ORIGINAL RESEARCH

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Association of Immunoscore, CD73 Expression and 53BP1 Expression with Neoadjuvant Chemoradiotherapy Efficacy in Patients with Locally Advanced Rectal Cancer

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ABSTRACT Objective: This study aimed to determine the relationship between immunoscore, CD73 expression, and P53 binding protein 1 (53BP1) expression in patients with locally advanced rectal cancer who received neoadjuvant chemoradiotherapy (nCRT) at the time of diagnosis and after treatment. **Material and Methods:** The patients with locally advanced rectal cancer treated with nCRT at diagnosis and after treatment were included and immunoscore, CD73 expression, and 53BP1 expression were evaluated. **Results:** A total of 53 patients were included in the study. Higher immunoscore and CD73 expression in the stroma (p=0.029 and p=0.011, respectively) were observed in the pathology specimen, in patients who responded well to nCRT before neoadjuvant treatment than that in patients who did not respond well. However, no association was noted between CD73 expression in tumor cells and pathological response (p=0.874). The immunoscore at diagnosis (p=0.087), 53BP1 expression (p=0.871), and CD73 expression in the stroma (p=0.053) did not correlate with 3-year disease-free survival (DFS). In contrast, an association between CD73 expression level in tumor cells (p=0.014), tumor regression grade (p=0.041), and pathological complete response (p=0.011) and 3-year DFS was found to be statistically significant. **Conclusion:** Our study revealed that in addition to tumor-related factors, host-related factors were associated with treatment response. A significant association was observed between immunoscore, CD73 expression, and neoadjuvant therapy response. Markers that can predict the response to nCRT are still needed, and more studies should be conducted to figure out how well immunomarkers work in this field.

Keywords: Rectal cancer; neoadjuvant therapy; pathological response; immunoscore; radiotherapy

Neoadjuvant chemoradiotherapy (nCRT) is recommended as the standard alternative for treating locally advanced rectal cancer.[1] In recent studies, the efficacy of total neoadjuvant therapy (TNT) has been demonstrated, with increased pathological complete response (pCR) rates.[2,3] However, in all neoadjuvant therapy studies, complete or near-complete response patients, as well as those who do not respond and develop distant metastases, have been reported. There is also concern regarding the overtreatment of patients with favorable prognostic characteristics possibly having a complete response to de-intensified therapy, in patients where TNT is used as a standard. Therefore, markers that can predict the response of patients to treatment are needed.

The DNA damage response (DDR) pathway has a role in tumor development and treatment resistance.[4] A tumor suppressor gene in the DDR pathway is P53 binding protein 1 (53BP1), and the relationship between its expression and rectal cancer prognosis has been demonstrated.[5,6] The loss of 53BP1 expression might reduce radiosensitivity.[7] In contrast, a connection exists between 53BP1 expression and the T cell function of the host, and 53BP1 expression has a substantial effect on the immune system.[8] Therefore, tumor-related mutations and factors and

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the host immune system will be determinants in the treatment response. The immunoscore for colon cancer developed by Pages et al. has proven to be a prognostic marker for adjuvant and palliative treatment response.[9] The CD3, CD8, and CD45 expressions are assessed both in the center of the tumor and invasive margin, and the immunoscore is calculated based on the expression rates. An association between immunoscore and neoadjuvant treatment response in rectal cancer has been demonstrated through a few retrospective studies.[6,10] Tumors with a high immunoscore respond better to nCRT. Consequently, assessing the parameters that might also alter the immune response will aid in the prediction of the treatment results.

Adenosine is an immunosuppressive molecule that directly influences both the innate and acquired immune responses.[11] The rate-limiting enzyme in adenosine synthesis is CD73.[12] An increase in adenosine inhibits the T cells, when CD73 expression is elevated, which negatively affects tissue homeostasis. Numerous types of cancers, including colon cancers, contain tumor cells expressing high levels of CD73.[13] Moreover, there is inadequate data regarding the association between CD73 expression in response to neoadjuvant treatment and immunoscore.

