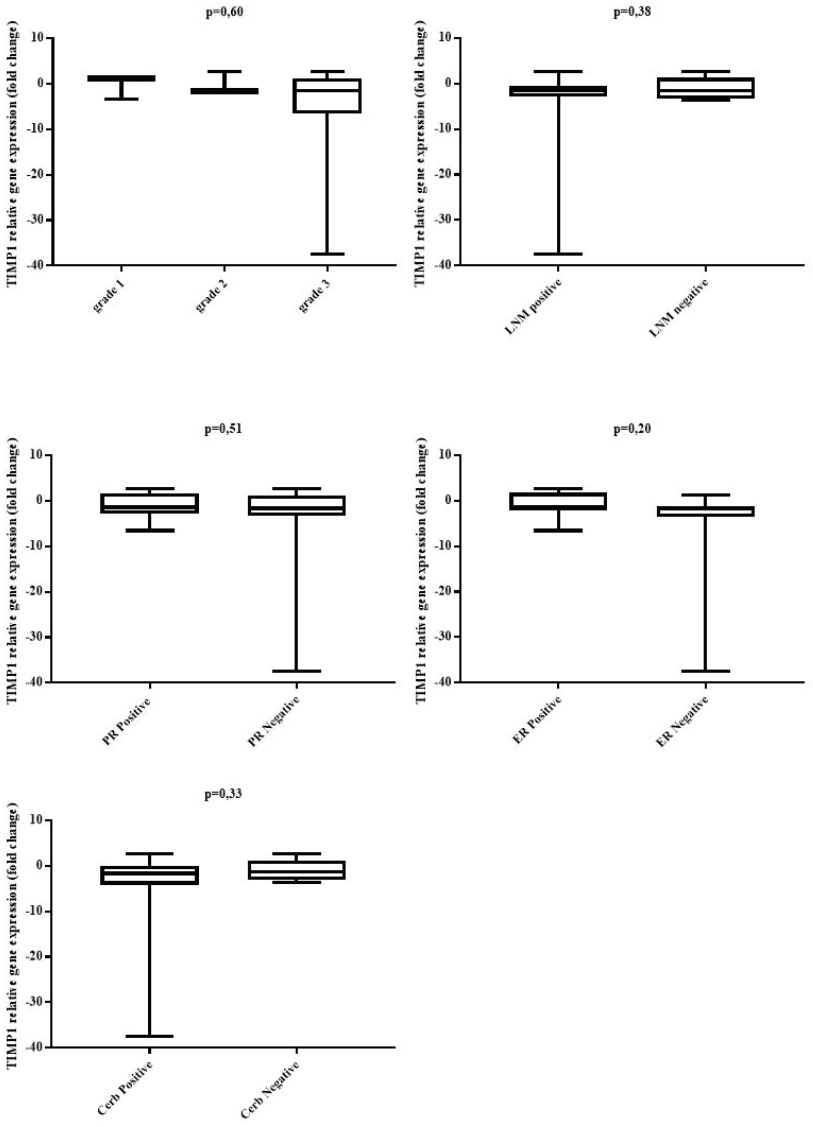

Figure 3. Box plots showing relative *TIMP-1* expression trends and the clinicopathological parameters.

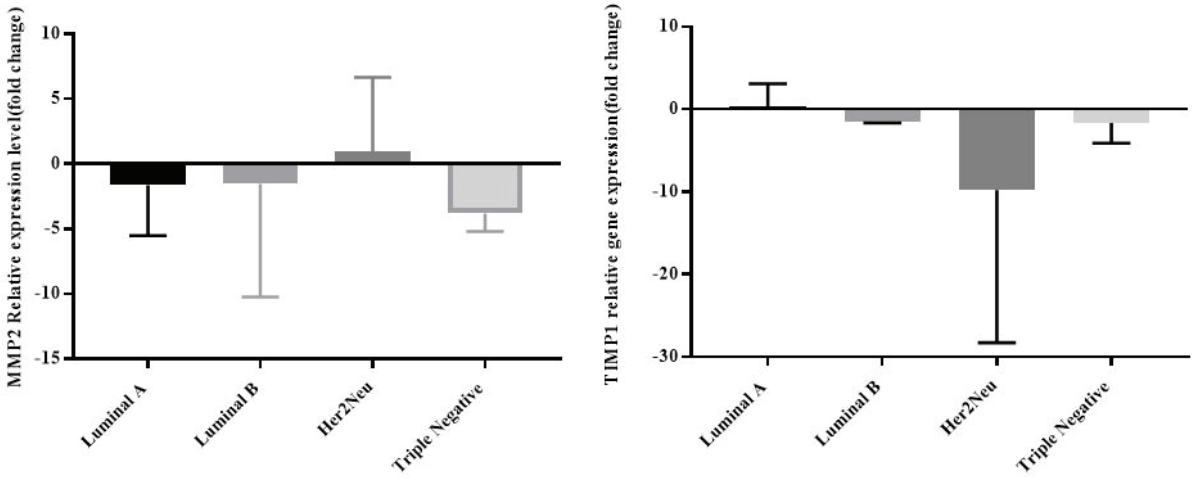

Figure 4. Box plots showing relative *MMP-2* and *TIMP-1* levels in each molecular grade. The line within the box plot represents the median value, and the lines extending from the box indicate the maximum and minimum expression levels.

