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The value of potential immunohistochemical biomarkers and clinicopathological findings in predicting response to neoadjuvant chemotherapy in breast cancer

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Abstract. – OBJECTIVE: Following neoadjuvant chemotherapy (NAC) in breast cancer (BC), complete treatment response is achieved in some patients, while treatment response is limited in others. Predicting non-responder patients can prevent exposure to adverse effects associated with chemotherapeutic agents and delays in selecting other treatment modalities. In this study, we aimed at identifying predictive factors related to tumor regression in patients with BC who received NAC.

PATIENTS AND METHODS: This single-center cohort included 91 patients with BC who underwent surgery following NAC based on pretreatment tumor biopsy. According to BC molecular subtype, tumor regression grade (TRG) was determined using the Miller-Payne scoring system in patients who received standard NAC. Immunohistochemical stainings for VEGFR3 and CD44 were applied to needle core biopsies obtained prior to NAC in these patients.

RESULTS: Pathological complete response (pCR) was achieved in 20 patients (22%). In univariate analysis, high Ki-67 expression, ER negativity, and HER2 positivity were determined to be predictive factors of TRG ($p < 0.05$). In multivariate analysis, Ki-67 was the single independent predictor of TRG, with a 1.05-fold effect size. CD44 and VEGFR3 levels did not affect TRG or survival ($p > 0.05$). There was a significant difference in TRG according to molecular subtype of BC ($p < 0.001$). The treatment response was 5.5-fold higher in HER2-positive patients compared with HER2-negative patients.

CONCLUSIONS: pCR rates were significantly higher in TNBC, HER2, and luminal HER2+ subtypes when compared with luminal HER2- subtype. Ki-67 >25% and ER negativity had a favorable effect on TRG after NAC. CD44 and VEGFR3 were not effective in predicting treatment response.

Key Words:
Breast cancer, Neoadjuvant chemotherapy, Tumor regression grade, CD44, VEGFR3, Hormone receptors, CerbB2/HER2, Ki-67, Molecular classification.

Introduction

Breast cancer (BC) is one of the most common cancers in women; it is the second most common cause of cancer-specific mortality in women worldwide[1]. BC with different biological and clinical behavior is a heterogeneous disorder classified into several subtypes based on histopathological type and molecular behavior[2].

Currently, it is known that neoadjuvant chemotherapy (NAC) in BC treatment is equivalent to adjuvant therapy[3]. The primary goal of NAC is to enhance the likelihood of breast-sparing surgery *via* regression of the size of the primary tumor. Furthermore, it may improve the quality of life by acting on metastatic lesions and reducing the need for axillary dissection in patients with lymph node metastasis[3-6]. Previous studies[7,8] have suggested that treatment response after NAC is strongly correlated with survival and prognosis.

While this treatment modality improves the quality of life in patients, a significant disadvantage is our inability to predict treatment response after NAC. Following NAC, a complete treatment response is achieved in some patients, while a treatment response is limited in others. The inability to predict treatment response leads to exposure to ineffective chemotherapy regimens and adverse effects in non-responders[9]. When treatment response can be predicted, the non-responders can

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directly continue with surgical treatment[7]. The search for new biomarkers to evaluate the diagnosis and prognosis of BC continues[10]. Several studies[7,10-13] have used molecular tumor type, tumor volume, histological grade, PDL1 expression, tumor-infiltrating leukocytes, and other biomarkers to predict tumor regression. In addition, several radiological imaging modalities have been investigated regarding their effectiveness in monitoring treatment response in patients[14,15]. However, there is no consensus on the prediction of tumor regression grade (TRG). Given the lack of predictive factors for treatment response following NAC, it is unclear how to identify patients with the highest likelihood of pCR.

In this study, we aimed at investigating the role of cluster of differentiation 44 (CD44, a cancer stem cell marker), vascular endothelial growth factor receptor 3 (VEGFR3, an angiogenic marker), estrogen receptor (ER), progesterone receptor (PR), HER2, Ki-67, histological grade, stage, and molecular subtype in predicting TRG following NAC.

Patients and Methods

Data and Sources

We identified patients diagnosed with BC between January 2015 and January 2021 from pathology archives using the electronic database of Kayseri Training and Research Hospital. Patients who underwent core needle biopsy before treatment, underwent surgery after NAC, and were followed up and treated at Kayseri Training and Research Hospital were included in the study. Overall, we included 91 patients fulfilling the inclusion criteria. Core needle slides obtained before NAC and slides obtained during surgery were re-assessed. This study was approved by the Kayseri City Training and Research Hospital Clinical Research Ethics Committee (Protocol No.: 651/2022). Informed consent was provided by each patient participating in the study.