This study aimed to determine the relationship between immunoscore, CD73 expression, and 53BP1 expression in patients who received nCRT for locally advanced rectal cancer at the time of diagnosis and their treatment response. The study secondarily aimed to determine the changes in immunoscore before and after CRT.

MATERIAL AND METHODS

Patients with locally advanced rectal cancer who received nCRT were included in the study. Locally advanced rectal cancer was determined as T3-T4 or N+ radiological stage. Patients whose locally advanced disease was diagnosed using magnetic resonance imaging and who were operated on after nCRT were included. The data of the patients treated between 2014 and 2019 at our center were analyzed retrospectively. The clinical and pathological patient characteristics were collected from the files and hospital operating system. Before data collection was initiated for the study was carried out in accordance with the Declaration of Helsinki. (Gazi University Ethics Committee, date: December 1, 2021; no: 77082166-604.01.02-224902).

The inclusion criteria were patients older than 18 years, with pathologically proven rectum adenocarcinoma, and with locally advanced disease at the time of diagnosis. The exclusion criteria were patients who were not operated on after nCRT or whose operative pathology specimens were not evaluated at our center. It was a prerequisite for the colonoscopic biopsy and magnetic resonance imaging for local staging at the time of diagnosis to be performed in our center. Patients with stage four disease at the time of diagnosis, those treated with TNT, or those receiving only radiotherapy were excluded from the study.

The demographic and clinical characteristics of the patients before treatment were determined. Data regarding age, sex, Eastern Cooperative Oncology Group performance status, tumor stage, tumor location, and post-treatment pathological evaluation were obtained from the patient files. The data of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9, and complete blood count, measured within 7 days before the start of nCRT, were recorded through the hospital operating system. Data on overall survival (OS), progression-free survival, and local relapse-free survival were evaluated. The time from diagnosis to death was defined as the OS. Disease-free survival (DFS) is defined as the time from diagnosis to progression or death. Local relapse-free survival time was the time from diagnosis to local recurrence.

IMMUNOHISTOCHEMISTRY

The patient list was evaluated through the pathology records and patients without residual tumors were excluded from the study. The hematoxylin and eosin (H&E) slides were reviewed. The slide with the maximum inflammatory reaction was selected for the immunohistochemical analysis.

The substances such as anti-CD73 (MA5-29454; rabbit monoclonal, Invitrogen, USA); anti-Maspin (polyclonal, Invitrogen, USA); anti-53BP1 (OTI2H6, mouse monoclonal, Invitrogen, USA); anti-CD8

(SP57, rabbit monoclonal, Roche, Switzerland); anti-CD3 (2GV6, rabbit monoclonal, Roche, Switzerland), and anti-CD45 (2B11&PD7/26, mouse monoclonal, Cell Marque, USA) were used to perform immunohistochemistry of formalin-fixed paraffin-embedded tissue blocks. The staining was performed using Ultraview DAB detection kits (Roche, Switzerland) on Ventana XT automatic strainers (Roche, Switzerland).

The area of interest within the tumor with the most inflammatory cell infiltrate for lymphocyte subtyping was micrographed (BX52 Olympus microscope with DP72 camera, Evident, Japan). First, H&E, then consecutively, CD45, CD3, and CD8 stained slides were captured. The CD3 to CD8 and CD3 to CD45 ratios were manually estimated. Immunoscores were assessed based on the method proposed by Anitei et al.[14]

The 53BP1 staining grades were determined with both percentages and intensities. The intensity of staining in tumor cells was scored as 1+ (weak), 2+ (moderate), or 3+ (strong).[15] CD73 staining was evaluated separately on the tumor gland surface and stroma.[16] The intensity of staining was scored as 1+ (weak), 2+ (moderate), or 3+ (strong). Based on the dual cytoplasmic-nuclear expression of maspin in tumor cells, maspin expression was assessed. Slides were considered to be as follows: negative (no staining); carcinomas with cytoplasm positivity (cytoplasmic positivity, without nuclear expression); or carcinomas with dual positivity (nuclear+cytoplasmic expression).[17]

TUMOR REGRESSION GRADE

Based on the 8th edition of the American Joint Committee on Cancer and the International Association for Cancer Control systems, tumor regression of the primary tumor after nCRT was evaluated in H&E stained preparations. Tumor regression grade (TRGs) were defined in the following manner: Grade 0, complete response, no residual cancer cells; Grade 1, moderate response, only small clumps or cells present as cancer cells; Grade 2, minimal response with predominant fibrosis and residual cancer cells; and Grade 3 was defined as a poor response with extensive residual cancer. Patients with TRGs 0 and 1, and 2 and 3 were evaluated as having a good response, and poor response, respectively.[18] Histopathological evaluations were reviewed by two gastrointestinal pathologists (MAİ and NA) independently.