and lymph node metastasis (Mahmood et al., 2015). Huang et al. measured serum *MMP-2* levels and reported a correlation between *MMP-2* and lymph node metastasis and higher TNM stage (Huang et al., 2014). Our study showed no statistical correlation between tumor grade, lymph node involvement, and hormone receptor status. Similar to our results, no correlation between *MMP-2* level and tumor grade, lymph node involvement, and ER/PR/Cerb-B2 status (Decock et al., 2005). These controversial reported results suggest that MMPs may provide independent prognostic foresight for BC progression.

MMP-2 has been extensively considered as a predictive biomarker for metastasis in BC. Both at the transcriptional level and protein level, most of the studies have reported an association between increased levels of *MMP-2* and lymph node metastasis (Huang et al., 2014). Similarly, to these reports, we found that the lymph node-positive group had higher *MMP-2* levels than the negative group. Daniele et al. looked at *MMP-2* expression in sentinel lymph nodes and serum in patients with metastatic and non-metastatic breast cancer. (Daniele et al., 2016). Their results showed that *MMP-2* was significantly decreased in the non-metastatic and control group compared to the metastatic group. These results emphasize the involvement of *MMP-2* in the metastatic process.

Despite the mean expression value of *MMP-2* being higher in the lymph node-positive group, a low level of *MMP-2* was observed in 4 patients.

An enzyme-linked immunosorbent assay (ELISA) study in lymph node-positive patients was performed by Leppaet al. (Leppa, Saarto, Vehmanen, Blomqvist&Eloma, 2004). They measured postoperative serum levels of *MMP-2* and followed five-year survival rates. They observed better overall survival (OS) and disease-free survival (DFS) rates in patients with low *MMP-2* levels; additionally, the frequency of bone and visceral metastasis was lower in the low *MMP-2* group than in the high *MMP-2* group. Their results suggested that the *MMP-2* level may be a predictive factor for DFS and OS and also may aid in dividing the node-positive group into two subgroups low risk and high risk. Thus, additional subgroups may be useful in identifying patients with a potentially favorable prognosis who could avoid toxic therapies. Although the negativity of lymph node involvement is a favorable prognosis factor in BC, some still suffer from metastasis. New prognostic biomarkers or subgroups are needed to overcome this dilemma. *MMP-2* has been considered a candidate prognostic biomarker and is associated with a favorable prognosis in node-positive BC (Daniele et al., 2016). Hirvonen et al. evaluated *MMP-2* expression by immunohistochemistry (IHC) staining in node-negative BC patients, and postoperative survival rates analysis was performed (Hirvonen, Talvensaari-Mattila, Pääkkö, &Turpeenniemi-Hujanen, 2003) Obtained data underlined potential correlation to MMP2 negativity and favorable prognosis in node-negative BC. Our sample group included seven lymph node-negative tumors, and 5 showed a low level of *MMP-2*. According to the study, these five patients should have better disease progression or relatively long survival than patients with high *MMP-2* levels. In the study of Lu et al., *MMP-2* expression levels were analyzed in primary tumors with metastasized lymph node samples by qRT-PCR in patients suffering invasive ductal BC (Lu, Chen, Ding, Li K, & Wu, 2012). The expression level of *MMP-2* in metastasized lymph nodes was higher compared with their primary tumors. These findings suggest that determining the level of *MMP-2* expression in metastatic tissue may help to clarify the role of *MMP-2* in lymph node metastasis. In all groups, the mean *TIMP-1* level was lower in tumors than in control tissues. Similar to our study, Figuera et al. found lower *TIMP-1* gene expression levels in tumors than in adjacent non-tumor tissues (Figuera et al., 2009)

Contrary to our study, Kousidou et al. and Zhang et al. reported higher expression levels of *TIMP-1* in BC tumors (Kousidou, Roussidis, Theocharis, &Karamanos, 2004; Zhang et al., 2013)

