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Evaluation of prognostic significance of histopathological characteristics and tumor-infiltrating lymphocytes for pancreatic cancer survival

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With a 5-year survival of < 10%, pancreatic cancer is one of the leading causes of cancer-related deaths. Given the role of the distribution of tumor-infiltrating lymphocyte (TILs) subtypes in the tumor and its microenvironment in predicting prognosis, the development of new targeted therapies based on T-cell adaptive response has gained considerable attention. This study aimed to examine the peritumoral spread of TILs and its relationship with other prognostic parameters and survival. This study included 60 patients with pancreatic cancer who had undergone surgery with follow-up between 2011 and 2021. Demographic characteristics, tumor histopathological features, peritumoral TILs counts, and intratumoral programmed cell death protein-1 (PD-1) and programmed death ligand – 1 (PD-L1) positivity were evaluated. Furthermore, overall survival and their efficacy in predicting survival according to TNM stage were analyzed. The number of cluster differentiation-3 positive (CD3 P) TILs increased with advancing pathological T stage. CD3 P and CD8 P TIL counts were higher in patients with peripancreatic fatty tissue invasion. Patients with PD-L1 positivity and higher TIL counts had better survival rates. PD-L1-negative patients with a low CD8 positive/total lymph node count (P/T) ratio had a longer survival. Moreover, patients with poorly differentiated tumors with low CD3 P/T and CD8 P/T ratios had a longer survival. The CD3 P/T and CD8 P/T ratios were compatible with the automatic and manual measurements. Age, tumor differentiation, N stage, and peritumoral TIL count and subtype, when evaluated together with the presence of PD-L1 in the tumor tissue, may have prognostic significance for survival in patients with pancreatic cancer.

Keywords CD3, CD8, Pancreatic cancer, PD-1, PD-L1, Survival, Tumor-infiltrating lymphocytes

Pancreatic cancer ranks as the tenth most prevalent type of cancer among men in Turkey[1]. Pancreatic cancer is usually diagnosed at an advanced stage owing to the lack of a routine screening program and asymptomatic clinical progression, leading to a poor prognosis. Although surgical resection offers survival advantages, most patients are diagnosed in advanced stages, and only a small subset of patients are eligible for surgery[2]. The 5-year survival rate of patients with an unresectable pancreatic cancer was 0.8% in 1975 and 0.9% in 2011. Pancreatic cancer has a poor 5-year survival rate, ranging from 2 to 9%, with negligible differences between high-, low-, and middle-income countries[3].

With a better understanding of the stage and histopathological features of pancreatic ductal adenocarcinoma (PDAC), targeted treatments for specific subgroups have become prominent. Consequently, other factors that affect prognosis have been investigated. Incorporating these new parameters could help predict a more accurate prognosis. Tumor-infiltrating lymphocytes (TILs) are associated with survival[4,5], and the exploration of the tumor microenvironment (TME) has highlighted the role of TILs in determining the prognosis. However, the

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mechanism of interaction between TILs and tumors is complex and remains unclear[6]. Studies investigating the association between TILs and prognosis in several types of cancer, especially colorectal cancer, have demonstrated the importance of TILs[7].

TILs refer to specific killer lymphocytes present in the TME. Tumors are generally infiltrated by immune cells such as T and B lymphocytes, natural killer (NK) cells, macrophages, dendritic cells, neutrophils, eosinophils, and mast cells. These cells secrete various substances with potential anti-tumor effects. The response of these cells to tumors forms the basis of tumor immunology. Cytotoxic T lymphocytes are crucial for inducing antitumor responses. The TME contains cells such as fibroblasts, stromal cells, immune cells, and adipocytes that surround the tumor. Cluster differentiation-3 (CD3) encodes a transmembrane protein complex that serves as a marker for T cells[8]. The CD3-T lymphocyte complex plays a crucial role in the T cell-mediated immune response by facilitating the activation of immunocompetent T lymphocytes. CD8 positive (CD8 P) T lymphocytes detect and destroy cancer cells[9]. Programmed cell death protein-1 (PD-1), which is found in many cells such as B lymphocytes, T lymphocytes, and NK cells, has two types: programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2). PD-L1 binds to cells expressing PD-1 to prevent hyperimmune responses and autoimmunization. Increased PD-L1 activation in cancer cells results in anergy or apoptosis, followed by immune system evasion. High PD-L1 levels have been associated with poor prognosis in several tumors, including PDAC[10]. A meta-analysis highlighted that PD-L1 expression may correlate with the T stage[11].

This study aimed to investigate the relationship between peritumoral TILs with tumor histopathological features and survival in PDAC patients who had undergone surgical resection.