STATISTICAL ANALYSIS

The IBM SPSS Statistics 22(IBM Corp., Armonk, NY, USA) program was used to analyze the data. program was used to analyze the data. The data distribution (parametric or nonparametric) was determined using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The paired t-test was used to compare two dependent groups when parametric test conditions were met; the Wilcoxon test was used in nonparametric conditions. The student's test was used to compare two independent groups under parametric conditions, while the Mann-Whitney U test was used under nonparametric conditions. The correlation between categorical variables was compared using the chi-square and Fisher's exact tests. The direction and strength of the association between the two variables were determined using Spearman's correlation test. Kaplan-Meier analysis was used to calculate DFS and OS times, and Kaplan-Meier curves were used to represent the results. The impact of independent variables on survival was assessed using a cox regression analysis. A p value of <0.05 was considered statistically significant in all analyses.

RESULTS

A total of 53 patients were included in the study. While 21 (40%) responded well to neoadjuvant therapy, 32 (60%) had a poor response. pCR was detected in 8 (15%) patients. The demographic and characteristic data and their distribution in patients according to the nCRT response are presented in Table 1. Patients who did not respond well to nCRT had greater extramural vascular invasion (EMVI) positivity in their magnetic resonance imaging at the time of diagnosis (p=0.027) and higher serum CEA levels at the time of diagnosis (p=0.029). Patients who responded well to nCRT had higher immunoscore and CD73 expression in the stroma (p=0.029 and p=0.011, respectively) in the pathology specimen before neoadjuvant treatment than that in those who did not respond well. However, no association existed be-

TABLE 1: Patient clinical characteristics and response to neoadjuvant chemoradiotheraphy.

	Total		Responder		Non-responder		
Total	**number**	**(%)**	**number**	**(%)**	**number**	**(%)**	**p value**
Total	53	100	21	40	32	60	
Age							0.264
<65 years	27	51	13	62	14	44	
≥65 years	26	49	8	38	18	55	
Gender							0.874
Female	17	32	7	33	10	31	
Male	36	68	14	67	22	69	
Distance to anal verge (cm)							0.259
<5	24	45	12	57	12	38	
≥5	29	55	9	43	20	62	
Tumor grade (differentiation)							0.259
Well-moderate	24	45	12	57	12	38	
Poor	29	55	9	43	20	62	
Clinical tumor (T) stage							0.200
cT1-2	6	11	4	33	2	6	
cT3-4	47	89	17	67	30	94	
Clinical nodal (N) stage							0.492
cN0	10	19	5	24	5	16	
cN+	43	81	16	76	27	84	
Pre-treatment MRI evaluation of CRM							0.159
CRM (-)	31	58	15	71	16	50	
CRM (+)	22	42	6	29	16	50	
Pre-treatment MRI evaluation of EMVI							0.027
EMVI (-)	38	72	19	90	19	59	
EMVI (+)	15	28	2	10	13	41	
Pre-treatment CEA level (ng/mL)							0.029
Low	39	74	19	90	20	62	
High	14	26	2	10	12	38	
Pre-treatment Ca19-9 level (U/mL)							0.118
Low	38	72	18	86	20	62	
High	15	28	3	14	12	38	
53BP1 expression level							0.269
Negative	3	6	0	0	3	9	
Positive	50	94	21	100	29	91	
CD73 expression level in stroma							0.011
Negative	25	48	5	24	20	62	
Positive	28	52	16	76	12	38	
CD73 expression level in tumor cells							0.874
Negative	36	68	14	67	22	69	
Positive	17	32	7	33	10	31	
Immunoscore							0.029
≤2	41	77	13	62	28	87	
>2	12	23	8	38	4	13	

MRI: Magnetic resonance imaging; CRM: Circumferential resection margin; EMVI: Extramural vascular invasion; CEA: Carcinoembryonic antigen; 53BP1: P53 binding protein 1.

tween CD73 expression in tumor cells and pathological response (p=0.874).

