Original paper

Clinical role of CD274 (PD-L1) and CD3+ lymphocytes in predicting high risk in advanced colorectal cancer patients receiving neoadjuvant chemotherapy

Suat Benek[1], Mehmet Zengin[2]

[1]Department of General Surgery, Tekirdağ University, Tekirdağ, Turkey
[2]Department of Pathology, Ankara Training and Research Hospital, Ankara, Turkey

In cancer research, the mechanism underlying the immune response to a tumour has been of great interest. In this study, we investigated the role of CD274 (programmed cell death-ligand 1 – PD-L1) and CD3+ tumour-infiltrating lymphocytes (TILs) in the prognosis of advanced colorectal cancer (CRC) patients treated with neoadjuvant chemotherapy.

We retrospectively examined primary tumour specimens from stage III/IV CRC patients operated on between 2008 and 2018.

We found a significant association between these biomarkers and pT stage (PD-L1, $p = 0.020$; CD3+TILs, $p = 0.025$), tumour grade (PD-L1, $p = 0.005$; CD3+TILs, $p = 0.004$), positive surgical margin (PD-L1, $p = 0.001$; CD3+TILs, $p = 0.001$), MSI (PD-L1, $p < 0.001$; CD3+TILs, $p < 0.001$), etc. We also discovered that these biomarkers are independent risk factors for MSI (PD-L1, OR = 1.84 [1.27–4.02], $p = 0.003$; CD3+TILs, OR = 1.92 [1.31–4.35], $p = 0.008$). Univariate analysis results revealed that patients with high PD-L1, low CD3+TIL, and both showed poor relapse-free survival (RFS) and poor overall survival (OS) (PD-L1: RFS, $p = 0.008$ and OS, $p = 0.001$; CD3+TILs: RFS, $p = 0.003$ and OS, $p = 0.005$; PD-L1 and CD3+TILs: RFS, $p < 0.001$ and OS, $p < 0.001$). The results of the multivariate analysis showed that the combined use of high PD-L1 and low CD3+TILs was a better predictor of poor RFS and OS (PD-L1 and CD3+TILs: RFS, hazard ratio – HR, = 2.85 [95% CI: 1.36–3.84], $p < 0.001$); OS, HR = 2.74 [1.32–3.71], $p < 0.001$). We also found a high PD-L1 parameter as another independent overall and relapse-free survival parameter.

Our findings suggest that a combination of high PD-L1 and low CD3+TIL can reliably predict poor survival in CRC patients receiving chemotherapy. Therefore, these biomarkers may be promising for the planning and execution of appropriate targeted therapies.

Key words: colorectal cancers, PD-L1, CD3+ lymphocytes, prognostic biomarkers, stage III/IV.

Introduction

Colorectal cancers (CRC) are among the deadliest tumours in the world [1]. There are many risk factors for CRC, including tobacco, alcohol, ulcerative colitis, sedentary lifestyle, ageing, microsatellite imbalance (MSI), chromosomal imbalances, and genetic mutations [1, 2]. In general, surgery alone is preferred in early-stage CRC patients, while chemo-radiotherapy is added in advanced stages [2]. Unfortunately, most CRC cases are diagnosed in advanced stages (stage III/IV). Although better results have been obtained in the treatment of CRC with the developments in diagnostic methods and treatment approaches, patients in advanced stages still face a very high rate of recurrence and death [2, 3]. The TNM system decides on treatment by considering only traditional pathological parameters [4]. However, some subpopulations receiving targeted therapies may yield beneficial results in terms of survival [4, 5]. Therefore, it seems necessary to investigate new survival markers to contribute to the clinical management of patients. Recent studies have highlighted the critical role of immunosuppression and the tumour environment in tumour development and the course of CRC, and programmed cell death-ligand 1 (PD-L1) and CD3+ tumour-infiltrating lymphocytes (TILs) have recently been shown to be promising markers [5].

The mechanism underlying cancer development is quite complex, and cancer cell-specific features alone may not be sufficient to explain the results [6]. That is, the clinical course and metastasis of cancers are highly affected by the tumour microenvironment [6, 7]. The tumour microenvironment contains a complex structure of various immune cells such as cancer-associated fibroblasts, lymphocytes, dendritic cells, and tumour-associated macrophages [7, 8]. Recently, there has been increasing evidence that TILs play a key role in the tumour microenvironment [8, 9], and diffuse TILs have been found in many malignant solid tumours [10–15]. Also, research has focused on how inflammatory cells and their subtypes in CRCs are distributed, and very promising findings have been obtained. For example, numerous studies have shown an association between a dominant CD3+TILs response and a favourable clinical course [16, 17]. That is, with the further elucidation of their molecular pathways, CD3+TILs can provide very useful information about the prognostic course of CRCs [18, 19]. On the other hand, CD274 (PD-L1) ranks first among the molecules that inhibit T-cells [20]. PD-L1 is expressed during the activation of T-cells and leads to reductions of T-cells [20, 21]. Many recent studies have investigated the PD-L1 pathway and have proven the effectiveness of this checkpoint protein for survival. For example, studies have shown that anti-PD-L1 agents function well in many advanced solid cancers [22–26]. However, research has mostly focused on the expression of PD-L1, and information on the relationship between this biomarker and lymphocyte subtypes and its therapeutic and prognostic value is very limited.