Materials and methods

The study was conducted in accordance with the Helsinki declaration with the informed consent of all patients and ethics committee approval was received from Haydarpaşa Numune Training and Research Hospital. This study was approved by the Health Sciences University Haydarpaşa Numune Training and Research Hospital Clinical Research Ethics Committee (approval no. HNEAH KAEK 2021/220 dated 06.09.2021). This study received funds from the Scientific Research Projects Coordination Department of the University of Health Sciences, which was used for immunohistochemical staining (Project Number: 2021/177). This retrospective study included 100 patients with PDAC who had undergone surgery at Health Sciences University Haydarpaşa Numune Training and Research Hospital between 2011 and 2021, with subsequent follow-up in the general surgery outpatient clinic. Patients aged > 18 years who had undergone R0 resection for primary ductal adenocarcinoma were included. Patients who received neoadjuvant treatment before the operation, those diagnosed with tumors other than primary ductal adenocarcinoma, those lost to follow-up or whose records could not be accessed, and patients who died within 30 days after the operation were excluded. Additionally, patients without tumor paraffin blocks in the pathology archive of our hospital were excluded. Finally, 60 patients who met the inclusion criteria were included in the final analysis. The clinicopathological characteristics of the patients, such as age, sex, tumor type, pathological tumor stage (pT stage), and lymph node metastasis (pN stage), were defined.

In our study, peritumoral CD3 and CD8 measurements were compared with PD-1 and PD-L1 measurements in tumor tissue using classical parameters that have prognostic importance, such as pathological stage, degree of tumor differentiation, perineural invasion (PNI), tumor diameter, and number of positive lymph nodes in PDAC, and their association with overall survival (OS) was analyzed to evaluate the prognostic significance of these factors.

Histopathological examination

Pathology reports of the resected specimens and hematoxylin and eosin (H&E)-stained preparations representing the tumor obtained from the pathology laboratory archives were examined by a pathologist.

Selection, preparation, and evaluation of tissue samples

Multiple H&E-stained preparations showing the tumor and surroundings of the resected specimens obtained from the pathology laboratory archives were re-evaluated under a conventional light microscope. Preparations that were not suitable for evaluation were re-stained with H&E using 4 µ thick sections taken from the paraffin blocks. These preparations were examined by a pathologist blinded to the patients' clinical results. Parameters such as the histological grade, pT stage, lymphovascular invasion, PNI, and N stage were reviewed, and the original pathology report was confirmed. Pathological staging of the tumors was performed according to the eighth edition of the American Joint Committee on Cancer[12]. For the immunohistochemical study, the H&E-stained preparations were examined under a light microscope with a 20× objective and a 40× objective. Areas that did not contain necrosis and/or hyalinization, artifacts, and inflammation were selected in the center of the tumor and at the invasive border of the tumor in microscopic sections for further evaluation[13]. To evaluate the peritumoral T lymphocytes at the tumor invasive border, a 5 mm wide region around the tumor was selected as the invasive border. Lymphoid aggregates were not evaluated[14].

Immunohistochemical staining

The formalin-fixed paraffin block of the selected tumor was kept on a cold plate (Histo-Line, Medite Inc., Orlando, FL, USA), which was set at -10 °C for 20 min. The positively charged slides used in this study were barcoded by printing the appropriate barcodes on a Leica Bond-Max model IHC/ISH fully automatic staining device (Nussloch, Germany). Sections of 3 µ thickness were obtained from the paraffin block containing the tissue sample and placed on positively charged slides using a water bath set at 44 °C. Positive control tissues were obtained from the tissue sections for each study. The prepared sections were physically deparaffinized for 20 min in a preset 80 °C oven. The studies were performed according to the specified protocol using the DS9800 Polymer DAB IHC Detection Kit on a Leica Bond-Max model IHC/ISH automatic staining device (Nussloch,