Histological Analysis and Staging

Histological grade and tumor type were determined *via* core needle biopsies. Histological tumor grade was evaluated using the Nottingham modification of the Bloom-Richardson criteria[16]. ER, PR, and Ki-67 status were assessed by immunohistochemistry (IHC). The routinely stained IHC slides were re-evaluated. Nuclear staining in more than 1% of tumor cells was considered positive for ER and PR. Ki-67 expression levels were expressed as the percentage (%) of cells with positive nuclear staining out of the total number of tumor cells. HER2 positivity (a score of 3+ membranous) was defined as strong, complete membrane staining in more than 10% of tumor cells; scores of 0 and 1+ were considered to be negative. The dual *in situ* hybridization (ISH) slides of cases with HER2 equivocal (score 2+) were re-assessed. The results were considered according to the recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). ISH results were assessed according to the ASCO/CAP Clinical Practice Guideline Focused Update[17].

Based on the results, patients were classified into molecular subtypes:

- Luminal A: ER+, HER2-, Ki-67 <15%; Luminal B: ER+, HER2- and Ki-67 ≥15%. These subtypes were categorized as luminal HER2-.
- Luminal HER2+: ER+, HER2+
- HER2+: ER-, PR-, HER2+
- Triple-Negative Breast Cancer (TNBC): ER-, PR- and HER2-

The following treatment regimens were given to the patients based on molecular subtypes:

- Luminal HER2- patients: four cycles of doxorubicin (Adriamycin) plus cyclophosphamide every 3 weeks, followed by paclitaxel for 12 weeks.
- Luminal HER2+ and HER2+ patients: four cycles of doxorubicin (Adriamycin) plus cyclophosphamide every 3 weeks, followed by paclitaxel plus trastuzumab plus pertuzumab for 12 weeks (trastuzumab and pertuzumab were given every 3 weeks).
- TNBC patients: four cycles of doxorubicin (Adriamycin) plus cyclophosphamide every 3 weeks, followed by paclitaxel for 12 weeks.

In all groups, the standard duration of NAC was 6 months. The patients underwent surgery within 4-6 weeks following the completion of neoadjuvant therapy.

To stain CD44 and VEGFR3 monoclonal antibodies in core needle biopsies obtained before treatment, 4-µm thick sections from paraffin-embedded formalin-fixed tissues were placed on lysine slides. The slides were deparaffinized in xylene. CD44 (1/50 dilution; Thermo Scientific, Lab Vision Corporation Fremont, USA) and VEFGR3 (1/70 dilution 60 minutes; Leica Biosystems, Novocastra Liquid Mouse monoclonal antibody, United Kingdom) monoclonal antibody

staining was performed using an automated staining device. Tonsillar tissue was used as a positive control for CD44, while placental tissue was used as a positive control for VEGFR3. The stained slides were assessed by a pathologist (EA) using a binocular microscope (Olympus BX53).

Cytoplasmic and membranous staining for CD44 was evaluated. During data analysis, the CD44-positive stained cells in the slides were divided into two groups: low-rate group (less than 80% of cancer cells were positive) and high-rate group (more than 80% of the cancer cells were positive). The intensity of CD44 staining was categorized into two groups: weak and strong[18].

Cytoplasmic staining was considered when assessing VEGFR3. During data analysis, the VEGFR3-positive stained cells in the slides were divided into two groups: low-rate group (less than 50% of cancer cells were positive) and high-rate group (more than 50% of the cancer cells were positive). The intensity of VEGFR3 staining was categorized into two groups: weak and strong.

Anatomical stage and BC subgroups were defined according to the American Joint of Cancer Classification System[19]. The Miller-Payne Tumor Regression Grade system was used to assess treatment response following NAC[20]. Patients with metastasis to lymph nodes despite lack of invasive cancer in the breast were categorized as Grade 4.

Statistical Analysis

The normal distribution of data was assessed using histograms, Q-Q plots, and the Shapiro-Wilk test. Categorical variables were compared using Pearson's χ^2 and Fisher's exact analyses. Kaplan-Meier curves (survival curves) expressed the likelihood of survival over time. Univariate and multivariate Cox regression analyses were used to assess the effects of the studied variables on survival. Variables found to be significant in univariate analysis ($p < 0.25$) were included in the multivariate model; independent risk factors for survival were investigated by forward selection using odds ratio. Univariate and multivariate logistic regression analyses were used to identify risk factors for neoadjuvant therapy. Variables found to be significant in univariate analysis ($p < 0.25$) were included in the multivariate model; independent risk factors were investigated by forward selection logistic regression. The risk ratio was presented with a 95% confidence interval. Data were analyzed using R 4.0.0 (available at: www.r-project.org). The level of significance was established at $p < 0.05$.

Results

All patients were female. The mean age at diagnosis was 50.24 ± 12.61 years (24-76 years). Of the patients, 45 (49.5%) were younger than 50 years old, while 46 (50.5%) were older than 50 years old.

Invasive ductal carcinoma was diagnosed in 79 patients (86.8%), invasive lobular carcinoma in 7 patients (7.7%), and miscellaneous carcinoma in 5 patients (5.5%; including 1 primary neuroendocrine carcinoma of the breast, 1 invasive papillary carcinoma, 1 micro-papillary carcinoma, 1 metaplastic carcinoma of breast, and 1 mix ductal and mucinous carcinoma). The tumor was localized to the right breast in 47 patients (51.6%), left breast in 43 patients (47.3%), and bilateral in 1 patient (1.1%). There was a unifocal tumor in 82 patients (90.1%) and a multifocal tumor in 9 patients (9.9%). In 29 patients, axillary lymph node biopsy was performed before treatment; there were metastatic lymph nodes in 28 patients and reactive lymph nodes in 1 patient. Clinicopathological characteristics are presented in Table I.

