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Association of Toll-Like Receptor 9 Expression with Prognosis in Breast Carcinoma

Meme Karsinomlarında Toll-Like Reseptör 9 Ekspresyonunun Prognozla İlişkisi

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Abstract

Aim: Breast cancer (BC) is the most common malignancy in women. Some molecules including TLR9 are still under investigation as potential prognostic factors in BC. In the present study, we aimed to determine the relation between TLR9 expression and clinicopathological prognostic parameters and survival in BC.

Material and Method: One hundred and thirty nine patients diagnosed as BC included the present study. Immuno-reactivity scoring (IRS) system was used to reveal the tissue TLR9 expression levels.

Results: We found higher TLR9 expression in tumors diagnosed as invasive carcinoma NOS, grade 3 tumors, tumors with necrosis, ER negative and Her2 positive tumors and triple negative molecular subtype. Furthermore, tumors with low TLR9 scores showed increased overall survival compared to tumors with high TLR9 scores.

Conclusion: TLR9 overexpression in BC is associated with some prognostic parameters including histologic type, tumor grade, tumor necrosis, ER and Her2 status and molecular subtype as well as overall survival. Further studies with larger patient series are needed to shed light on the use of TLR9 as a clinical and therapeutic target in BC.

Keywords: Breast cancer, pathology, TLR9, immunohistochemistry, prognosis

Öz

Amaç: Meme kanseri (MK) kadınlarda en sık görülen malignitedir. Toll-like reseptör 9 (TLR9) dahil bazı moleküller, MK'de potansiyel prognostik faktörler olarak halen araştırılmaktadır. Bu çalışmada, MK'de TLR9 ekspresyonu ile klinikopatolojik prognostik parametreler ve sağkalım arasındaki ilişkiyi belirlemeyi amaçladık.

Gereç ve Yöntem: Bu çalışmaya MK tanısı konulan 139 hasta dahil edildi. Doku TLR9 ekspresyon seviyelerini ortaya koymak için immüno-reaktivite skorlama (IRS) sistemi kullanıldı.

Bulgular: İnvaziv karsinom NOS tanısı alan tümörlerde, derece 3 tümörlerde, nekrozlu tümörlerde, Östrojen reseptörü (ER) negatif ve Human epidermal growth factor 2 (Her2) pozitif tümörlerde ve üçlü negatif moleküler alt tipte TLR9 ile yüksek düzeyde ekspresyon tespit edildi. Ayrıca, TLR9 skoru düşük olan tümöre sahip hastaların genel sağkalımı, yüksek TLR9 skoru olan tümörlü hastalara kıyasla daha fazla idi.

Sonuç: MK'de TLR9 aşırı ekspresyonu, genel sağkalımın yanı sıra histolojik tip, tümör derecesi, tümör nekrozu, ER ve Her2 durumu ve moleküler alt tip gibi bazı prognostik parametrelerle ilişkilidir. TLR9'un MK'de klinik ve terapötik bir hedef olarak kullanımına ışık tutmak için daha geniş hasta serileri ile daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: Meme kanseri, patoloji, TLR9, immunohistokimya, prognoz

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INTRODUCTION

Breast cancer (BC) is the most common malignancy in women with increased mortality rates in the last 50 years worldwide.[1,2] Though well-known prognostic parameters exist in BC, it is difficult to predict the biological behavior since BC is a heterogeneous disorder consists of complex pathologic entities.[3]

Toll-like receptors (TLRs) are pattern recognition receptors primarily expressed by cells of the immune system as well as epithelial tumor cells.[4] Recent studies have also demonstrated expression of TLR9, one of the TLRs, in various normal epithelial cells and in cancer cells, including breast, brain, gastric, lung and prostate cancers.[5,6]

TLRs activate the production of many biological factors, causing an inflammatory response and inducing type 1 interferons and other cytokines that activate the adaptive immune system.[3,7]

TLRs are like a double-edged sword that exhibits both antitumor and protumor activities with many features, and further studies are needed to understand their effects in the tumor mechanism. A better understanding of the working mechanism and effects of this promising structure will enable us to use the relevant existing treatments in more appropriate combinations and in appropriate cases, to prevent future undesirable effects and wrong treatments, and to better plan new treatments to be developed.