In this study, we aimed to investigate the expression of PD-L1 and CD3+TILs in stage III/IV CRCs and to examine their effects on survival.

Materials and methods

We designed our study according to the recommendations of REMARK [27]. Figure 1 presents the flowchart of the study. We searched for two biomarkers with promising results in the literature. We tried to explain the complex processes in the tumour microenvironment. We discussed CRC, which are among the deadliest tumours. We studied a relatively homogeneous sample. We examined stage III/IV tumours, which are the most common tumour stages in CRC patients.

Background

Kırıkkale University Health Research Ethics Committee gave ethical approval to our study (Protocol no: 2019.06.05). Also, we carried out all the proce-

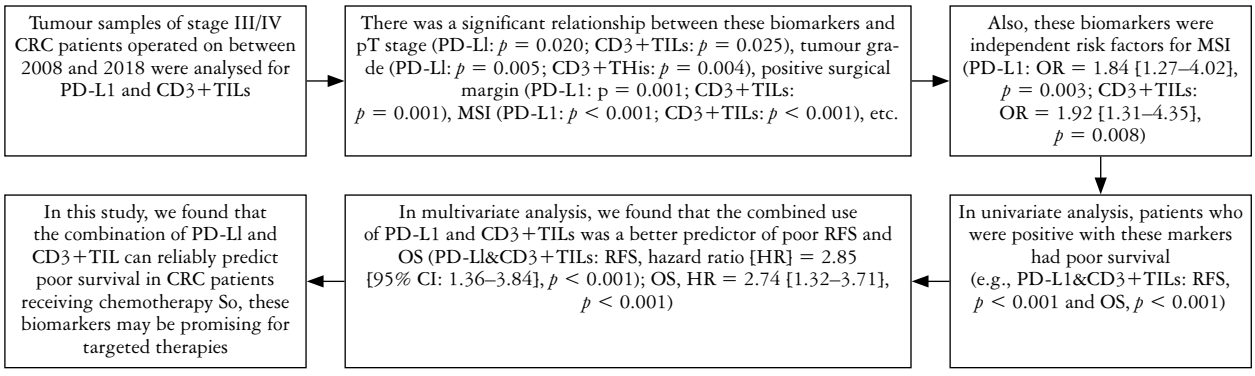

Fig. 1. Schematic summary of the study

CI – confidence interval, CRC – colorectal cancer, HR – hazard ratio, MSI – microsatellite instability, OR – odds ratio, OS – overall survival, PD-L1 – programmed cell death ligand-1, RFS – relapse-free survival, TILs – tumour infiltrating lymphocytes

dures in the study in accordance with the 1964 Helsinki Declaration and the relevant national/institutional ethical standards.

We performed the study retrospectively in Kırıkkale University Faculty of Medicine, Department of Pathology. We obtained the medical history of 343 patients operated on for CRC between 2008 and 2018 from the Kırıkkale University Hospital digital database. We excluded a total of 26 patients with postoperative mortality within one month, multiple tumours, and neoadjuvant therapy. In addition, 13 cases had scarce tumour tissue in paraffin blocks, we could not reach paraffin blocks in 12 cases, and 64 cases had different tumour stages. As a result, we conducted the study with a total of 228 patients.

Patients

We used the university's archive records to collect clinical, pathological, and survival information of the participants. We categorized CRCs according to the following criteria: age (< 68 and ≥ 68), gender (female and male), tumour location (right and left), tumour size (< 5.5 cm and ≥ 5.5 cm), pT stage (pT I–II and pT III–IV), tumour grade (low/moderate and high grade), lymphatic-perineural invasion (yes and no), white blood cells (high and low), tumour budding (high and low), positive surgery border (yes and no), lymphocytes (high and low), and microsatellite instability (yes and no).

Haematological evaluation

Archival records of lymphocytes ($\times 10^9$/l) and white blood cells ($\times 10^9$/l) were used for haematological values. These records were obtained from blood samples taken from patients preoperatively into standard tubes with ethylenediaminetetraacetic acid. These blood samples were counted using an automated haematology analyser and evaluated by an experienced infectious disease specialist. Ranges accepted as normal by our hospital laboratory were taken as reference values.

Histopathological evaluation

We collected paraffin blocks and haematoxylin-eosin (H&E) stained sections from the archive room of the Department of Pathology. We found that the patients had tumour blocks ranging 3–22. We re-analysed the sections and selected one block appropriately representative of the tumours. We took 4 μm thick sections from these blocks and stained them with PD-L1, CD3 and H&E. We determined MSI status based on immunohistochemistry (IHC) and divided the conditions into two groups: MSI-proficiency (MSI-P) and MSI-deficiency (MSI-D). All evaluations were made by two expert pathologists blinded to the clinical and pathological information of the patients, in accordance with the guidelines of the American Joint Cancer Classification Committee [20].We evaluated tumours semi-quantitatively using a conventional light microscope (Nikon Eclipse E600, Switzerland) and a 20× objective, in accordance with the recommendations of the International Working Group on Tumor-Infiltrating Lymphocytes [28]. First, we scanned each section with a 10× objective to identify differences in inflammatory cell distribution within the tumour and selected a tumour area containing predominantly inflammatory cells. We evaluated lymphocytes with membranous staining (even if focal or weak) as positive for PD-L1 and CD3. However, we considered false IHC staining in the absence of clearly stained blue nuclei in lymphocytes. In this way, we divided the patients into two groups as positive and negative (Fig. 2).