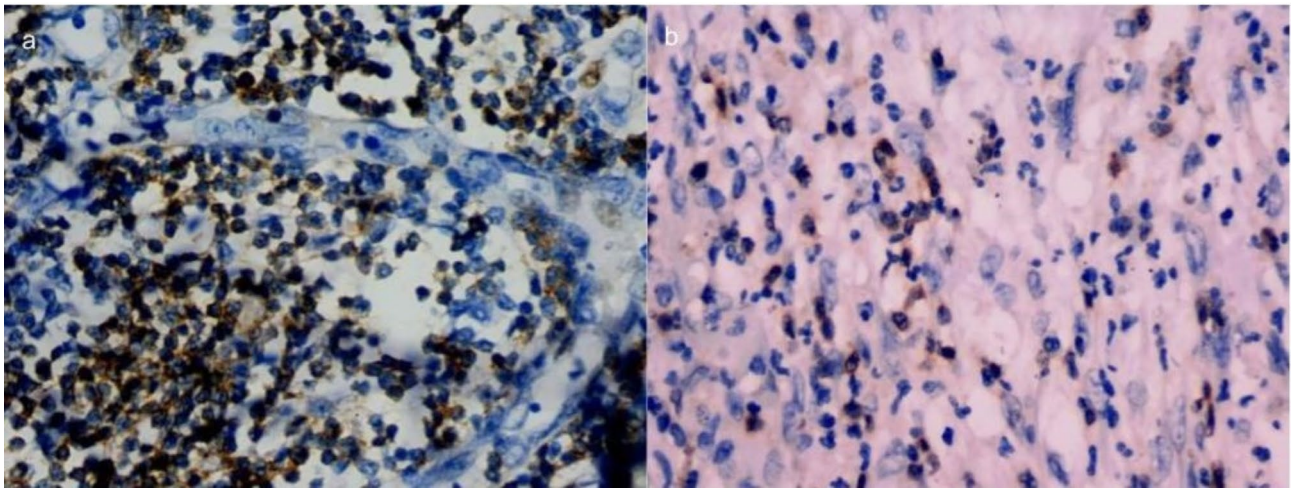

Fig. 1. Cluster differentiation-3 (CD3)-stained peritumoral area (viewed with Olympus BX51 (Tokyo, Japan) at 40× magnification). (**a**) Preparation with high-density of CD3-positive tumor-infiltrating lymphocyte (TILs) (61-year-old Male with 44-month overall survival). (**b**) Preparation with low-density of CD3-positive TILs (47-year-old woman with 18-month overall survival).

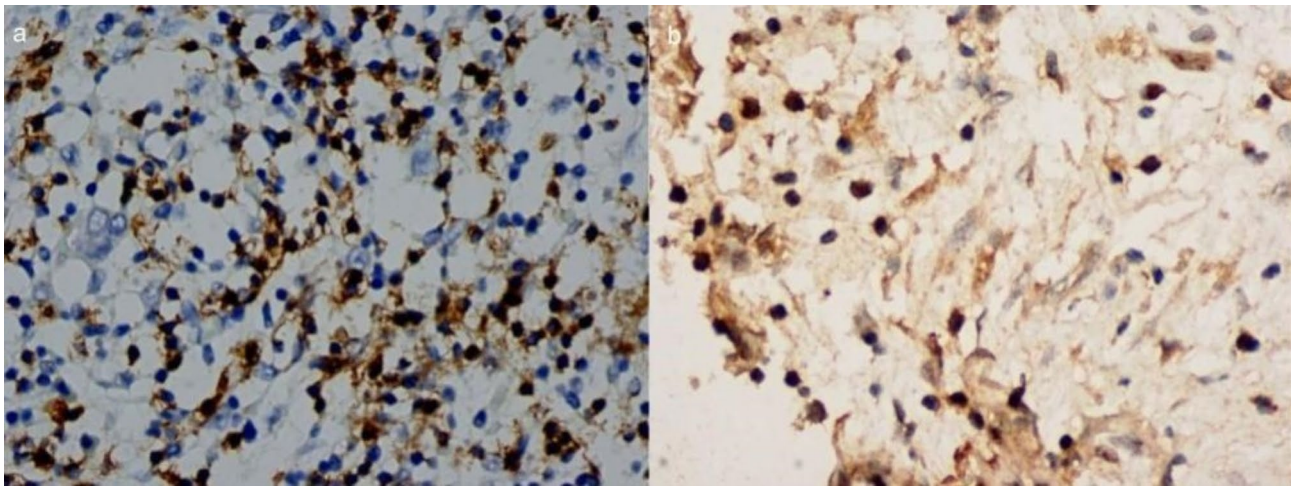

Fig. 2. Cluster differentiation-8 (CD8)-stained peritumoral area (Viewed with Olympus BX51 (Tokyo, Japan) at 40× magnification). (**a**) Preparation with high density of CD8-positive TILs (60-year-old male with 30-month overall survival). (**b**) Preparation with low density of CD8-positive TILs (60-year-old woman with 16-month overall survival).

Germany). The completed preparations were processed in 96% alcohol and xylene for 2 min each. Subsequently, the lamellar closure was performed as follows: CD3 (LN10 clone: Tonsil, technique: Leica BOND-MAX IHC/ISH modulate, Leica Biosystems, Nussloch, Germany); CD8 (4B11 clone: Tonsil, technique: Leica BOND-MAX IHC/ISH modulate, Leica Biosystems, Nussloch, Germany); PD-1 (NAT105 clone: Tonsil, technique: Leica BOND-MAX IHC/ISH modulate, Leica Biosystems, Nussloch, Germany); and PDL-1 (73 – 10 clone: Tonsil, technique: Leica BOND-MAX IHC/ISH modulate, Leica Biosystems, Nussloch, Germany).