The median patient follow-up duration was 47.9 months. Upon data analysis, recurrence was identified in 22 of 53 (42%) patients, while 14 (26%) patients died. The median value could not be reached in both DFS and OS. Local recurrence was detected in only three of 22 relapsed patients. The immunoscore obtained from the biopsy at the time of diagnosis of 3 patients with local recurrence was

evaluated as 0. The 3-year DFS rate in the entire patient group was determined as 62% (n=33). The 3-year DFS rates of subgroups are presented in Table 2. The immunoscore at diagnosis (p=0.087), 53BP1 expression (p=0.871), and CD73 expression in the stroma (p=0.053) did not correlate with 3-year DFS. In contrast, an association between CD73 expression level in tumor cells (p=0.014), TRG (p=0.041), and pCR (p=0.011) and 3-year DFS was found to be statistically significant. Figure 1 illustrates the Kaplan-Meier graphs illustrating the relationship between DFS and immunoscore, 53BP1 expression level, and CD73 expression level. The rate of the 3-year OS was 81%. The 3-year OS rates for subgroups are summarized in Table 3. While only one of 12 patients with a high immunoscore at the time of diagnosis died within the first 3 years, 11 patients were still alive (p=0.228). The only variable that affected 3-year OS was the CD73 expression in tumor cells (p=0.010).

The immunoscore was evaluated on the diagnostic and postoperative pathology specimens. Immunoscore could not be assessed in the postoperative pathology of 8 patients owing to the complete pathological response. Twelve pathologies had a high immunoscore at the time of diagnosis. In contrast, the immunoscore was found to be high in only 5 of 45 patients whose postoperative pathology was evaluated. Two of five patients with a high postoperative immunoscore had a good TRG, while three had a poor TRG. There was no statistically significant correlation between the immunoscore measured in the diagnostic biopsy and postoperative pathology (p=0.281, r=-168).

DISCUSSION

The nCRT is the standard treatment approach for locally advanced rectal cancer, and markers to predict patients who will respond well to nCRT are required. In our study, the relationship between some immune markers known to be closely related to the immune system of the host and TRG was determined. A significant relationship was found between immunoscore and CD73 expression and TRG. CD73 can be used along with immunoscore for selecting patients who will respond well to nCRT, and this result will add to the scientific literature.

The rate of pCR after capecitabine has been reported to be between 13 to 18% with standard long-course radiotherapy.[19-21] In our study, TNT was applied for any patient, and pCR rates were similar. Although the 3-year DFS and OS rates appear to be low in comparison to current studies, survival times are increasing owing to the development of radiotherapy techniques and the increased use of nCRT. Additionally, circumferential resection margin and EMVI involvement is associated with a poor prognosis during preoperative radiological staging.[22] In our study, the pathological responses were worse in patients with EMVI involvement than that in those without involvement. Therefore, additional studies evaluating the immunoscore, CD73, and 53BP1 expressions in patients with the same radiological prognostic features may elucidate the prognostic power of these markers.

The 53BP1 is one of the DNA repair genes and was closely related to TRG in a study published by Huang et al. in 2019.[5,6] In their study, 53BP1 was stained positively in 28 (82%) of 34 patients with locally advanced rectal cancer. In our study, 53BP1 staining was observed in only 6% of patients, and no significant conclusion could be drawn with such a small sample size. Although 53BP1 expression was examined immunohistochemically in both studies, the difference in results may be owing to the differences in test kits and the inclusion of patients of different races. However, to evaluate the association between 53BP1 expression and nCRT response, additional studies involving larger patient populations are needed. The relationship between CD73 expression and rectal cancer has been assessed previously.[13,23] It has been demonstrated, particularly in mouse models that CD73 expression can be elevated in rectal cancer, and a high expression level is associated with a poor prognosis.[24] Patients with metastatic colorectal cancer had a poor prognosis when their expression levels were high. CD73 expression was also associated with a poor prognosis in a recent study involving only patients with rectal cancer.[13] In contrast, while 50% of the patients in this study were at an early stage, the other 50% had a locally advanced or metastatic stage of rectal cancer. The patients included in these studies were extremely

TABLE 2: Three-year disease free survival rates in all patient groups and subgroups.