The purpose of this study is to investigate the relationship of TLR9, which has a remarkable place in cancer researches recently due to its relationship with tumor progression, with prognostic parameters in BC.

MATERIAL AND METHOD

The study was approved by Selçuk University Ethics Committee (Date: 13.01.2021, Decision no:2021/13). One-hundred and thirty nine consecutive patients with BC who underwent modified radical mastectomy between January 2009 and December 2018 were included in this study. Relevant data such as age, tumor diameter, multifocality, tumor stage, lymph node metastasis, distant metastasis, survival, and hormone receptor status were obtained from patient records. Hematoxylin-eosin stained pathology preparations were examined by 2 pathologists to evaluate pathological prognostic parameters such as histological subtype, tumor grade and presence of tumor necrosis, and to determine the appropriate tissue block for immunohistochemical staining.

The clinical and pathological staging of the cases was re-evaluated according to the American Joint Cancer Committee (AJCC) 2018 TNM BC staging system.

The molecular subtypes of the cases were determined according to the ki67 proliferation index, Her2 and hormone receptor status of the tumors. Accordingly, five different subtypes were obtained. Luminal subtype (A and B) is hormone receptor positive BC. The most common type is luminal A, which is low grade, has a low ki67 score, and has a good prognosis. Luminal B subtype expresses more proliferation and Her2 gene and less ER related gene. This subtype can be divided into two categories based on Her2 positivity or negativity. Her2 and triple-negative BC subtypes are high-grade and more aggressive types and show a high risk of systemic and local recurrence.[1]

To evaluate the association of TLR9 expression with progression-free survival (PFS) and overall survival (OS), tumors were analyzed in 6 categories, 1, 2, 3, 4, 6 and 9 based on IRS scores. Progression-free survival was defined as the interval from the date of completion of primary therapy to the date of clinical or radiological evidence of recurrent disease (confirmed by biopsy). Overall survival (OS) was accepted as the time from the date of diagnosis to death or last follow-up, without any restriction on the cause of death. These were calculated from follow-up records and the National Death Registry, last checked on December 20, 2018.

Immunohistochemical Staining Procedure

Sections were deparaffinized for 1 hour in an oven at 60°C. Immunohistochemical staining was performed using an automatic staining machine (Sequenza Immunostaining Center Each 73300001 Shandon / Thermo). For antigen retrieval, 1/10 diluted EDTA Buffer (PH: 8) (AP-9004-999 Thermo scientific) was applied in the PT Module (A80400012 Lab Vision). Sections were washed with PBS for 5 minutes.

Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide (TA-125-HP ThermoScientific). After washing with PBS for 10 minutes, protein blocking (TA-125-PBQ ThermoScientific) was performed and then sections were incubated for 60 minutes with a 1:100 dilution of primary antibody: anti-TLR9 antibody (ab37154, Abcam). Then the sections were incubated with Amplifier Quanto (TL-125-QPB ThermoScientific) for 20 minutes and with HRP Polymer Quanto (TL-125-QPH ThermoScientific) for 30 minutes, respectively. Washing was done with PBS at each step. To identify positive cells, staining was performed with DAB chromogen and finally counterstained with hematoxylin for 30 seconds. Spleen tissue was considered as positive control.

Evaluation and Scoring of Immunohistochemically Stained Slides

Preparations stained with immunohistochemical TLR9 were evaluated and scored by a pathologist who was blinded to the patients' data. In the light microscopic evaluation, the entire cross-section of the glass was reviewed at 100X magnification. The IRS system was used to determine TLR9 expression levels. This system,[8]

previously used by Wang et al., was dependent on staining intensity and percentage of positive cells. The IRS system is explained in **Table 1**.

Table 1: Immunorectivity scoring (IRS) system of TLR9

Percentage of Positive Cells (A)	Staining Intensity (B)
0: No positive cells	0: Negative
1: 1% to 33% positive cells	1: Mild
2: 34% to 66% positive cells	2: Moderate
3: 67% to 100% positive cells	3: Severe

Statistical Analysis

SPSS v25.0 package program was used for statistical analysis. The Kolmogorov-Smirnov Z test was used to test the normality of the distribution. Parametric analysis methods were used for normal distributions, non-parametric tests were preferred for abnormal distributions. Independent t-test was used to determine statistical significance when two groups were compared. Analysis of Variance (ANOVA) and non-parametric Kruskall-Wallis tests were used to compare the mean scores of more than two groups. Statistical significance was considered as $p<0.05$. PFS (progression-free survival) was defined as the time from diagnosis to recurrence or progression. OS (disease-free survival) was measured from the date of enrollment to the date of death from any cause. OS and PFS were estimated using the Kaplan-Meier method.