The correlation between main clinical parameters and *TIMP-1* levels was examined in this study, and no statistically significant correlation was discovered. Some authors reported an association between *TIMP-1* level and several clinicopathological parameters (Lipton et al., 2008; Sieuwerts et al., 2007, Figuera et al., 2009, Nakopoulou et al., 2002, Abdollahi et al., 2019). Lipton et al. showed the correlation between serum *TIMP-1* level and Cerb-B2 status, liver metastasis, and soft tissue metastasis (Lipton et al., 2008; Sieuwerts et al., 2007) found the relationship between tissue *TIMP-1* mRNA level and tumor size, lymph node involvement, tumor grade, age, ER/PR. Figuera et al. reported a significant correlation between *TIMP-1* and only PR status (Figuera et al., 2009). Nakopoulou et al. reported that high *TIMP-1* mRNA expression levels were associated with lymph node metastases and increased c-erbB-2 expression (Nakopoulou et al., 2002). Abdollahi et al. found *TIMP-1* gene expression levels in patients with lymph node metastasis and without metastasis using RT-PCR. They also emphasized the significant role of upregulated *TIMP-1* in lymph node involvement. (Abdollahi et al., 2019)

TIMP-1 levels in different samples, including tumor and serum, have been previously linked to a poorer outcome in BC at both transcriptional and protein levels (Nakopoulou et al., 2002; Würtz et al., 2008). In 2003, in contrast to those reports, they reported that an increased level of *TIMP-1* may be a sign of a favorable prognosis in BC (Nakopoulou et al., 2003).

Several publications investigated the prognostic value of *TIMP-1* in BC and showed a correlation between recurrence-free survival (RFS) (Dechaphunkul et al., 2012; Wu et al., 2008), DFS (Nakopoulou et al., 2003), OS (Nakopoulou et al., 2003; Dechaphunkul et al., 2012; Wu et al., 2008). In our study, increased *TIMP-1* expression was also observed in grade 1, lymph node-negative, and ER/PR/Cerb-B2 positive tumors, which were expected to present a better prognosis. Positivity of hormone receptors (ER/PR/Cerb-B2), low grade of the tumor (grade 1), and absence of lymph node involvement are considered favorable prognostic factors in BC. Patients with low risk are given less aggressive treatments. Talvensaari et al. conducted a prospective study on *TIMP-1* levels in serum using ELISA before surgery in primary node-negative patients (Talvensaari-Mattila &Turpeenniemi-Hujanen, 2005). Results suggested that cases with preoperative low TIMP-1 levels had longer RFS time than patients with high *TIMP-1* levels. Additionally, they found that

preoperative high serum *TIMP-1* elevated the risk of recurrence in primary node-negative breast carcinoma. In another study, Dechaphunkul et al. *TIMP-1* level was analyzed by IHC in early-stage primary BC patients treated with standard adjuvant therapy (Dechaphunkul et al., 2012). Increased *TIMP-1* levels in early-stage tumors were linked to early recurrence and short survival, according to their findings. These findings suggest that new independent prognostic factors for low-risk BC should be used to identify patients who require more aggressive treatment. The coexistence of high histological grades (grades 2 and 3) and lymph node involvement is considered a sign of high risk and poor prognosis, and more aggressive treatments are applied to patients with high risk.

To reduce the side effects of toxic chemotherapeutics, further effective prognostic biomarkers for the high-risk group are needed. Paula Kuvajaa and coworkers performed IHC staining of *TIMP-1* in node-positive and high-grade tumors (Kuvaja, Talvensaari-Mattila, Pääkkö, &Turpeenniemi-Hujanen, 2005). They discovered that the *TIMP-1* negative group had better disease-specific survival than the *TIMP-1* positive group. They hypothesized that lack of *TIMP-1* is associated with a better prognosis in patients with breast cancer due to *TIMP-1's* pro-apoptotic function. Our group examined low-level *TIMP-1* in 2 of 7 high-grade lymph node-positive samples. A more favorable prognosis is expected for this group.

CONCLUSION

In conclusion, alternative prognostic biomarkers for BC are required to clarify disease risk in both high-risk and low-risk groups. These prognostic markers may help enlarge subgroups related to previously defined risk groups. Our study and relevant reports have supported that *MMP-2* and *TIMP-1* are promising independent markers for proper risk stratification and predicting prognosis. Additionally, inhibition of metastasis-related biomarkers such as *MMP-2* and *TIMP-1* via drug-based inhibitors is a novel strategy for targeted therapy. Furthermore, we suggest that analyzing *MMP-2* and *TIMP-1* in different tissues with various methods at different times can be a part of the methodology approach for the diagnosis and follow-up.

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Author Contributions: Conception/Design of Study- H.S., N.Ç., N.Z.Ç.; Data Acquisition- S.A.G., T.Ç.; Data Analysis/Interpretation- D.S.A., S.E.K., M.Y.T.; Drafting Manuscript- D.S.A., S.E.K., M.Y.T.; Critical Revision of Manuscript- H.S., N.Ç., N.Z.Ç. S.A.G., T.Ç .; Final Approval and Accountability- D.S.A., S.E.K., M.Y.T., S.A.G., T.Ç.

Conflict of Interest: The authors have no conflict of interest to declare.

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