CD3 and CD8: Digital image analysis was performed using a computer-connected camera (EasyPath, Argenit, Version: 1.4.1.0, Istanbul, Turkey) (URL: https://argenit.com/easypath-easyshot/) and a light microscope (Olympus BX51, Tokyo, Japan) to quantify the intensity of peritumoral T lymphocyte infiltration that exhibited positive staining at the invasive margin of the tumor. The preparations were viewed using an EasyScan digital slide scanner and a 40× objective with the Argenit EasyPath program. The digital slide scanner was set to 0.175 µ pixels to establish standard thresholds for cell density in the selected regions. After calibration, two consecutive areas were selected on a 40× objective to determine the number of T lymphocytes in 1 mm^2 for each case, covering a total area of 1 mm^2. Total T lymphocytes (positively stained and unstained) and CD3-positive (CD3 P) and CD8 P T lymphocytes in the selected areas were counted separately, automatically, and manually by a blinded pathologist using a previously controlled cell counting method (Figures 1 and 2).

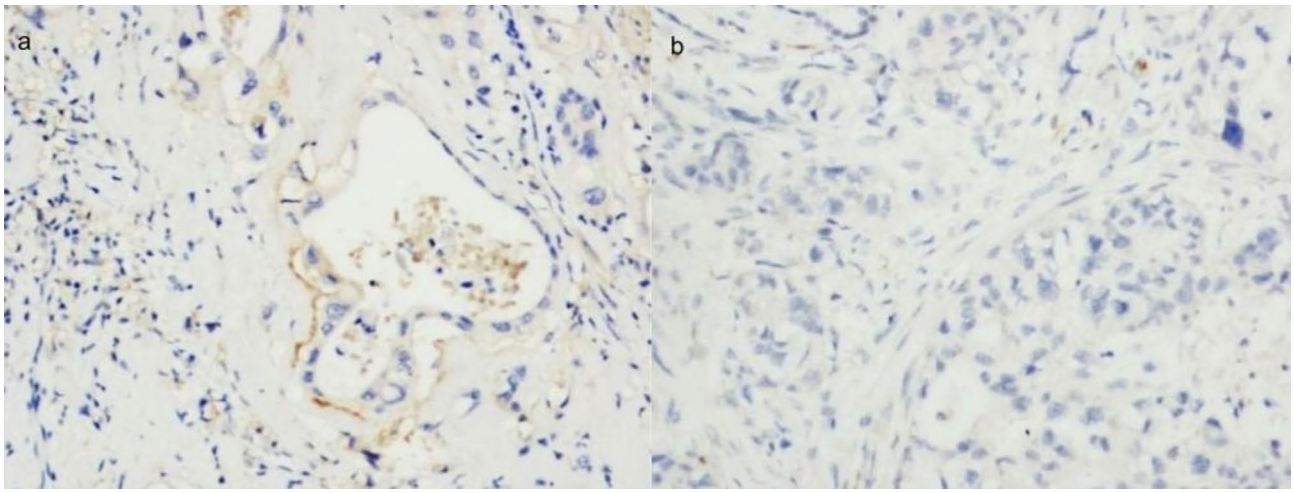

Fig. 3. PD-L1-stained intratumoral area (viewed with Olympus BX51 (Tokyo, Japan) at 20× magnification). (**a**) Preparation with high programmed death ligand-1 (PD-L1) concentration (61-year-old male with 24-month overall survival). (**b**) Preparation with low PD-L1 concentration (93-year-old male with 5-month overall survival).

		N	%
Sex	Female	25	41,7
	Male	35	58,3
Age (years)	Median (min–max); mean ± SD	63.5 (34–93)	64.52 ± 11.55
	< 65 years	32	53.3
	≥ 65 years	28	46.7
Survival (months)	Median (min–max); mean ± SD	14 (4–60)	18.10 ± 14.15

Table 1. Distribution of demographic characteristics. Abbreviations: SD, standard deviation.

PD-1 and PD-L1: In the evaluation of immunohistochemical staining, plasma cell staining was used as an internal control, and tonsil tissue taken in the same preparation was used as an external positive control. Immunohistochemical staining was performed under a light microscope (Olympus BX51; Tokyo, Japan) with a 20× objective. Membrane staining in tumor cells was evaluated, and > 1% staining was considered positive[15,16] (Fig. 3).

Statistical analysis

All statistical analyses were performed using the IBM SPSS statistics software 23.0 (IBM SPSS, Turkey). Descriptive statistical methods (mean, standard deviation, median, frequency, ratio, minimum, maximum, and quartiles) were used to evaluate the study data. The normality of the distribution was analyzed using the Kolmogorov–Smirnov test, Shapiro–Wilk test, skewness-kurtosis test, and graphical evaluations. The Mann–Whitney U test was used for two-group comparisons of non-normally distributed data. Spearman's correlation analysis was used to evaluate the relationships between quantitative variables. The intraclass correlation coefficient (ICC) was used to evaluate the compatibility of CD3 and CD8 measurements between automatic and manual methods. A p-value < 0.05 indicated statistical significance.