	Total		Progression		Non-progression		
Total	**number**	**(%)**	**number**	**(%)**	**number**	**(%)**	**p value**
Total	53	100	20	38	33	62	
Age							0.577
<65 years	27	51	9	45	18	55	
≥65 years	26	49	11	55	15	45	
Gender							0.225
Female	17	4	7	20	13	39	
Male	36	68	16	80	20	61	
Distance to anal verge (cm)							0.242
<5	24	45	7	35	17	52	
≥5	29	55	13	65	16	48	
Tumor grade (differentiation)							0.082
Well-moderate	24	45	6	30	18	55	
Poor	29	55	14	70	15	45	
Clinical tumor (T) stage							0.390
cT1-2	6	11	1	5	5	15	
cT3-4	47	89	19	95	28	85	
Clinical nodal (N) stage							0.870
cN0	10	19	4	20	6	18	
cN+	43	81	16	80	27	82	
Pre-treatment MRI evaluation of CRM							0.156
CRM (-)	31	58	9	45	22	67	
CRM (+)	22	42	11	55	11	33	
Pre-treatment MRI evaluation of EMVI							0.831
EMVI (-)	38	72	14	70	24	73	
EMVI (+)	15	28	6	30	9	27	
Pre-treatment CEA level (ng/mL)							0.645
Low	39	74	14	70	25	76	
High	14	26	6	30	8	24	
Pre-treatment Ca19-9 level (U/mL)							0.058
Low	38	72	11	55	27	82	
High	15	28	9	45	6	18	
53BP1 expression level							0.871
Negative	3	6	1	5	2	6	
Positive	50	94	19	95	31	94	
CD73 expression level in stroma							0.053
Negative	25	48	13	65	12	36	
Positive	28	52	7	35	21	64	
CD73 expression level in tumor cells							**0.014**
Negative	36	68	18	90	18	55	
Positive	17	32	2	10	15	45	
Immunoscore							0.087
≤2	41	77	18	90	23	70	
>2	12	23	2	10	10	30	
Pathologic complete response							**0.019**
Yes	8	15	0	0	8	24	
No	45	85	20	100	25	76	
Tumor regression grade							**0.041**
Good responder	21	42	4	20	17	52	
Poor responder	32	58	16	80	16	48	

MRI: Magnetic resonance imaging; CRM: Circumferential resection margin; EMVI: Extramural vascular invasion; CEA: Carcinoembryonic antigen; 53BP1: P53 binding protein 1.