Immunohistochemistry Results

When CD44 expression was assessed based on the extent of staining, 48 patients (52.7%) had a low rate of expression, and 43 patients (47.3%) had a high rate of expression. When CD44 expression was assessed based on intensity, 15 patients (16.5%) showed weak staining and 76 patients (83.5%) showed strong staining.

When VEGFR3 expression was assessed based on the extent of staining, 63 patients (69.2%) had a low rate of expression, and 28 patients (30.8%) had a high rate of expression. When VEGFR3 expression was assessed based on intensity, 61 patients (67%) showed weak staining and 30 patients (33%) showed strong staining.

There was a significant association between CD44 intensity and clinical T stage ($p = 0.022$). Weak CD44 staining values were higher than strong CD44 staining values for cT1 and cT3. The most vital staining was observed in cT2, possibly due to the clustering of patients.

There was a significant difference in VEGFR3 intensity between the HER- group and the HER2+ group ($p = 0.018$). The difference was due to more vital staining in the HER2+ group than in the HER2- group.

There were no significant correlations between the extent of CD44 and VEGFR3 staining and cT,

Table I. Clinicopathological characteristics.

		No. of cases	Percentage (%)
Age (years)	< 50 years	46	50.5
	≥ 50 years	45	49.5
ER	Negative (< 1%)	16	17.6
	Positive (≥ 1)	75	82.4
PR	Negative (< 1%)	46	50.5
	Positive (≥ 1)	45	49.5
HER2	Negative	57	62.6
	Positive	34	37.4
Ki-67	< 25%	35	38.5
	≥ 25%	56	61.5
Tubular formation	1	1	1.1
	2	24	26.4
	3	66	72.5
Nuclear Grade	1	6	6.6
	2	54	59.3
	3	31	34.1
Mitotic Score	1	31	34.1
	2	44	48.4
	3	16	17.6
Nottingham Grade	1	9	9.9
	2	54	59.3
	3	28	30.8
Histological subtype	Ductal	79	86.8
	Lobular	7	7.7
	Another	5	5.5
Miller-Payne TRG	1	24	26.4
	2	12	13.2
	3	20	22.0
	4	15	16.5
	5	20	22.0
Tumor localization	Upper outer quadrant	47	51.6
	Upper inner quadrant	17	18.7
	Lower outer quadrant	10	11.0
	Lower inner quadrant	7	7.7
	Retroareolar	10	11.0
Molecular subtype	Luminal HER2-	53	58.2
	Luminal HER2+	22	24.2
	HER2+	12	13.2
	TNBC	4	4.4
Clinical T stage	cT1	14	15.4
	cT2	64	70.3
	cT3	13	14.3
Clinical N stage	cN0	16	17.6
	cN1	66	72.5
	cN2	9	9.9
Mortality	No	86	94.5
	Yes	5	5.5

Data are expressed as n (%). ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human Epidermal Growth Factor Receptor 2, TRG: Tumor Regression Grade TNBC: Triple- negative breast cancer, cT: Clinical T stage, cN: Clinical N stage.

cN, histological grade, histological type, molecular subtype, or age (Table II).

There was a significant difference in the HER2+ group and pathological response in the correlation between TRG and clinicopathological findings and IHC results after NAC ($p < 0.001$). This significant difference was due to better treatment response in HER2+ patients compared with HER2- patients. There was no significant correlation between cN stage and pathological response, while there was a significant correlation between yPN stage and pathological response ($p = 0.015$). This difference was due to a reduction in yPN stage with increasing pathological response.

Table II. The relationship between CD44 and VEGFR3 expression status and clinicopathological findings in pre-NAC needle biopsy samples.