Results

This study included 60 patients, among whom 25 (41.7%) were women, who had undergone surgery at the Haydarpaşa Numune Training and Research Hospital General Surgery Clinic of the University of Health Sciences between 2011 and 2021. Of the included patients, 32 (53.3%) were aged ≤ 65 years, with an average age of 64.52 (34–93). All of our patients had exitus at the end of the follow-up period. The average survival time is 18.10 ± 14.15 months (4–60 months) (Table 1). When tumor localization was examined, 8.3% ($n = 5$), 88.3% ($n = 53$), 1.7% ($n = 1$), and 1.7% ($n = 1$) of the tumors were located in the uncinate process, head, body, and cauda, respectively (Table 2). When the comorbidities of the patients were evaluated; 19(% 31,6) patients had hypertension, 13 (% 21,6) had diabetes mellitus, 6(%10) had thyroid gland disorders (1 had papillary thyroid cancer), 6 (%10) had heart disease (4 had coronary artery disease, 2 had congestive heart failure). In addition, 1 patient had a history of nephrectomy due to renal cell carcinoma 30 years ago and 1 patient had a history of colectomy due to colon tumor 10 years ago. PD-L1 was identified in 7 patients (11.7%), and only one case was PD-1 positive. The median age of PD-L1 positive cases was 62 (range 46–83). The median overall survival of PD-

		N	%
Differentiation	Well differentiated	2	3.3
	Moderately differentiated	43	71.7
	Poorly differentiated	15	25.0
Pathological stage	Stage 1B	2	3.3
	Stage 2 A	12	20.0
	Stage 2B	39	65.0
	Stage 3	7	11.7
Stage T	pT1	1	1.7
	pT2	7	11.7
	pT3	52	86.7
Stage N	pN0	14	23.3
	pN1	39	65.0
	pN2	7	11.7
Perineural invasion	No	7	11.7
	Yes	53	88,3
Tumor diameter (cm)	Median (min–max); mean ± SD	3.5 (1.5–6)	3.53 ± 1.00
	< 3.5 cm	27	45,0
	≥ 3.5 cm	33	55,0
Total lymph node counts	Median (min-max); mean ± SD	18 (5–54)	19.48 ± 8.67
Positive lymph node counts	Median (min-max); mean ± SD	2.5 (0–15)	3.12 ± 3.00
Negative lymph node counts	Median (min-max); mean ± SD	14 (4–49)	16.40 ± 8.54
Tumor localization	Uncinate process	5	8.3
	Head	53	88.3
	Body	1	1.7
	Cauda	1	1.7
PD-1	No	59	98.3
	Yes	1	1.7
PD-L1	No	53	88.3
	Yes	7	11.7
Peripancreatic fatty tissue invasion	No	10	16.7
	Yes	50	83.3
Common bile duct invasion	No	15	25.0
	Yes	45	75.0
Wirsung invasion	No	25	41.7
	Yes	35	58.3
Duodenal invasion	No	22	36.7
	Yes	38	63.3
Ampulla of vater invasion	No	38	63.3
	Yes	22	36.7
Vascular invasion	No	58	96.7
	SMV	1	1,7
	Vporta	1	1.7

Table 2. Distribution of prognostic features related to the disease. Abbreviations: PD-1, programmed cell death protein-1; PD-L1, programmed death ligand-1; SMV, superior mesenteric vein; Vporta, vena portae; SD, standard deviation.

L1 positive cases was 14 (range 5–63) months. The patient with PD1 positivity was a 61-year-old male. Overall survival time was 24 months. Exitus occurred due to hematemesis.

Survival time decreased with an increase in the number of positive lymph nodes ($p < 0.05$). Patients aged < 65 years had a lower survival probability than those aged ˃ 65 years ($p < 0.05$) (Table 3). Patients with poorly differentiated tumors exhibited lower survival than those with well- or moderately differentiated tumors ($p < 0.05$). Our findings indicated that among the factors affecting survival, only age, degree of tumor differentiation, and the number of positive lymph nodes were associated with survival. Survival time was not correlated with T stage, N stage, tumor diameter, PD-L1 presence, or peripancreatic fatty tissue invasion ($p > 0.05$).