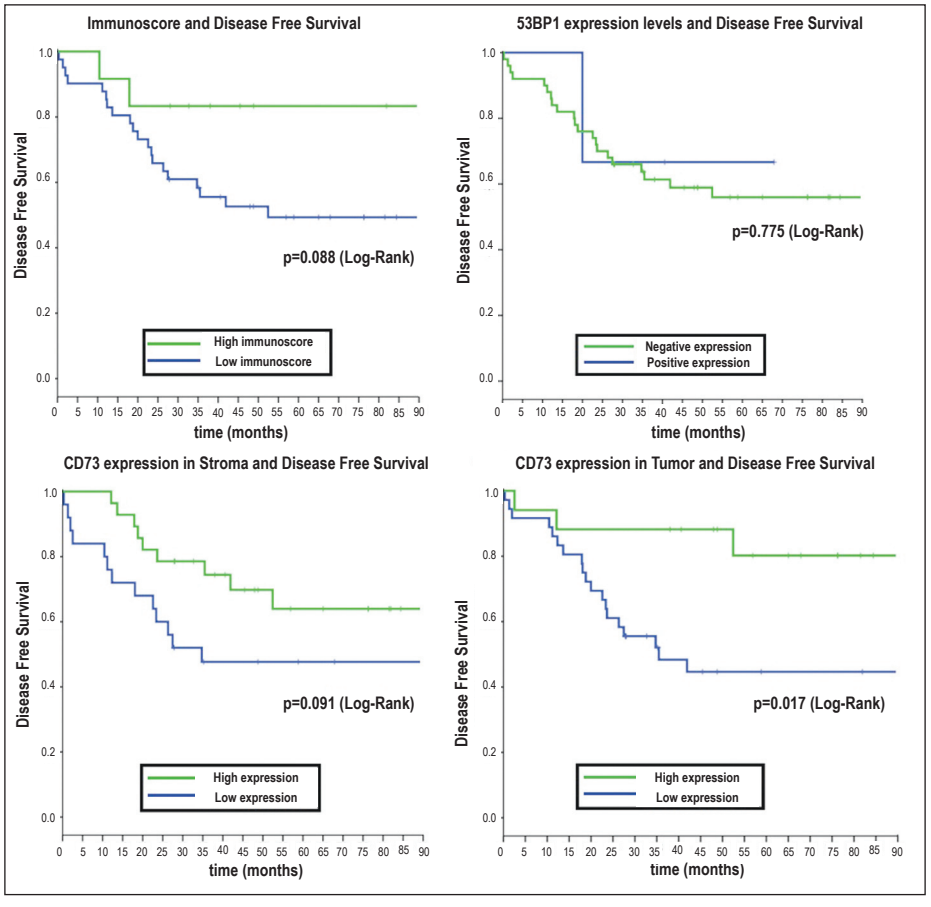

FIGURE 1: Immunoscore, 53BP1 expression, CD73 expression and disease-free survival.
53BP1: P53 binding protein 1.

diverse. One of these studies was conducted on mice, another on patients with metastatic colorectal cancer, and the third on those with rectal cancer of any stage. In our study, no relationship was observed between higher CD73 expression in tumor cells and 3-year DFS. Moreover, contrary to previous research, increased expression was associated with improved median DFS. The difference between our results and those found in the literature can be attributed to our patients being more similar and receiving exactly equal treatments. Additionally, every patient in our study underwent R0 resection; therefore, it can be concluded that tumor-related immunosuppression disappeared after neoadjuvant therapy and surgery. Therefore, contradictory results may have been found between CD73 expression and DFS or OS in the literature. The results of studies involving more patients must be conducted in the same patient population. In contrast, our DFS and OS data should be confirmed using a statistical analysis conducted after a longer follow-up period.

The immunoscore was developed by Galon et al., and its predictive and prognostic power in colon cancer has been demonstrated in numerous studies.[9,25-27] It was suggested after evaluating the immunoscore of patients enrolled in the International Duration Evaluation of Adjuvant Therapy study that the immunoscore should be assessed during the adjuvant treatment period.[27] Studies have examined the linkage between immunoscore and response to nCRT in patients with locally advanced rectal cancer.[6,10,28,29] In these studies, patients with a high immunoscore at the time of diagnosis exhibited better treatment responses that were supported by our study. Additionally, immunoscore was found to be associated with DFS in the study conducted by Huang et al.[6] In our study, there was no correlation between immunoscore and DFS or OS; however, patients with higher im-

TABLE 3: Three-year overall survival rates in all patient groups and subgroups.