Immunohistochemistry

Tonsil and colon tissues were used as positive and negative controls, respectively. First, we boiled the sections in citrate buffered solution in the microwave for 10 minutes. Then, we kept the sections in 0.3% hydrogen peroxide-methanol solution for 10 minutes at room temperature. We used rabbit monoclonal PD-L1 antibody (Abcam, 1 : 100, ab205921, Cambridge, UK) and rabbit monoclonal CD3 antibody (Abcam, 1 : 50, ab16669, Cambridge, UK) as primary antibodies. We incubated the sections with these antibodies overnight at room temperature, and with the secondary antibody (Dako) for one hour the next day. Finally, we applied haematoxylin (Merck, Darmstadt, Germany) to the sections and closed with Pertex (Histolab, Gothenburg Sweden). PD-L1 expression was scored according to the proportion of PD-L1+ tumour cells (TC 0 for $< 1\%$, TC 1 for 1–4%, TC2 for 5–49%, and TC 3 for $\geq 50\%$). TC < 2 and ≥ 2 were regarded as tumours with low and high expression, respectively [15]. The CD3 densities were assigned one of four scores: 0 (no positive cells), 1 (a few scattered individual positive cells), 2 (small positive cell clusters with approximately 5% of all cells staining positively), and 3 (more abundant and organized staining with more than approximately 10% of all cells staining positively). For the final statistical analysis, the $CD3^T$ and $CD8^T$ densities were grouped as 'low' (score 0–1) and 'high' (scores 2–3) [29].

Follow-up

In this study, we considered survival and recurrence rates as outcome measures. To calculate these rates, we calculated the time from the day of primary surgery to the event. We followed each patient (range 12.5–128.5 months) for ten years to make a more reliable judgment about clinical outcomes. However, we censored any event after sixty months of follow-up