RESULTS

One-hundred and thirty nine patients diagnosed with BC were included in this study. All patients were female and the mean age of the patients was 55 (24-88).

We observed cytoplasmic staining in tumor cells with TLR9 (**Figure 1, 2**). TLR9 expression was reduced in normal acini compared to tumor cells (**Figure 1**).

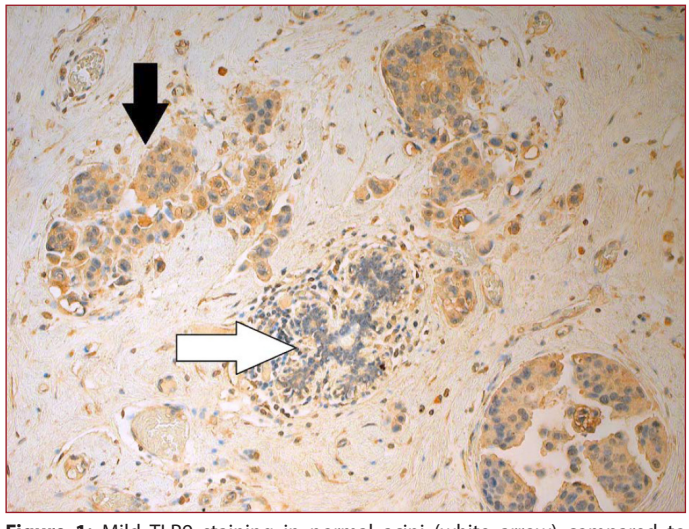

Figure 1: Mild TLR9 staining in normal acini (white arrow) compared to adjacent tumor tissue (black arrow) (TLR9, Original Magnification, X200).

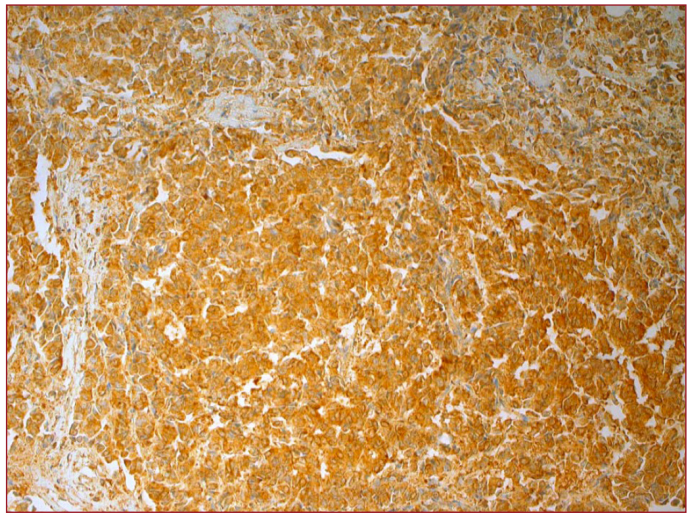

Figure 2: Strong and diffuse cytoplasmic TLR9 staining in a grade 3 tumor (TLR9, Original Magnification, X200).

Clinicopathological features of the patients are summarized in **Table 2**. One-hundred and seventeen cases were diagnosed with invasive carcinoma, not otherwise specified (NOS) and its variants. Of the variants, 4 (3.4%) were carcinoma with apocrine differentiation, 2 (1.7%) were invasive carcinoma with neuroendocrine differentiation. Other invasive carcinoma category included 13 (11.1%) invasive lobular carcinoma, 2 (1.7%) invasive papillary carcinoma, 2 (1.7%) mucinous carcinoma, 1 (0.8%) secretory carcinoma, 2 (1.7%) medullary carcinoma and 2 (1.7%) tubular carcinoma cases. A statistically significant difference was found between the histological subtypes of breast carcinoma in terms of TLR9 expression, and TLR9 expression was found to be significantly higher in patients with invasive carcinoma (NOS) than other invasive carcinomas (p=0.027).