A comparison of the TIL measurements and survival times demonstrated that an increase in manually measured CD8 P, CD8 P/T (positive/total TIL ratio) and automatically measured CD8 P decreased the survival time ($p < 0.05$) (Table 4). CD8 P/T levels (manually measured) were higher in patients aged ≥ 65 years than

		Survival (months)			P
		N	Median	Mean ± SD	
Age (years)	< 65	32	16	20.81 ± 13.98	0.010*
	≥ 65	28	12	15.00 ± 13.95	
Differentiation	Well or moderate	45	16	21.11 ± 15.06	0.001**
	Poor	15	9	9.07 ± 3.86	
Stage T	pT1 or pT2	8	15.5	14.50 ± 6.63	0.983
	pT3	52	13.5	18.65 ± 14.94	
Stage N	pN0 or pN1	53	14	18.75 ± 14.78	0.526
	pN2	7	15	13.14 ± 6.69	
Tumor diameter (cm)	< 3.5 cm	27	14	19.30 ± 15.15	0.582
	≥ 3.5 cm	33	14	17.12 ± 13.44	
PD-L1	No	53	14	17.42 ± 13.20	0.729
	Yes	7	14	23,29 ± 20,60	
Peripancreatic fatty tissue invasion	No	10	16	20.60 ± 14.29	0.152
	Yes	50	12.5	17.60 ± 14.22	

Table 3. Evaluation of survival duration (months) according to demographic data and disease characteristics. Mann–Whitney U test *$p < 0.05$, ** $p < 0.01$. Abbreviations: PD-LI, programmed death ligand-1; SD, standard deviation.

	Survival time (months)	
	r	*p*
Automatic counts		
CD3 P	-0.026	0.846
CD3 P/T	-0.154	0.241
CD8 P	-0.100	0.448
CD8 P/T	-0.176	0.179
Manual counts		
CD3 P	-0.080	0.545
CD3 P/T	-0.087	0.508
CD8 P	-0.236	0.069
CD8 P/T	-0.274	0.034*

Table 4. Relationship between survival time (months) and CD measurements. *$p < 0.05$. Abbreviations: CD, cluster differentiation; r, Spearman's correlation coefficient P, positive lymph node counts; P/T, positive/total lymph node counts ratio.

in those aged ≤ 65 years ($p < 0.05$). Survival time in patients with poorly differentiated tumors increased with a decrease in the manually measured CD3 P/T and CD8 P/T ratios ($p < 0.05$) (Table 5).

Patients were grouped according to the pT stage, and manually measured CD3 P and CD8 P counts in TILs were statistically significant between patients with pT1 and pT2 and patients with pT3 ($p < 0.05$). Manually measured CD3 P and CD8 P counts were higher in patients with pT3 stage (Table 6). Patients with pN0 and pN1 exhibited prolonged survival with a decrease in the manual CD8 P/T ratio ($p < 0.05$) (Table 6). A higher automatic CD3 P count and manual CD3 P/T ratio were associated with longer survival times among PD-L1 positive patients ($p < 0.05$). Similarly, PD-L1-negative patients with a lower manual CD8 P/T ratio exhibited a longer survival ($p < 0.05$). (Table 6) Manual CD3 P and CD8 P counts and automatic CD8 P counts were higher in patients with peripancreatic fatty tissue invasion than in those without ($p < 0.05$). Patients without peripancreatic fatty tissue invasion with a low automatic CD 8 P and CD 8 P/T ratio had a longer survival ($p < 0.05$).

The automatic and manual measurements of CD3 and CD8 counts are summarized in Table 7. The CD3 P/T and CD8 P/T ratios were consistent between the automatic and manual measurements ($p < 0.01$) (Table 7).

Discussion

Pancreatic cancer is the seventh most common cause of cancer-related death worldwide[2]. According to the 2022 cancer statistics in the United States, pancreatic cancer is the third leading cause of death in both sexes[17]. The 5-year survival rate of patients with PDAC using a multidisciplinary approach is approximately 8%, even in the most experienced centers[18]. The poor prognosis associated with pancreatic cancer has highlighted the

		Differentiation	
		Well or moderate ($n=45$)	Poor ($n=15$)
Automatic counts			
CD3 P & survival time (months)	r	-0.150	-0.121
	p	0.325	0.668
CD3 P/T & survival time (months)	r	-0.178	-0.191
	p	0.243	0.495
CD8 P & survival time (months)	r	-0.119	-0.195
	p	0.436	0.487
CD8 P/T & survival time (months)	r	-0.175	-0.259
	p	0.251	0.350
Manual counts			
CD3 P & survival time (months)	r	-0.218	0.074
	p	0.151	0.793
CD3 P/T & survival time (months)	r	-0.019	-0.598
	p	0.902	0.018*
CD8 P & survival time (months)	r	-0.287	-0.249
	p	0.056	0.372
CD8 P/T & survival time (months)	r	-0.110	-0.636
	p	0.474	0.011*

Table 5. Evaluation of the relationship between survival duration and CD measurements according to the degree of differentiation. *$p<0.05$. Abbreviations: CD, cluster differentiation; r, Spearman's correlation coefficient; P, positive lymph node counts; P/T, positive/total lymph node counts ratio.