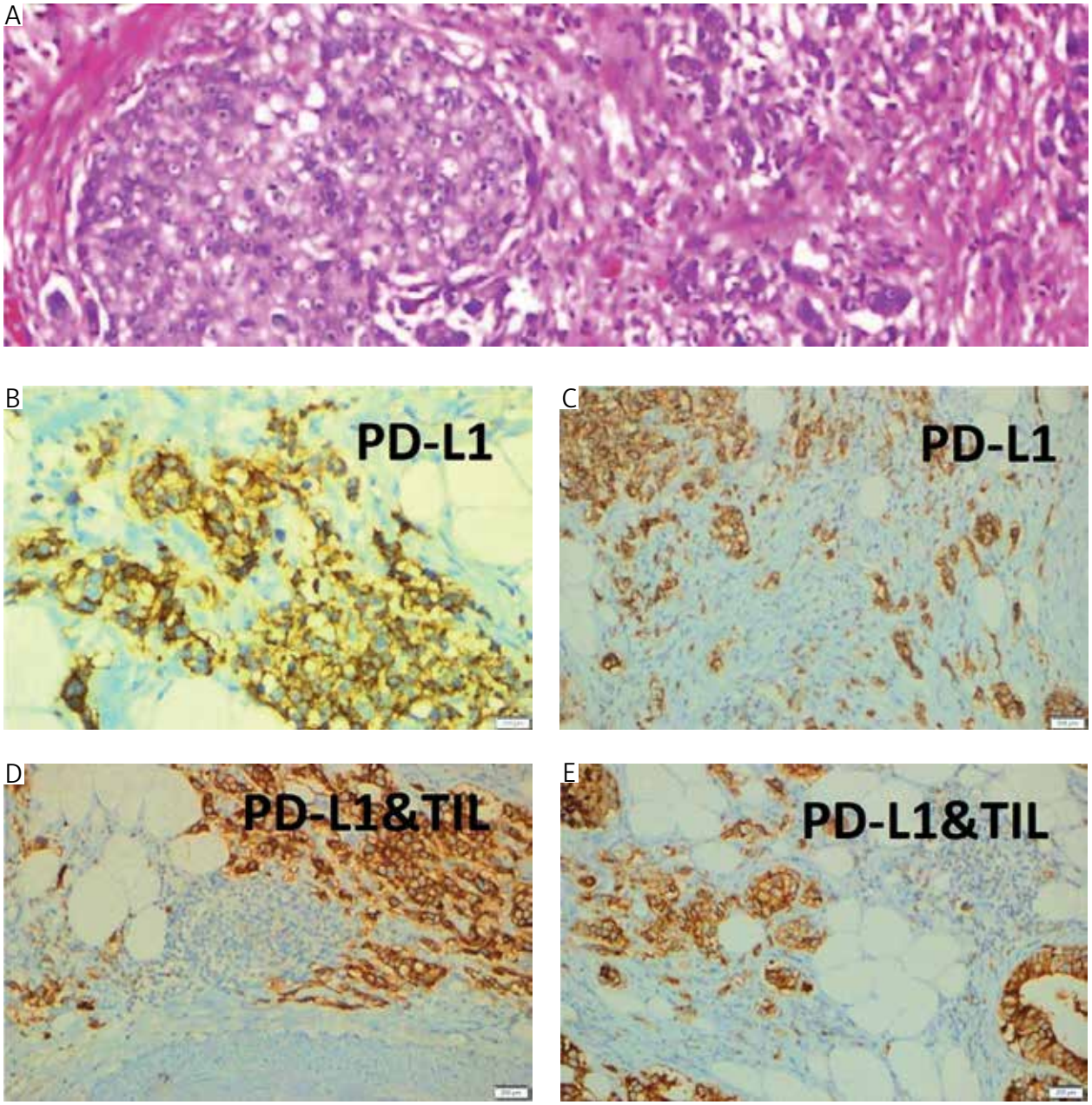

Fig. 2. Microscopic images of PD-L1 and CD3+TILs. After scanning all sections using H&E stained sections (A), PD-L1 (B, C) and PD-L1&CD3+TILs (D, E) evaluation was semi-quantitatively performed on IHC stained slides

H&E – haematoxylin and eosin, IHC – immunohistochemistry, PD-L1– programmed cell death ligand-1, TIL – tumour infiltrating lymphocyte

at sixty months. We also censored patients who were diagnosed with a secondary malignancy during follow-up at the time of diagnosis of this new cancer. We defined overall survival (OS) as the time between the date of first surgery and the date of death from any cause or date of the last follow-up. We defined recurrence-free survival (RFS) as the time between the date of first surgery and the date of death from any cause or date of distant/regional recurrence.

We confirmed the survival data of the patients by contacting them from the contact numbers in the hospital records. We checked patients quarterly for the first two years, every six months for the next three years, and annually thereafter. When the patients came to the examination during the postoperative period, we performed a complete physical examination and evaluated the tumour markers. We performed a follow-up colonoscopy on the patients one year after the operation. If we did not see any pathology, we called the patients for a second colonoscopy after three years at the earliest. For the first two years, we invited patients for chest X-rays every three months and a computed tomography scan of the abdomen every six months.

Statistical analysis

We presented the data as a percentage, frequency, range, median and standard deviation. We used χ^2 and logistic regression tests (95% CI and 1.0 odds ratio – OR) to investigate univariate and multivariate prognostic parameters. As mentioned earlier, we used the κ test for interobserver agreement, the Spearman test and Wilcoxon signed-rank test for correlations/differences between estimates, respectively. We used log-rank and Cox regression tests (95% CI and 1.0 hazard ratio – HR) for univariate and multivariate survival analyses. We used Kaplan-Meier analysis to present the survival curves. We performed all statistical analyses with SPSS 21.0 (IBM Institute, North Castle, USA) and considered $p < 0.05$ statistically significant.

Results

General features

Of the patients, 141 (61.8%) were male and 87 (38.2%) were female. The median age was 68 (34–89 years), and the median tumour size was 5.5 cm (3–10 cm). Low differentiated tumours were detected in 90 patients (39.4%), and moderately/poorly differentiated tumours in 138 patients (60.6%). Eighty-three (36.4%) patients were pT I–II, 145 (63.6%) patients were pT III–IV. Considering the location of the tumours, the tumour was located in the right colon in 83 (36.4%) cases and in the left colon in 145 (63.6%) cases.

Histopathological research

We scanned all sections at low power magnification and found that most staining was heterogeneously distributed and more pronounced in invasive areas. As a result of statistical analysis, we found that there was a significant difference between these markers and poor prognostic parameters (for PD-L1: pT stage [$p = 0.020$], tumour grade [$p = 0.005$], positive surgical margin [$p = 0.001$], lymphocytes (blood) [$p = 0.005$], MSI [$p < 0.001$]; for CD3: pT stage [$p = 0.025$], tumour grade [$p = 0.004$], positive surgical margin [$p = 0.001$], lymphocytes (blood) [$p = 0.002$], MSI [$p < 0.001$]). Also, we concluded by logistic regression analysis that PD-L1 expression and CD3+TILs are independent risk factors for MSI (PD-L1: OR = 1.84 [1.27–4.02], $p = 0.003$; CD3+TILs: OR = 1.92 [1.31–4.35], $p = 0.008$) (Table I, II).

Patient follow-up

During follow-up, we found that 158 patients died (PD-L1 positive = 124, CD3+TILs = 122, PD-L1&CD3+TILs = 128) and 171 patients relapsed (PD-L1 positive = 127, CD3+TIL positive = 124, PD-L1&CD3+TILs = 134). The 5-year OS and RFS rates were 22% and 26% in PD-L1 positive patients, 23% and 28% in CD3+TIL positive patients, and 19% and 22% in PD-L1&CD3+TIL positive patients, respectively (Table III).

Survival analyses

In the univariate survival analysis, we found that there was a significant difference between the groups in terms of PD-L1(RFS, $p = 0.008$; OS, $p = 0.001$), CD3+TILs (RFS, $p = 0.0033$; OS, $p = 0.005$), and PD-L1&CD3+TILs (RFS, $p < 0.001$; OS, $p < 0.001$). We concluded that using these parameters together in multivariate survival analysis is a more successful independent poor survival parameter for RFS (HR = 2.85 [95% CI: 1.36–3.84], $p < 0.001$) and OS (HR = 2.74 [1.32–3.71], $p < 0.001$). We also found that another independent poor prognostic parameter was PD-L1 (Table IV, Fig. 3).