Thirty-six (25.8%) of the cases were grade 1, 68 (48.9%) grade 2, 36 (25.8%) grade 3. TLR9 expression was found to be significantly higher in grade 3 tumors than in grade 1 and grade 2 tumors (p=0.001).

Twenty tumors had necrosis. Tumors with necrosis showed higher TLR9 expression than tumors without necrosis (p=0.03)

Twenty-seven (19.4%) of the cases were estrogen receptor (ER) negative, 27 (19.4%) were under 50% expression with ER, and 85 (61.1%) were over 50% with ER. A statistically significant correlation was found between the ER status of the cases and TLR9 expressions. TLR9 expression was found to be significantly higher in ER negative group than in the other two groups (p=0.004).

Seventy-eight (56.1%) of the cases were Her2 negative and 61 (43.9%) were Her2 positive. A statistically significant correlation was found between Her2 status and TLR9 expressions of the cases, and TLR9 expression was found to be significantly higher in the Her2 positive group than in the Her2 negative group (p=0.015).

Table 2: Relation of TLR9 expression with clinicopathological characteristics

Parameter	n	Mean IRS (±std)	P-value
Age (years)			0.131
<55	72	4.83±2.188	
≥55	67	5±2.146	
Histologic subtype			0.027
Invasive carcinoma (NOS) and variants	118	5.09±2.192	
Other invasive carcinomas	21	3.90±1.7	
Tumor grade			0.001
1	36	4.58±1.746	
2	68	4.28±2.094	
3	35	6.49±1.931	
DCIS			0.078
Absent	72	5.24±2.236	
Present	67	4.57±2.039	
Pathological T Stage			0.102
T1	45	4.3±0.286	
T2	73	5.30±0.257	
T3	19	4.68±0.502	
T4	2	6.5±2.50	
Pathological N Stage			0.345
N0	49	4.69±0.293	
N1	40	5.4±0.387	
N2	29	4.45±0.353	
N3	21	5.14±0.459	
Distant metastasis			0.962
M0	122	4.91±0.198	
M1	17	4.94±2.015	
Clinical stage			0.053
1	78	4.55±2.043	
2	26	6±2.4	
3	24	4.83±1.926	
4	11	5.09±2.3	
Tumor diameter			0.1
<2 cm	25	4.24± 1.832	
≥2 cm	114	5.06±2.207	
Molecular subtype			0.018
Luminal A	41	4.73±2.0.37	
Luminal B (Her2 negative)	31	3.94±1.999	
Luminal B (Her2 positive)	47	5.34±2.150	
Her2-rich	14	5.64±2.134	
Triple negative	6	6.17± 2.483	
Tumor focality			0.740
Unifocal	121	4.93±2.186	
Multifocal	18	4.78±2.045	
Tumor necrosis			0.030
Absent	20	4.73±2.04	
Present	119	6±2.406	
Vascular invasion			0.292
Absent	42	4.62±2.141	
Present	97	5.04±2.169	
Extracapsular extension			0.175
Absent	101	4.76±0.219	
Present	38	5.32±0.327	
Estrogen receptor			0.004
Negative	27	5.78±2.082	
<%50	25	5.58±2.120	
≥%50	85	4.44±2.084	
Progesteron receptor			0.148
Negative	45	5.18±2.149	
<%50	36	5.22±2.044	
≥%50	58	4.5±2.226	
Her2			0.015
Negative	78	4.53±2.118	
Positive	61	5.41±2.132	
Ki67			0.310
<%14	45	4.62±2.208	
%14-49	27	4.48±1.929	
≥%50	67	4.70±2.263	

n: Number of cases, Std: Standart deviation, NOS: Not otherwise specified, DCIS: Ductal carcinoma in situ

A statistically significant relationship was also found between molecular subtypes and TLR9 expressions (p=0.018). The lowest TLR9 expression was found in the luminal B Her2 negative group, and the highest expression was found in the triple-negative group. Furthermore, TLR9 expression was found to be significantly higher in the luminal B Her2 positive group and group 4, Her2 positive groups, compared to the luminal B Her2 negative group.

No statistically significant relationship was determined between TLR9 expression and tumor diameter and pathological tumor stage (p=0.1 and 0.102, respectively).

Table 3 summarizes the association of TLR9 expression with the site of metastasis. No statistically significant correlation was determined between TLR9 expression and metastasis site (p> 0.05).