		Stage T		Stage *N*		PD-L1	
		pT1 or pT2 ($n=8$)	pT3 ($n=52$)	pN0 or pN1 ($n=53$)	pN2 ($n=7$)	No ($n=53$)	Yes ($n=7$)
Automatic counts							
CD3 P & survival (months)	r	-0.476	-0.018	-0.077	0.577	-0.133	0.857
	p	0.233	0.897	0.583	0.175	0.343	0.014*
CD3 P/T & survival (months)	r	-0.357	-0.160	-0.211	0.414	-0.204	0.250
	p	0.385	0.256	0.130	0.355	0.143	0.589
CD8 P & survival (months)	r	-0.714	-0.013	-0.082	-0.523	-0.155	0.536
	p	0.047*	0.925	0.558	0.229	0.268	0.215
CD8 P/T & survival (months)	r	-0.595	-0.158	-0.234	0.523	-0.198	0.179
	p	0.120	0.265	0.091	0.229	0.154	0.702
Manual counts							
CD3 P & survival (months)	r	-0.333	-0.049	-0.110	0.396	-0.140	0.270
	p	0.420	0.729	0.434	0.379	0.319	0.558
CD3 P/T & survival (months)	r	-0.690	0.007	-0.078	-0.144	-0.188	0.821
	p	0.058	0.960	0.577	0.758	0.177	0.023*
CD8 P & survival (months)	r	-0.766	-0.175	-0.220	-0.445	-0.260	0.107
	p	0.027*	0.213	0.114	0.317	0.060	0.819
CD8 P/T & survival (months)	r	-0.333	-0.257	-0.315	0.036	-0.319	-0.143
	p	0.420	0.066	0.022*	0.939	0.020*	0.760

Table 6. Evaluation of the relationship between survival time and cd measurements according to stage T, stage N and PD-L1 status. *$p<0.05$. Abbreviations: CD, cluster differentiation; r, Spearman's correlation coefficient ; P, positive lymph node counts; P/T, positive/total lymph node counts ratio; PD-LI, programmed death ligand-1.

need for new treatment options, such as immunotherapy, which has been extensively investigated. However, the clinical efficacy of immunotherapy for PDAC is suboptimal, primarily due to the TME, which plays a critical role in treatment resistance to immunotherapy[19]. T cells, in particular, can be used in immunotherapy to eliminate tumor cells[20]. The accumulation of TILs in the TME is important for PDAC prognosis[4,5,21,22]. Immune checkpoint-directed therapies regenerate T cells and block immune escape caused by the gradual activation of tumor-specific immune checkpoints, such as the PD-1–PD-L1 complex[23].

	Median (min-max)	Mean ± SD
Automatic counts		
CD3 P	422.5 (8–1091)	444.25 ± 249.60
CD3 T	1015.5 (456–2242)	1065.27 ± 344.58
CD3 P/T	0.4 (0–0.7)	0.40 ± 0.17
CD8 P	361.5 (150–804)	362.62 ± 139.32
CD8 T	847 (461–1395)	841.22 ± 198.51
CD8 P/T	0.4 (0.2–0.7)	0.43 ± 0.13
Manual counts		
CD3 P	53 (2–200)	60.55 ± 35.13
CD3 T	106.5 (20–300)	113.52 ± 53.92
CD3P/T	0.6 (0.1–0.8)	0.53 ± 0.15
CD8 P	50 (16–135)	53.95 ± 22.76
CD8 T	94.5 (35–662)	107.63 ± 79.87
CD8 P/T	0.6 (0.1–0.8)	0.54 ± 0.14
	Comparison of automatic and manual counts	
	ICC (95% CI)	**P**
CD3 P	0.272 (-0.218–0.565)	0.112
CD3 T	0.224 (-0.299–0.537)	0.166
CD3 P/T	0.704 (0.505–0.823)	0.001**
CD8 P	0.313 (-0.150–0.590)	0.076
CD8 T	0.078 (-0.543–0.449)	0.377
CD8 P/T	0.744 (0.572–0.847)	0.001**

Table 7. Distribution of CD measurements and compliance of CD measurements according to measurement method. $^{**}p < 0.01$. Abbreviations: CD, cluster differentiation; ICC: intraclass correlation coefficient; P, positive lymph node counts; P/T, positive/total lymph node counts ratio; SD, standard deviation; CI, confidence interval.

A 2021 review reported an average survival time of 25–30 months[2]. In another cohort of 96 patients, the mean survival time was 14.9 months[18]. A study conducted in the Netherlands reported an OS of 26.8 months in patients who were followed up with active surveillance and surgery[24]. In our study, the survival times varied between 4 and 60 months, (mean: 18.10 ± 14.15 months).