Discussion

In this study, we aimed to investigate whether PD-L1 and CD3+TILs have an effect on the survival of patients with stage III/IV CRC. Our results revealed that the combined use of these parameters can quite successfully predict poor prognosis in CRC. We also found that these parameters are very interestingly related to MSI.

Cancer cells use a variety of mechanisms to hide from the immune system, including down-regulating foreign tumour antigens, secreting anti-inflammatory cytokines, and expressing negative regulators of the immune system. And so they create a microenvironment that suppresses the immune system [6–9]. Although the host resists many solid tumours with the immune response, the immune response factors regulated by cancer offset this resistance [10–15]. PD-1 is one of the most important elements of this interaction between cancer and the immune system. Although there are many ligands in the PD-1 pathway, the most critical one is CD274 (PD-L1, B7-H1) [20]. When PD-L1 complexes with PD-1, the effector functions of T cells are reduced [20, 21]. It is known that PD-L1 expression in many epithelial cancer cells paves the way for cancer development and inhibits the antitumour response. For example, anti-PD-L1 agents have been reported to be successful in head and neck, cervical, and small cell lung carcinoma [22–24]. Studies on the relationship between CRC and prognosis are limited in the literature and controversial results are also encountered [25]. Although many studies have demonstrated a significant relationship between PD-L1 expression and low survival rates of CRC patients, there are also studies that have failed to detect any association [25, 26].

Table I. Statistical analysis of demographic and clinicopathological characteristics of patients

Parameters		PD-L1		*p*-value	CD3+TILs		*p*-value
		Low	High		High	Low	
Age				0.766			0.746
	< 68, *n* (%)	42 (39)	46 (37)		39 (39)	49 (37)	
	≥ 68, *n* (%)	64 (61)	76 (63)		59 (61)	81 (63)	
Gender				0.331			0.320
	Female, *n* (%)	44 (41)	43 (35)		41 (42)	46 (35)	
	Male, *n* (%)	62 (59)	79 (65)		57 (58)	84(65)	
Tumour location				0.475			0.306
	Right, *n* (%)	36 (33)	47 (38)		32 (32)	51 (39)	
	Left, *n* (%)	70 (67)	75 (62)		66 (68)	79 (61)	
Tumour size				0.264			0.550
	< 5.5 cm, *n* (%)	45 (42)	43 (35)		40 (41)	48 (37)	
	≥ 5.5 cm, *n* (%)	61 (58)	79 (65)		58(59)	82 (63)	
Lymphatic-perineural invasion				0.803			0.746
	No, *n* (%)	40 (37)	48 (39)		39 (39)	49 (37)	
pT stage	Yes, *n* (%)	66 (63)	74 (61)		59 (61)	81 (63)	
				0.020*			0.025*
	pT I–II, *n* (%)	47 (44)	36 (32)		44 (45)	39 (29)	
	pT III–IV, *n* (%)	59 (56)	86 (68)		54 (55)	89 (71)	
Tumour grade				0.005*			0.004*
	Low/moderate, *n* (%)	52 (49)	38 (29)		49 (50)	41 (31)	
	High, *n* (%)	54 (51)	84 (71)		49 (50)	89 (69)	
WBC				0.069		0.083	
	Low, *n* (%)	49 (46)	42 (34)		45 (46)	45 (34)	
	High, *n* (%)	57 (54)	80 (66)		53 (54)	85 (66)	
Tumour budding				0.865			0.838
	Low, *n* (%)	42 (39)	47 (38)		39 (39)	50 (38)	
	High, *n* (%)	64 (61)	75 (62)		59 (61)	80 (62)	
Positive surgical margin				0.001*			0.001*
	No, *n* (%)	54 (52)	37 (29)		51 (54)	40 (30)	
	Yes, *n* (%)	52 (48)	85 (71)		47 (46)	90 (70)	
Lymphocytes (blood)				0.005*			0.002*
	High, *n* (%)	51 (48)	37 (29)		49 (50)	39 (29)	
	Low, *n* (%)	55 (52)	85 (71)		49 (50)	91 (71)	
MSI				< 0.001*		32 (23)	< 0.001*
	MSI-P, *n* (%)	50 (47)	30 (23)		47 (48)	98 (77)	
	MSI-D, *n* (%)	56 (53)	92 (77)		51 (52)		

MSI – microsatellite instability, MSI-D – microsatellite instability-deficiency, MSI-P – microsatellite instability-proficiency, PD-L1 – programmed cell death ligand-1, TIL – tumour infiltrating lymphocytes, WBC – white blood cells
** The significance level for the p-value was 0.05. Significant results are shown in italics.*

Table II. Multivariate regression analysis of the five parameters that were statistically significant

Parameters	PD-L1	*p*-value	CD3+TILs	*p*-value
	OR (95% CI)		OR (95% CI)	
pT stage	3.47 (0.71–4.45)	0.416	3.76 (0.68–4.32)	0.424
Tumour grade	3.63 (0.73–4.88)	0.235	3.28 (0.74–3.62)	0.235
Positive surgical margin	2.25 (0.84–5.32)	0.284	2.11 (0.79–5.46)	0.246
Lymphocytes (blood)	3.15 (0.68–4.87)	0.316	3.26 (0.64–3.92)	0.364
MSI status	1.84 (1.27–4.02)	0.003*	1.92 (1.31–4.35)	0.008*