Clinicopathologic findings	CD44 percentage			CD44 intensity			VEGFR3 percentage			VEGFR3 intensity		
	Low	High	*p*	Weak	Strong	*p*	Low	High	*p*	Weak	Strong	*p*
Clinical T stage												
cT1	8 (16.7)	6 (14.0)	0.135	5 (33.3)	9 (11.8)	**0.022**	12 (19.0)	2 (7.1)	0.314	10 (16.4)	4 (13.3)	0.697
cT2	30 (62.5)	34 (79.1)		6 (40.0)	58 (76.3)		43 (68.3)	21 (75.0)		41 (67.2)	23 (76.7)	
cT3	10 (20.8)	3 (7.0)		4 (26.7)	9 (11.8)		8 (12.7)	5 (17.9)		10 (16.4)	3 (10.0)	
cN												
cN0	10 (20.8)	6 (14.0)	0.456	2 (13.3)	14 (18.4)	0.379	10 (15.9)	6 (21.4)	0.346	10 (16.4)	6 (20.0)	0.910
cN1	32 (66.7)	34 (79.1)		10 (66.7)	56 (73.7)		45 (71.4)	21 (75.0)		45 (73.8)	21 (70.0)	
cN2	6 (12.5)	3 (7.0)		3 (20.0)	6 (7.9)		8 (12.7)	1 (3.6)		6 (9.8)	3 (10.0)	
AJCC Stage												
2	33 (68.8)	35 (81.4)	0.169	9 (60.0)	59 (77.6)	0.078	46 (73.0)	22 (78.6)	0.918	44 (72.1)	24 (80.0)	0.599
3	13 (27.1)	5 (11.6)		6 (40.0)	12 (15.8)		13 (20.6)	5 (17.9)		14 (23.0)	4 (13.3)	
4	2 (4.2)	3 (7.0)		0 (0.0)	5 (6.6)		4 (6.3)	1 (3.6)		3 (4.9)	2 (6.7)	
HER2												
Negative	30 (62.5)	28 (65.1)	0.830	10 (66.7)	48 (63.2)	0.796	43 (68.3)	15 (53.6)	0.268	44 (72.1)	14 (46.7)	**0.018**
Positive	18 (37.5)	15 (34.9)		5 (33.3)	28 (36.8)		20 (31.7)	13 (46.4)		17 (27.9)	16 (53.3)	
Ki-67												
< 25	15 (31.3)	20 (46.5)	0.201	2 (13.3)	33 (43.4)	0.058	24 (38.1)	11 (39.3)	0.999	24 (39.3)	11 (36.7)	0.986
≥ 25	33 (68.8)	23 (53.5)		13 (86.7)	43 (56.6)		39 (61.9)	17 (60.7)		37 (60.7)	19 (63.3)	
Nottingham Grade												
1	3 (6.3)	6 (14.0)										
0.417	0 (0.0)	9 (11.8)	0.417	6 (9.5)	3 (10.7)	0.479	6 (9.8)	3 (10.0)	0.729			
2	31 (64.6)	23 (53.5)		10 (66.7)	44 (57.9)		36 (57.1)	18 (64.3)		39 (63.9)	15 (50.0)	0.454
3	14 (29.2)	14 (32.6)		5 (33.3)	23 (30.3)		21 (33.3)	7 (25.0)		16 (26.2)	12 (40.0)	
Histological subtypes												
Ductal	43 (89.6)	36 (83.7)	0.417	12 (80.0)	67(88.2)	0.362	52 (82.5)	27 (96.4)	0.161	51 (83.6)	28 (93.3)	0.304
Lobular	2 (4.2)	5 (11.6)		1 (6.7)	6(7.9)		6 (9.5)	1 (3.6)		5 (8.2)	2 (6.7)	
Another	3 (6.3)	2 (4.7)		2 (13.3)	3(3.9)		5 (7.9)	0 (0.0)		5 (8.2)	0 (0.0)	

Continued

Table II *(Continued)*. The relationship between CD44 and VEGFR3 expression status and clinicopathological findings in pre-NAC needle biopsy samples.

Clinicopathologic findings	CD44 percentage			CD44 intensity			VEGFR3 percentage			VEGFR3 intensity		
	Low	High	*p*	Weak	Strong	*p*	Low	High	*p*	Weak	Strong	*p*
Miller- Payne TRG												
1	14 (29.2)	10 (23.3)	0.320	6 (40.0)	18(23.7)	0.306	15 (23.8)	9 (32.1)	0.342	18 (29.5)	6 (20.0)	0.666
2	5 (10.4)	7 (16.3)		1 (6.7)	11 (14.5)		9 (14.3)	3 (10.7)		9 (14.8)	3 (10.0)	
3	8 (16.7)	12 (27.9)		1 (6.7)	19 (25.0)		16 (25.4)	4 (14.3)		13 (21.3)	7 (23.3)	
4	11 (22.9)	4 (9.3)		4 (26.7)	11 (14.5)		12 (19.0)	3 (10.7)		10 (16.4)	5 (16.7)	
5	10 (20.8)	10 (23.3)		3 (20.0)	17 (22.4)		11 (17.5)	9 (32.1)		11 (18.0)	9 (30.0)	
Molecular subtypes												
Luminal HER2-	29 (60.4)	24 (55.8)		9 (60.0)	44 (57.9)	0.969	39 (61.9)	14 (50.0)		41 (67.2)	12 (40.0)	0.058
Luminal HER2+	11 (22.9)	11 (25.6)	0.713	3 (20.0)	19 (25.0)		16 (25.4)	6 (21.4)	0.178	13 (21.3)	9 (30.0)	
HER2+	7 (14.6)	5 (11.6)		2 (13.3)	10 (13.2)		5 (7.9)	7 (25.0)		5 (8.2)	7 (23.3)	
TNBC	1 (2.1)	3 (7.0)		1 (6.7)	3 (3.9)		3 (4.8)	1 (3.6)		2 (3.3)	2 (6.7)	
Age (years)												
≥ 50 years	28 (58.3)	17 (39.5)	0.114	9 (60.0)	36 (47.4)	0.541	29 (46.0)	16 (57.1)	0.452	28 (45.9)	17 (56.7)	0.334
< 50 years	20 (41.7)	26 (60.5)		6 (40.0)	40 (52.6)		34 (54.0)	12 (42.9)		33 (54.1)	13 (43.3)	

Data are expressed as n (%), VEGRF3: Vascular endothelial growth factor receptor 3, CD44: Cluster of differentiation 44, cT: Clinical T stage, cN: Clinical N stage, AJCC: American Joint Committee on Cancer (2018-8th edition), HER2: Human Epidermal Growth Factor Receptor 2, TRG: Tumor Regression Grade, TNBC: Triple-negative breast cancer.