Table 3: Relation of Site of Metastasis With TLR9 Expression

Parameter	n	Mean IRS (±std)	P- value
Bone metastasis			0,501
Absent	128	4,88±2,163	
Present	11	5,36±2,203	
Lung metastasis			0,739
Absent	135	4,93±0,187	
Present	4	4,5±0,866	
Liver metastasis			0,828
Absent	137	4,92±2,170	
Present	2	4,5±2,121	
Adrenal gland metastasis			0,290
Absent	136	4,89±2,180	
Present	3	6±0,01	

n: Number of cases, Std: Standart deviation.

Median follow-up was 54 (1-120) months. To evaluate the relationship of TLR9 expression with PFS and OS, cases were categorically evaluated as IRS 1, 2, 3, 4, 6 and 9. Accordingly, the mean OS in the groups with TLR9 scores 1 and 2 was significantly higher than those with TLR9 scores 3, 4, 6 and 9 (p=0.014). In terms of PFS, no difference was observed between TLR9 scores (p> 0.05) (**Table 4**, **Figure 3, 4**).

Table 4: Comparison of PFS and OS of patients in different TLR9 categories.

	TLR9 Score	Mean	Std. Error	Median	Min	Max	IQR	P*
PFS	1	99,17	22,931	121,50	6	147	101	0,166
	2	108,00	9,256	117,00	63	146	67	
	3	81,97	5,691	80,00	0	143	46	
	4	86,27	15,716	109,00	5	139	88	
	6	84,02	4,289	67,00	12	150	56	
	9	80,87	9,937	98,00	0	135	43	
OS	1[a]	120,50	13,635	129,50	56	147	42	0,014
	2[a]	112,92	9,063	128,00	63	146	61	
	3[b]	82,53	6,495	66,50	14	148	49	
	4[ab]	83,00	15,356	66,00	2	139	88	
	6[b]	82,65	3,767	68,00	5	148	48	
	9[ab]	85,87	5,872	97,00	54	121	38	

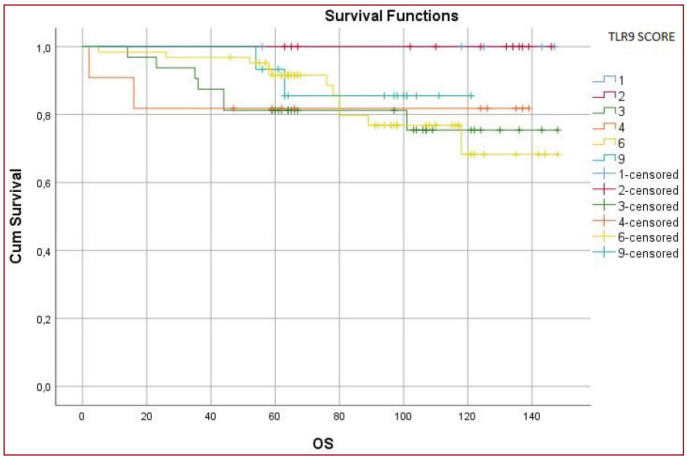

Figure 3: Kaplan Meier curves for overall survival (OS) of TLR9 expressions.

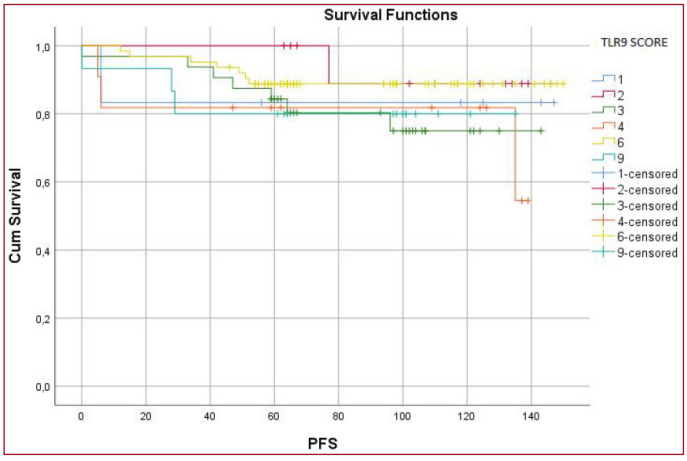

Figure 4: Kaplan Meier curves for progression free survival (PFS) of TLR9 expressions.