CI – confidence interval, MSI – microsatellite instability, OR – odds ratio, PD-L1 – programmed cell death ligand-1, TIL – tumour infiltrating lymphocytes
** The significance level for the p-value was 0.05. Significant results are shown in italics.*

Different molecular pathways may have led to these different prognostic effects of PD-L1 on CRCs, which we see in the literature. For example, some studies have reported alternative secondary receptors for PD-L1 [20, 30]. Although the function of PD-L1 in suppressing the T-lymphocyte response has been demonstrated in many studies [31, 32], its effect may vary due to the variability in the immune background of tissue types and cancers. It has not yet been determined which transcription factors are involved in the interaction between PD-L1 and the immune system and which inhibitor molecules are mediated. Furthermore, it is unclear what overlapping mechanisms exist in the intracellular pathways of these parameters. In our study, we found a significant association between CRC patients with PD-L1 expression and poor prognosis. In addition, this significant relationship was more evident in cases with low CD3+TILs. In this context, our findings may bring a different perspective to PD-L1. For example, cancer therapy has now met with promising immune checkpoint-related agents, and CD3+TILs can be a helpful marker in patient selection. In addition, elucidating the molecular infrastructure of this relationship may open the door to new therapeutic agents. In conclusion, further studies are needed in CRC to reveal the PD-L1 molecular pathways.

It has been shown in the literature that lymphocytes are very important prognostically in patients with CRC. Lymphocytes generate an effective immune response by attracting different cell types into the tumour microenvironment [8, 9]. Studies have shown that significant T-cell infiltration is an independent survival parameter [10–15]. Also, investigations have demonstrated that solid T cell infiltration is among the most critical outcome markers in CRC [12, 33]. The clinical significance of TIL subpopulations has been the subject of many studies, although it is not fully known whether subtypes of lymphocytes display specific host characteristics or affinity for various tumours [18, 19, 34, 35]. For example, Turksma *et al*. [34] examined TILs in CRC and found that the presence of CD3+ lymphocytes plays a positive role in survival. Deschoolmeester performed multivariate analyses on a range of prognostic factors and found CD3+ lymphocytes to be among the most informative markers. In this study, we found a strong association between low CD3+ lymphocytes and poor prognosis [34]. We also found that tumours expressing PD-L1 had significantly fewer CD3+ lymphocytes. This finding may help to significantly broaden the perspective on current prognostic indicators. For example, since tumours use these pathways during the invasion, co-inhibiting them may also be a treatment option in CRC. With regard to clinical practice, these findings may provide important prognostic information in patient selection for chemoradiotherapy. More comprehensive studies can contribute to the subject.

Although the development of many CRCs is affected by chromosomal instability, MSI is associated with approximately 12–15% of these [34, 36, 37]. MSI-associated CRCs usually present as right-sided, mucinous, lymphocyte-rich neoplasms and have a better prognosis than others [38]. Such tumours often show elevated T-lymphocyte infiltration and upregulation of T-cell immune checkpoints [37, 38]. Immune checkpoints have been demonstrated to be a very important step in preventing tumour invasions. Inhibition of these molecules can enhance the immune response, inhibit tumour progression, and promote tumour regression [38, 39]. However, few studies are available on the prognostic effects of PD-L1 in patients with MSI-associated CRC, with differing results. Le Dung *et al*. [39] found that approximately one-third of MSI-related CRC cases receiving anti-PD-1 agents responded favourably to treatment. In the same study, PD-L1 expression was also shown to be associated with the survival of MSI-associated CRC patients. We also obtained similar findings in our study. Such a finding, which overlaps with those found in the literature, is unexpected and striking, because it shows that different PD-L1 pathways may use different mechanisms of action within the heterogeneous CRC spectrum. Further and comprehensive research may clarify this issue.

Table III. Univariate survival analysis

Parameters		Overall survival	*p*-value	Relapse-free survival	*p*-value
		5-year survival (%)		5-year survival (%)	
Age			0.474	51	0.463
	< 68	52			
	≥ 68	41		40	
Gender			0.445		0.452
	Female	55		54	
	Male	45		44	
Tumour location			0.276		0.283
	Right	49		50	
	Left	36		35	
Tumour size			0.224		0.212
	< 5.5 cm	48		50	
	≥ 5.5 cm	35		37	
Lymphatic-perineural invasion			0.618		0.638
	No	51		50	
	Yes	45		45	
pT stage			0.025*		0.038*
	pT I–II	48		49	
	pT III–IV	35		32	
Tumour grade			0.033*		0.047*
	Low/moderate	50		50	
	High	33		35	
WBC			0.598		0.579
	Low	51		50	
	High	44		45	
Tumour budding			0.417		0.384
	Low	52		52	
	High	43		42	
Positive surgical margin			0.008*		0.035*
	No	46		47	
	Yes	26		29	
Lymphocytes (blood)			0.030*		0.042*
	High	45		46	
	Low	30		30	
MSI			0.027*		0.038*
	MSI-P	47		46	
	MSI-D	29		28	
PD-L1			0.001*		0.008*
	Low	45		47	
	High	22		26	
CD3+TILs			0.005*		0.033*
	High	46		47	
	Low	23		28	
PDL1&CD3+TILs***			< 0.001*		< 0.001*
	No	45		44	
	Yes	19		22	