There was a significant difference between molecular subtype and pathological response ($p < 0.001$). This difference was due to poorer NAC response in the luminal HER2- group than in other groups. The HER2+, luminal HER2+, and TNBC groups had better response to neoadjuvant treatment when compared with the luminal HER2- group. No significant difference between pathological response and extent and intensity of CD44 and VEGFR3 staining was observed (Table III).

Cox and Logistic Regression Analysis Results

In the Cox regression analysis of risk factors for survival, Ki-67 proliferation index was the only significant risk factor associated with survival in univariate analysis ($p = 0.019$). Survival decreased with increasing Ki-67 proliferation index.

In univariate Cox regression analysis, Ki-67 had a significant effect on survival, with a 1.05-fold effect size ($p = 0.019$). Tumor size, VEGFR3 staining extent (%), CD44 staining extent (%), ER, PR, and age group had no significant risk effect on survival ($p > 0.05$). In the multivariate Cox regression model, Ki-67 proliferation was the only significant risk factor for survival, with a 1.05-fold effect size (Table IV).

In univariate logistic regression analysis, we found that ER, HER2, and Ki-67 proliferation had a significant risk effect on TRG ($p < 0.05$) (Table V). ER increased the risk for TRG by 1.02-fold. The treatment response was 5.5-fold higher in HER2+ patients compared with HER2- patients. TRG was 2.5-fold higher in patients with Ki-67 >25%. In the univariate logistic regression analysis, it was found that PR, tumor diameter, mitotic index, histological grade, VEGFR3 staining extent and intensity, CD44 staining intensity, cT stage, and age group had no significant risk effects on TRG ($p > 0.05$) (Table V).

In multivariate logistic regression analysis, HER2 was the only significant variable ($p < 0.05$); treatment response was 5.5-fold higher in HER2+ patients compared with HER2- negative patients (Table V).

Discussion

In this study, we investigated the correlation between TRG after NAC and CD44, VEGFR3, ER, PR, HER2, Ki67 expression status, clinicopathological factors, and BC patient prognosis.

The HER2-encoding tyrosine kinase receptor belongs to the epidermal growth factor receptor family and is expressed/amplified in 20-40% of invasive BCs[21,22]. In our study, the rate of HER2+ cases (including luminal HER2+) was 37.4%. HER2 positivity was previously associated with metastatic disease risk, increased recurrence rate, and increased mortality. The introduction of targeted therapies has changed the course of the disease. With adjuvant therapy, survival and quality of life have increased in HER2+ BC patients[23]. When TRG was evaluated after NAC in our study, the treatment response was 5.5-fold higher in HER2+ cases compared with HER2- cases. This result reveals the eligibility of these patients for NAC. Although HER2 was initially considered a poor prognostic marker for BC, its true benefit is its role in predicting response to anti-HER2 treatment in neoadjuvant/adjuvant therapy[21].

The World Health Organization (WHO) classifies BCs into four molecular subtypes, luminal B subtype: ER+ and/or PR+ and/or HER2+ and high Ki-67. The heterogeneity in the definition of luminal B patients may lead to inconsistent outcomes[22,24]. Luminal HER2+ patients have been assessed within the HER2 subtype in many studies[6-8,12,13,24-26], while the luminal HER2+ subtype was categorized separately in many others, as in our study. It has been reported that 10-year survival was 70.0% in patients with luminal A tumors, 54.4% in patients with luminal B tumors, 46.1% in luminal HER2+ tumors, and 48.1% in patients with HER2+ tumors[26].

In our study, among the luminal groups, we observed that luminal HER2+ subtype was associated with a TRG more like the HER2+ subtype rather than the luminal HER2- subtype (Figure 1). However, pCR was lower in the luminal HER2+ subtype (36.4%) compared with the HER2+ subtype with a negative hormone receptor (58.4%). Treatment response following NAC decreased in ER+ patients, and there was a significant correlation between TRG and the risk effect of ER ($p = 0.002$). Based on these results, we concluded that decreased pCR, despite HER2 positivity, was associated with hormone receptor positivity. Our results support the results of previous studies[6,7,21,25,27] suggesting that ER and PR positivity has an unfavorable effect on pCR.

Given the survival and TRG outcomes following NAC, the classification of luminal HER2+ as a distinct subtype may prevent contradictory results and ensure the classification of luminal

Table III. The relationship between Tumor Regression Grade and clinicopathological variables.