DISCUSSION

In the present study, we analyzed immunohistochemical expression of TLR9 in 139 BC patients, which is one of the largest patient series in the literature, and revealed that TLR9 expression is associated with some prognostic parameters such as histologic type, tumor grade, presence of tumor necrosis, ER and Her2 status, molecular subtype as well as overall survival in BC.

It is well known that cancer and inflammation are related entities, consequently, persistent inflammatory events can lead to carcinogenesis.[3,9-12] In the current literature, several studies exist to investigate relation of TLRs with both inflammation and cancer. TLRs are important components of the innate immune system not only defense host against several infectious agents, but also shown to be expressed by many epithelial tumor cells including BC.[4,11]

Among TLRs, TLR9 has shown to be highly expressed in BC cells and capable of promoting cellular invasion in vitro by increasing matrix metalloproteinase activity.[13,14] It was revelad that TLR9 assists in the progression of BC and patients with BC have higher circulating levels of TLR9 compared to normal.[11,13]

In their study, González-Reyes et al.[3] suggested that BCs with high TLR9 expression by fibroblast-like cells were associated with low probability of metastasis. In contrast, Jukkola-Vuorinen et al.[5] revealed that BCs metastasized to axillar lymph nodes at the time of diagnosis had slightly higher TLR9 expression compared with tumors with no axillary lymph node metastasis. Similarly, Qiu et al.[13] found that TLR9 expression was significantly higher in patients with BC displaying lymph node metastasis or advanced pathological stage. In our study, no relation was detected between TLR9 expression and lymph node metastasis status and stage, as well as distant metastasis.

We also evaluated the possible relationship between TLR9 expression and tumor grade, histologic and molecular subtypes, and hormone receptor status. Invasive carcinoma (NOS) subtype, formerly known as invasive ductal carcinoma, showed higher TLR9 expression compared to other subtypes. This finding differed from Jukkola-Vuorinen et al. study, who revealed the highest TLR9 expression levels in tumors with mucinous morphology.[5] In the same study, grade 3 tumors had higher TLR9 expressions than lower grade tumors as revealed by Meseure et al.[4] Our results regarding to relation of TLR9 expression with tumor grade was consistent with the literature and highest in grade 3 tumors.

Although very little is known about the relationship between TLR9 expression and ER function, previous studies revealed that estradiol and especially progesterone inhibits TLR9-mediated inflammation in both human and mouse plasmocytoid dendritic cells.[5,15] It was also suggested that these steroids could affect downstream signaling proteins in the TLR pathway.[15] In a previous study, it was shown that ER-negative tumors had higher expression with TLR9 compared to ER-positive tumors.[5] In another study, high TLR9 levels were associated with favorable outcome in triple-negative tumors.[4] In our study, similarly, higher TLR9 expression was detected in ER-negative tumors compared to ER-positive groups but on the contrary, the highest TLR9 expression was present in triple-negative subgroup, supporting that high TLR9 expression is a poor prognostic parameter in BC.

According to the current literature, there is also a relationship between TLR9 expression and the survival of patients with BC. Tuomela et al.[16] suggested that low tumor TLR9 expression is associated with significantly shortened disease-specific survival in patients with triple-negative BC. Meseure et al.[4] revealed better metastasis free survival in triple-negative BC patients with higher TLR9 levels. However, in the present study, low TLR9 expression was also found to be associated with better overall survival, supporting the association of other favorable prognostic parameters with low TLR9 expression in BC.

There are also some limitations to our study. This study was conducted in a single center with patients who did not have uniform or randomized treatment or follow-up. Prospective studies with larger patient series are needed to use TLR9 as a prognostic biomarker in BC.

CONCLUSION

TLR9 expression in BC is associated with some prognostic parameters such as the histologic type of tumors, histologic grade, presence of tumor necrosis, ER and Her2 status, molecular subtype as well as overall survival. Thus, we can conclude that low TLR9 expression is associated with better prognosis in patients with BC. Further studies with larger patient series are needed to shed light on the use of TLR9 as a clinical and therapeutic target in BC.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was approved by Selçuk University Ethics Committee (Date: 13.01.2021, Decision no:2021/13)

Informed Consent: Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Referee Evaluation Process: Externally peer-reviewed.

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

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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