MSI – microsatellite instability, MSI-D – microsatellite instability-deficiency, MSI-P – microsatellite instability-proficiency, PD-L1 – programmed cell death ligand-1, TIL – tumour infiltrating lymphocytes, WBC – white blood cell
** The significance level for the p-value was 0.05. Significant results are shown in italics.*
**** PD-L1-High and CD3+TILs-Low*

Table IV. Multivariate survival analysis

Parameters	Overall survival		Relapse-free survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
pT stage	3.48 (0.63–4.39)	0.464	3.64(0.71–4.63)	0.466
Tumour grade	3.52 (0.68–4.42)	0.273	3.55 (0.70–4.71)	0.273
Positive surgical margin	1.23 (1.07–2.76)	0.043*	3.32 (0.85–5.86)	0.257
Lymphocytes (blood)	4.35 (0.61–3.42)	0.449	3.26 (1.15–4.53)	0.354
MSI status	3.72 (0.73–5.52)	0.286	3.83 (0.79–5.28)	0.329
PD-L1	2.56 (1.19–3.43)	0.005*	2.48 (1.18–3.67)	0.042*
CD3+TILs	2.63 (1.23–3.69)	0.042*	2.34 (0.89–5.55)	0.248
PDL1&CD3+TILs	2.74 (1.32–3.71)	< 0.001*	2.85 (1.36–3.84)	< 0.001*

CI – confidence interval, HR – hazard ratio, MSI – microsatellite instability, PD-L1 – programmed cell death ligand-1, TIL – tumour infiltrating lymphocytes
** The significance level for the p-value was 0.05. Significant results are shown in italics.*
**** PD-L1-High and CD3+TILs-Low*

The low standardization and reproducibility of the methods can be considered as a disadvantage of the TIL assessment. Visualization, scoring, and staining are the main sources of variability in the evaluation of TIL, and the literature is home to hundreds of methodologically diverse studies. Some studies in the literature have investigated inflammatory cells in the stromal and intraepithelial compartments [34]. Others have evaluated inflammatory cells in the centre and invasive front of tumours [40]. Also, some have studied inflammatory cells in the neoplastic epithelial cells [41]. In this study, we took into account the recommendations of the International Tumor-Infiltrating Lymphocytes Study Group [28]. In conclusion, unlike the studies above, we used a reliable method in this study and thus greatly increased standardization and reproducibility.

The present work has notable strengths. First, we investigated two biomarkers that had previously proven to be promising parameters. Second, we discussed CRC, which is among the most common lethal tumours. Next, we examined a relatively homogeneous patient sample and performed a standardized assessment. Finally, we performed the study following the REMARK guidelines.

On the other hand, this was a retrospective study and it was not possible to control for the sample differences. Also, we were only able to examine small portions of tumours, and this sample may not be representative of the entire tumour. Finally, participants' receiving treatment in accordance with pre-2015 guidelines may differ from current approaches.

Conclusions

We found that PD-L1 and CD3+TILs are independent predictors of poor prognosis in stage III/IV CRC patients. Also, a significant association of these