Clinicopathological Characteristics and IHC	Tumor regression grade		
	Good response	Partial response	*p*
cT			
cT1	4 (11.4)	10 (17.9)	0.633
cT2	25 (71.4)	39 (69.6)	
cT3	6 (17.1)	7 (12.5)	
cN			
cN0	7 (20.0)	9 (16.1)	0.799
cN1	24 (68.6)	42 (75.0)	
cN2	4 (11.4)	5 (8.9)	
AJCC Stage			
2	25 (71.4)	43 (76.8)	0.685
3	7 (20.0)	11 (19.6)	
4	3 (8.6)	2 (3.6)	
HER2			
Negative	14 (40.0)	44 (78.6)	**< 0.001**
Positive	21 (60.0)	12 (21.4)	
Ki-67			0.079
< 25%	9 (25.7)	26 (46.4)	
≥ 25%	26 (74.3)	30 (53.6)	
Nottingham Grade			
1	1 (2.9)	8 (14.3)	0.203
2	22 (62.9)	32 (57.1)	
3	12 (34.3)	16 (28.6)	
Histological subtype			
Ductal	33 (94.3)	46 (82.1)	0.192
Lobulary	2 (5.7)	5 (8.9)	
Another	0 (0.0)	5 (8.9)	
Molecular subtype			**< 0.001**
Luminal HER2-	10 (28.6)	43 (76.8)	
Luminal HER2+	14 (40.0)	8 (14.3)	
HER2	8 (22.9)	4 (7.1)	
TNBC	3 (8.6)	1 (1.8)	
yPN			
ypN0	25 (71.4)	22 (39.3)	**0.015**
ypN1	8 (22.9)	24 (42.9)	
ypN2	2 (5.7)	6 (10.7)	
ypN3	0 (0.0)	4 (7.1)	
Multicentricity			
No	34 (97.1)	48 (85.7)	0.145
Yes	1 (2.9)	8 (14.3)	
VEGFR3 percentage			
Low	23 (65.7)	40 (71.4)	0.566
High	12 (34.3)	16 (28.6)	
VEGFR3 intensity			
Weak	21 (60.0)	40 (71.4)	0.259
Strong	14 (40.0)	16 (28.6)	
CD44 percentage			
Low	21 (60.0)	27 (48.2)	0.273
High	14 (40.0)	29 (51.8)	
CD44 intensity			
Weak	7 (20.0)	8 (14.3)	0.475
Strong	28 (80.0)	48 (85.7)	
Age (years)			
≥ 50	16 (45.7)	29 (51.8)	0.573
< 50	19 (54.3)	27 (48.2)	

Data are expressed as n (%). IHC: immunohistochemistry cT: Clinical T stage, cN: Clinical N stage, AJCC: American Joint Committee on Cancer (2018-8th edition), HER2: Human Epidermal Growth Factor Receptor 2, yPN: postoperative pathological N stage, VEGRF3: Vascular endothelial growth factor receptor 3, CD44: Cluster of differentiation 44.

Table IV. Cox regression analysis of risk factors on survival.

Variables	Univariate		Multivariate	
	HR (95% CI)	p	HR (95% CI)	p
Ki-67 %	1.05 (1.01-1.10)	**0.019**	1.05 (1.01-1.10)	**0.019**
Tumor size before treatment	0.94 (0.85-1.04)	0.226	-	-
VEGFR3 (%)	0.97 (0.93-1.02)	0.200	-	-
CD44 (%)	1.01 (0.98-1.03)	0.551	-	-
ER %	1.00 (0.98-1.02)	0.987	-	-
PR %	0.98 (0.95-1.02)	0.357	-	-
Age (years)				
< 50 years	1.00	-		
≥ 50 years	1.55 (0.26-9.25)	0.634		

HR: Hazard ratio. CI: Confidence Interval. VEGRF3: Vascular endothelial growth factor receptor 3, CD44: Cluster of differentiation 44, ER: Estrogen receptor, PR: Progesterone receptor.

B subtype into a more well-defined subgroup. A pCR rate of 0.3-7.5% has been reported in luminal A subtype and 14.6-16.2% in luminal B HER2- subtype[6,25]. In our study, the pCR rate was 3.8% in luminal HER2- subtype (luminal A and luminal B HER2- patients). It should be recognized that patients with luminal HER2- subtype are not eligible for NAC and that surgery should

Table V. Logistic regression analysis result of risk factors for Miller-Payne Tumor Regression Grading System.

Variates	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age (years)	1.02 (0.99-1.05)	0.286		
< 50 years	1.00	-		
≥ 50 years	1.28 (0.55-2.97)	0.573		
ER%	1.02 (1.01-1.03)	**0.002**		
PR %	1.01 (1.00-1.03)	0.070		
Ki-67 %	0.98 (0.95-1.00)	**0.031**		
HER2				
Negative	1.00	-	1.00	-
Positive	5.50 (2.17-13.94)	**< 0.001**	5.50 (2.17-13.94)	**< 0.001**
Mitotic score				
1	2.04 (0.64-6.51)	0.227		
2	3.14 (0.90-11.03)	0.074		
3	1.00	-		
Clinical T stage				
T1/2	1.00	-		
T3/4	1.45 (0.44-4.73)	0.540		
VEGFR3 percentage				
Low	1.30 (0.53-3.23)	0.566		
High	1.00	-		
VEGFR3 intensity				
Weak	1.67 (0.68-4.06)	0.261		
Strong	1.00	-		
CD44 percentage				
Low	1.00	-		
High	1.61 (0.69-3.79)	0.275		
CD44 intensity				
Weak	1.00	-		
Strong	1.50 (0.49-4.58)	0.477		

OR: Odds ratio. CI: Confidence Interval. ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human Epidermal Growth Factor Receptor 2, VEGRF3: Vascular endothelial growth factor receptor 3, CD44: Cluster of differentiation 44.