	Total		Dead		Alive		
Total	**number**	**(%)**	**number**	**(%)**	**number**	**(%)**	**p value**
Total	53	100	11	21	42	79	
Age							0.745
<65 years	27	51	5	45	22	52	
≥65 years	26	49	6	55	20	48	
Gender							0.701
Female	17	4	3	27	14	33	
Male	36	68	8	73	28	67	
Distance to anal verge (cm)							0.735
<5	24	45	4	36	20	48	
≥5	29	55	7	64	22	52	
Tumor grade (differentiation)							0.308
Well-moderate	24	45	3	27	21	50	
Poor	29	55	8	73	21	50	
Clinical tumor (T) stage							0.324
cT1-2	6	11	0	0	6	14	
cT3-4	47	89	11	100	36	86	
Clinical nodal (N) stage							0.667
cN0	10	19	1	9	9	21	
cN+	43	81	10	91	33	79	
Pre-treatment MRI evaluation of CRM							0.765
CRM (-)	31	58	6	55	25	60	
CRM (+)	22	42	5	45	17	40	
Pre-treatment MRI evaluation of EMVI							0.482
EMVI (-)	38	72	9	82	29	69	
EMVI (+)	15	28	2	18	13	31	
Pre-treatment CEA level (ng/mL)							0.645
Low	39	74	8	73	31	74	
High	14	26	3	27	11	26	
Pre-treatment Ca19-9 level (U/mL)							0.942
Low	38	72	8	73	30	71	
High	15	28	3	27	12	29	
53BP1 expression level							0.361
Negative	3	6	0	0	3	7	
Positive	50	94	11	100	39	93	
CD73 expression level in stroma							0.056
Negative	25	48	8	73	17	40	
Positive	28	52	3	27	25	60	
CD73 expression level in tumor cells							0.010
Negative	36	68	11	100	25	60	
Positive	17	32	0	0	17	40	
Immunoscore							0.228
≤2	41	77	10	91	31	74	
>2	12	23	1	9	11	26	
Pathologic complete response							0.102
Yes	8	15	0	0	8	19	
No	45	85	11	100	34	81	
Tumor regression grade							0.116
Good responder	21	42	2	18	19	45	
Poor responder	32	58	9	82	23	55	

MRI: Magnetic resonance imaging; CRM: Circumferential resection margin; EMVI: Extramural vascular invasion; CEA: Carcinoembryonic antigen; 53BP1: P53 binding protein 1.

munoscore had numerically longer survival times. After the survival data has matured, the impact of the immunoscore on DFS and OS must be re-evaluated. In contrast, it may be rational to combine CD73 with CD3, CD8, and CD45, which comprise the immunoscore.

There are some limitations to our study. First, its retrospective design and the small number of patients. Additionally, the effect of the markers used on neoadjuvant radiotherapy could not be determined completely. Considering that radiotherapy provides local control, local recurrence was detected in only 3 patients; therefore, we could not make additional statistics on the parameters that affect local recurrence. Our study also had a few strengths. All patients were given long-course radiotherapy and received capecitabine in the presence of radiotherapy. These patients, who received the same treatment and were staged by the same radiologists and pathologists, could eliminate individual differences in the results. However, further studies should be conducted to evaluate the immunoscore in patients receiving TNT with a larger number of patients.

CONCLUSION

In conclusion, our study revealed that host- and tumor-related factors, were associated with treatment response. An association existed between immunoscore and CD73 expression and the response to neoadjuvant therapy. The markers to predict the nCRT response are needed, and additional research is required to evaluate the immunomarkers' effects.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Osman Sütcüoğlu, Mehmet Arda İnan, Nuriye Özdemir;* ***Design:*** *Osman Sütcüoğlu, Mehmet Arda İnan;* ***Control/Supervision:*** *Nalan Akyürek, Ozan Yazıcı, Nuriye Özdemir, Ahmet Özer;* ***Data Collection and/or Processing:*** *Osman Sütcüoğlu, Nazan Günel, Aytuğ Üner, Hüseyin Bora, Gözde Savaş, Nuriye Özdemir;* ***Analysis and/or Interpretation:*** *Osman Sütcüoğlu, Mehmet Arda İnan, Ozan Yazıcı;* ***Literature Review:*** *Osman Sütcüoğlu, Mehmet Arda İnan;* ***Writing the Article:*** *Osman Sütcüoğlu, Mehmet Arda İnan;* ***Critical Review:*** *Ahmet Özer, Nazan Günel, Aytuğ Üner, Hüseyin Bora, Gözde Savaş, Nuriye Özdemir;* ***References and Fundings:*** *Osman Sütcüoğlu, Nuriye Özdemir;* ***Materials:****Osman Sütcüoğlu, Mehmet Arda İnan.*

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