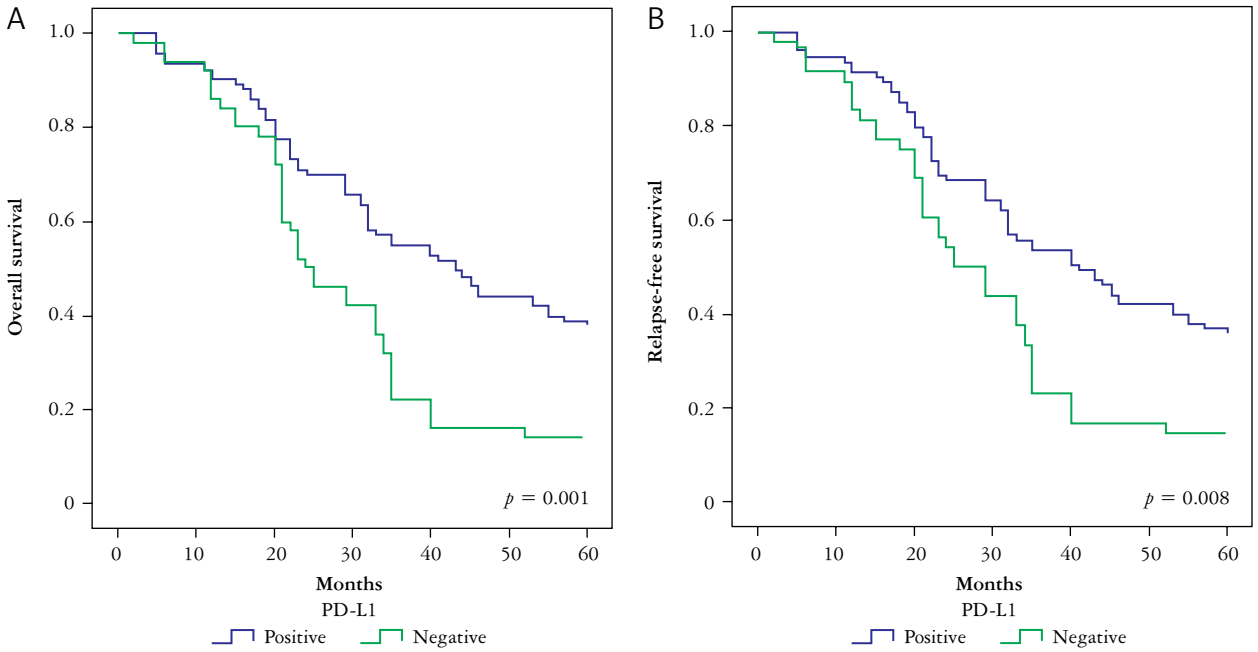

Fig. 3. Kaplan-Meier survival curves of PD-L1 and CD3+TILs

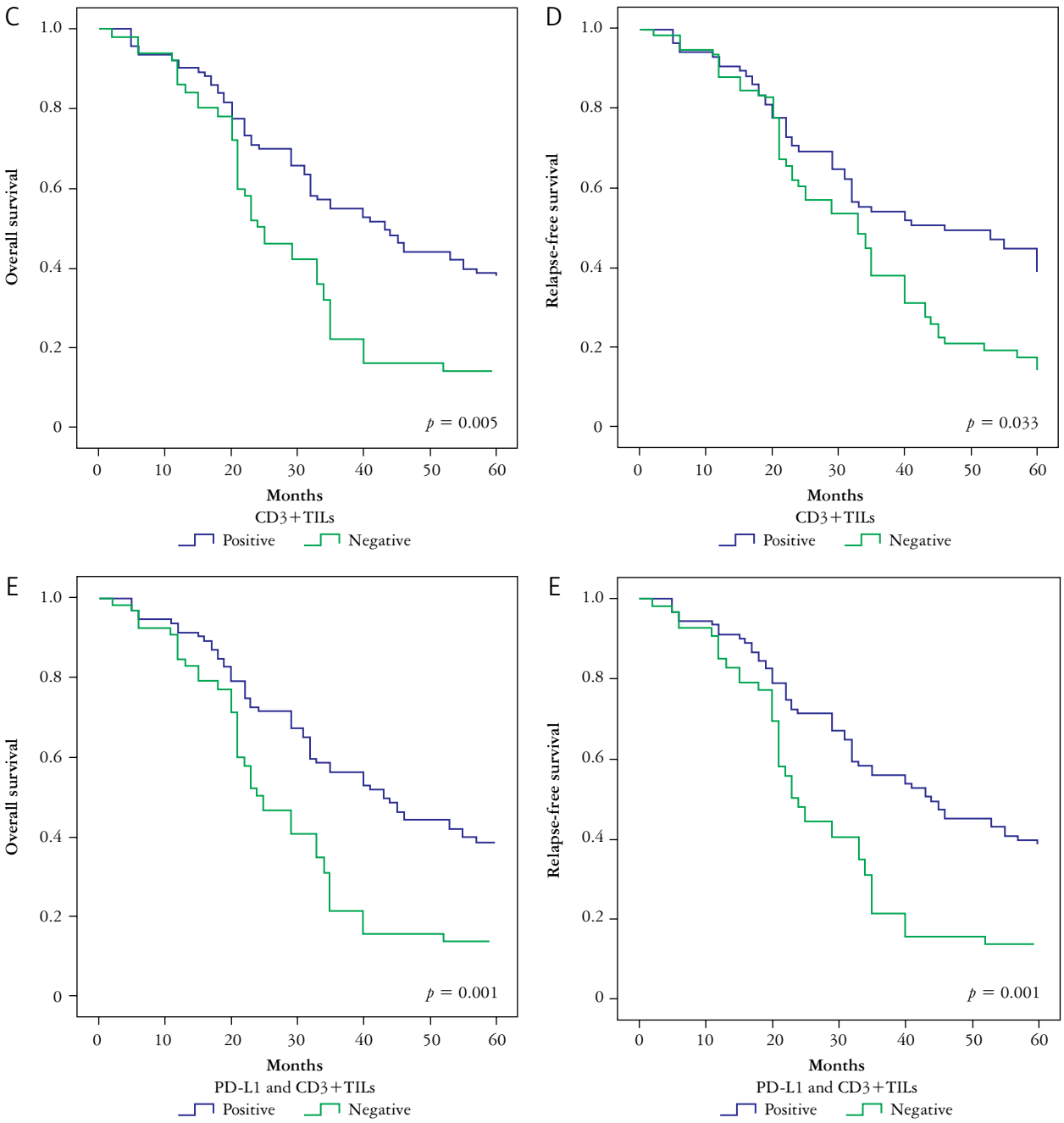

Fig. 3. Cont.

parameters with MSI may contribute to the elucidation of the tumour microenvironment and targeted therapies. In addition, these biomarkers can be very useful in daily practice as they offer the desired reproducibility in predicting prognosis.

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Address for correspondence

Assistant Professor **Suat Benek**
Department of General Surgery
Tekirdağ University
Tekirdağ, Turkey
e-mail: cerrahsbenek@gmail.com