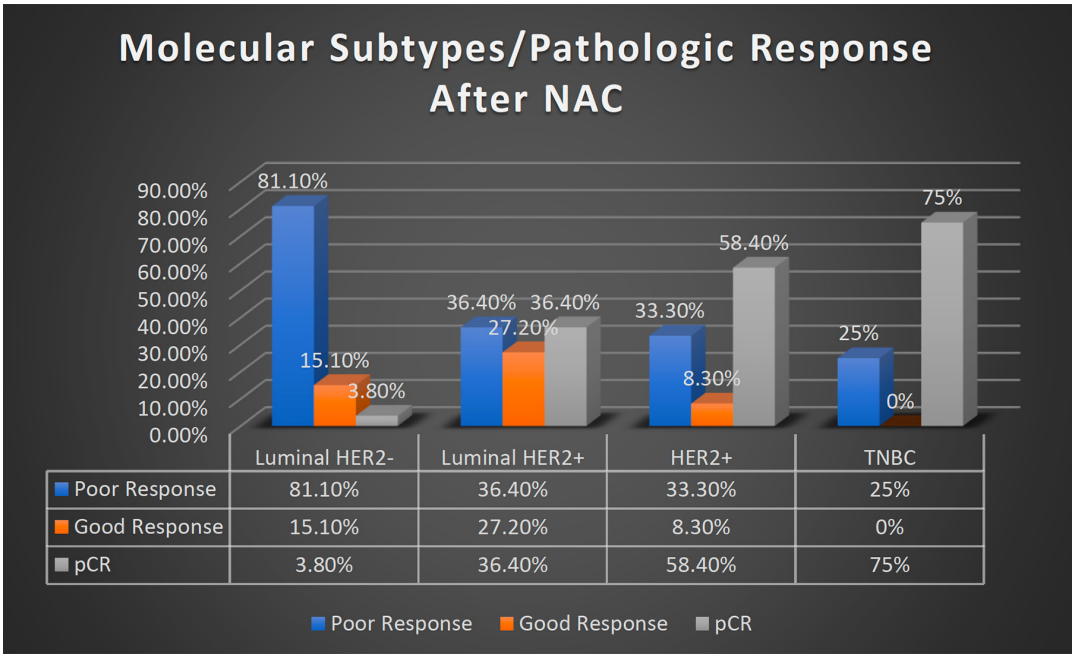

Figure 1. The lowest response was Luminal HER2-, and the best response rate was in the TNBC subtype. The post-NAC pathological response rate of the Luminal HER2+ subgroup is closer to the HER2+ group. Poor therapeutic response: Miller Payne Grade 1,2,3, Good therapeutic response: Miller Payne Grade 4, pCR: pathologic complete response: Miller Payne Grade 5, TNBC: Triple- negative breast cancer.

be the first treatment option in these patients despite advanced stage and diffuse lymph node involvement.

The risk for recurrence and mortality is higher in ER+/PR- patients than in ER+/PR+ patients. The mean Ki-67 proliferation index was 38% in ER+/PR- and 23.3% in ER+/PR+ patients in our study. The Ki-67 proliferation index is important in patients with luminal molecular subtypes. Ki-67 is a biomarker expressed in all active phases (G1, S, G2, and M) other than the resting phase (G0) of the cell cycle. The percentage of Ki-67-positive cells is an independent prognostic factor in primary BC[21,28]. Ki-67 levels have been reported[29] to be higher in young patients. Although there is no consensus regarding the cut-off value for Ki-67, it has been suggested that Ki-67 > 25% is associated with a poorer prognosis[6,28-31]. In our study, the only significant factor in univariate Cox regression analysis was Ki-67 proliferation index, with decreased survival being associated with increasing Ki-67 proliferation index (p = 0.019). In addition, in logistic regression analysis, we found that patients with Ki-67 > 25% had 2.5-fold better treatment response. Some studies suggest[4,5,7,32] that high Ki-67 expression is associated with poor NAC response; however, there is also evidence that it indicates better response in tumors with high Ki-67 expression. Our results indicate that high Ki-67 proliferation index can be used as a marker to identify BC patients with a higher likelihood of responding to NAC but poorer survival.

In tumors with a high proliferation rate, hypoxia is anticipated due to vascularization and nutritional problems in the center of the tumor tissue. This situation results in the induction of angiogenesis, which is characterized by increased VEGF/VEGFR expression[33]. Angiogenesis is an essential process for tumor growth, invasion, and metastatic capacity[4,33]. VEGF, localized on chromosome 6, is one of the primary factors involved in the formation of vascularity that supplies neoplastic tissue[21,33]. Lymphatic endothelial cells are thought to originate from a subpopulation of vascular endothelial cells and are responsible for tumor lymphangiogenesis. The crucial role of the VEGF-C/VEGF-D/VEGFR3 signaling pathway in lymphangiogenesis has previously been described[34,35]. Our study investigated the association between VEGFR3 and treatment response and histopathological findings following NAC. VEGFR3 overexpression was found in 30.8% of cases. Increased VEGF expression has been

linked to poor prognosis in patients with BC[4,36]; however, our study detected no correlation between VEGFR3 and survival or TRG. HER2 positivity was the single factor that was significantly correlated with the intensity of VEGFR3 expression ($p = 0.018$). It has been reported that the frequency of ER/PR negativity and HER2 positivity was higher in tumors with increased VEGFR3 expression[4,35]. These findings suggest that HER2 activation is one of the several mechanisms favoring angiogenesis[32]. In BC, increased expression of a member of the VEGF family may be associated with more aggressive cancer phenotypes and thus more aggressive tumor biology. However, as seen in our study, the association of VEGFR3 with an aggressive tumor profile has no unfavorable prognostic effect on response to NAC or survival[35].

BC was the first human tumor in which a putative stem cell subpopulation was identified. Cancer stem cells (CSCs), also called "cancer-initiating cells", have the ability of self-regenerate, proliferate, and form heterogeneous tumor cell lineages[37]. CSCs are implicated in recurrence, metastasis, and refractoriness to anti-tumor treatments in BC[38,39]. CD44 is an adhesion molecule in the form of transmembrane glycoprotein and a critical CSC marker localized on chromosome 11p13[39].

In our study, we investigated the role of CD44 in predicting TRG following NAC. Although a significant difference was found between the intensity of CD44 expression and clinical T stage, we determined that the difference was due to a larger number of patients with cT2 stage; therefore, they were excluded from analysis ($p = 0.022$) (Table II). In our study, there was a high level of CD44 expression in 47.3% of BCs despite the absence of a significant correlation between CD44 and TRG or clinicopathological findings. There are inconsistent results regarding the correlation between CD44 and clinicopathological findings in the literature. Some publications[33,39-42] suggest a correlation between CD44 expression and advanced stage, higher histological grade, ER negativity, higher cell proliferation, risk for lymph node metastasis, and shorter survival. In contrast, other studies[43] suggest no significant correlation between CD44 expression and tumor size, lymph node status, or hormone receptors. Moreover, some studies[43] suggest a correlation between high CD44 expression and smaller tumor size, lack of axillary metastasis, and earlier stages. In a study attempting to identify chemoresistant cells in luminal cancers, Tang et al[43] demonstrated that CD44+CD24+ cells were expressed in chemoresistant cells, suggesting that the presence of CD44+CD24+ cells could predict chemoresistance in luminal BCs[43]. Our patient group included all subtypes, rather than luminal-type BC alone, and no significant association was found between CD44 expression and treatment response following NAC.

Limitations

This study presents several limitations. The sample size was small due to patients who failed to complete treatment or underwent surgery or needle core biopsy at other facilities. In our study, pCR rate following NAC was found to be relatively high due to the limited number of tumors of the TNBC subtype[7,8,12,13,20,27]. However, this did not change the fact that TNBC and HER2 subtypes have better pCR when compared with other molecular subtypes. Another limitation was that, due to financial issues, we failed to assess CD44 plus CD24, which were assessed together in some studies[42,43] reporting that simultaneous increases in these stem cell markers were significant in chemoresistance. In our study, the lack of significant correlation between CD44 and NAC may have been due to the assessment of CD44 alone.

Conclusions

Our study demonstrated that ER negativity positively affected TRG following NAC. In addition, Ki-67 proliferation index appears to be a valuable biomarker for identifying patients with a higher likelihood of response following NAC. Ki-67 proliferation index is a biomarker that can be used to identify BC patients with poor survival. It appears that high Ki-67, negative hormone receptors, and HER2+ and TNBC subtypes are major predictive factors of response to NAC.

Based on our results and literature, metastatic capacity seems to be associated with VEGFR3 in HER2+ tumors; however, VEGFR3 expression does not influence treatment response following NAC. Our study showed that CD44 and VEGFR3 could not be used to predict treatment response following NAC. Identifying biomarkers that can predict response to NAC will allow appropriate management of patients. Therefore, further studies will be necessary to assess NAC activity markers accurately and effectively.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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Informed Consent
Informed consent was provided by each patient participating in the study.

Ethics Approval
This study was approved by the Kayseri City Training and Research Hospital Clinical Research Ethics Committee (Protocol No.: 651/2022).

Authors' Contribution
Ebru Akay: concept and design of the work and protocol, paper draft, and data acquisition. Saliha Karagöz Eren, Nail Özhan: data acquisition, statistical analysis, the paper's conception, draft, and critical review. Alaettin Arslan, Hatice Karaman: critical review, approval of the final version of the manuscript. All authors read and approved the final manuscript.

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