Lin et al. identified advanced age, poor differentiation, and the number of metastatic lymph nodes as poor prognostic factors for survival[9]. Our findings indicated that the total number of lymph nodes was not associated with survival, and the number of positive lymph nodes and survival were negatively correlated, which is consistent with the findings of another study[25]. Furthermore, survival time and the number of positive lymph nodes demonstrated a negative association[26]. Our findings elucidate a significant association between age and survival time ($p < 0.05$). Compared with patients with moderately or well-differentiated tumors, those with poorly differentiated tumors exhibited lower survival rates. Our findings demonstrated an inverse relationship between the number of positive lymph nodes and survival time ($p < 0.05$).

High CD3 P and CD8 P T lymphocyte infiltrations were positive prognostic indicators[4]. In a 2019 study involving 57 patients, high infiltration of peritumoral CD3 P and CD8 P T lymphocytes was associated with survival in univariate analyses. Moreover, CD3 P TILs infiltration is a predictor of survival, independent of classical histopathological parameters[5]. A 2017 study that included 90 patients showed that tumor stage and peritumoral CD8 infiltration were correlated; low peritumoral CD8 infiltration was observed in stage 1 patients, and high peritumoral CD8 infiltration was observed in advanced-stage patients[27]. High infiltration of CD8 P T lymphocytes in the TME is associated with better patient survival[28,29]. Our findings that patients with poorly differentiated tumors and a low CD3 P/T ratio (manually measured) exhibited a longer survival ($p < 0.05$) highlighted the degree of differentiation as a more important prognostic indicator than CD3 expression. If the manually measured CD8 P/T ratio was low, survival was longer ($p < 0.05$). Moreover, the manually measured CD8 P count was lower in patients with pT1 and pT2 tumors, and survival was longer ($p < 0.05$). Studies examining the relationship between CD8 P T lymphocyte infiltration and survival according to N stage are lacking. In our study, survival was longer when the automatically measured CD8 P count and CD8 P/T ratio were low in patients without peripancreatic fatty tissue invasion ($p < 0.05$). Studies examining the relationship between peripancreatic fatty tissue invasion and survival are lacking.

High CD8 expression in PD-L1 positive patients was associated with positive survival outcomes[30]. PD-L1 positivity is an independent poor prognostic factor in patients with high CD8 P TILs density[31]. Hwang et al.

reported that a high CD8 T lymphocyte count was associated with longer survival; however, this association was not statistically significant[32]. Blocking the PD-1/PD-L1 pathway is a promising treatment option for various tumors, such as esophageal, breast, and gastrointestinal tract cancers. PD-L1 expression induced by tumor cells facilitates immune surveillance evasion[11]. High PD-L1 expression is a poor prognostic factor in pancreatic cancer[31,33,34]. Our findings elucidated that PD-L1 positive patients with an increased CD3 P/T ratio (manually measured) exhibited a longer survival ($p < 0.05$). Conversely, PD-L1 negative patients with a decreased CD8 P/T ratio (manually measured) had a longer survival ($p < 0.05$). However, irrespective of the PD-L1 positivity, CD3 and CD8 were not significantly correlated ($p > 0.05$).

Studies comparing the automated and manual counting of CD3, CD8, and PD-1 cells are scarce[35,36]. However, no meta-analyses or reviews have been conducted on automatic cell counting methods in digital pathology. Our findings demonstrated no statistically significant agreement in CD3 and CD8 numbers between the automated and manual CD measurements. However, the CD3 and CD8 P/T ratios were comparable between automated and manual measurements ($p < 0.05$).

In pancreatic cancer, cancer-associated fibroblasts (CAF) and other immune cells also play an important role around the tumor[37–40]. Since pancreatic cancer is one of the cancer types that benefit less from immunotherapy, combined treatments have been introduced to increase the effectiveness of the treatment[7]. PD-L1 is expressed on T cells as well as B cells, dendritic cells, macrophages and tumoral cells[41]. This situation is horizon-opening for the development of alternative treatments to break the resistance mechanisms in the T-cell response.

In addition to the parameters we looked at in our study, there are new studies in the literature aimed at increasing PD1/PDL1 activity[42]. In an experimental study conducted in Germany, it was mentioned that insulin could increase the effect of immunotherapy by increasing PD-L1 expression[43]. Another study mentioned that pancreatic cancer patients with high PD-L1 expression and high B7-H4 molecule had low response to immunotherapy[44]. In animal experiments on immunotherapies, it has been mentioned that inhibition of HDAC5 protein, which is a histone deacetylase, may increase antitumoral activity in resistant cases[45]. A promising experimental study that suggests that PD-L1-targeted chimeric antigen receptor (CAR) T cell therapies can overcome resistance to naked molecules to increase treatment efficacy is noteworthy[46]. All these studies are promising for overcoming the poor prognosis of pancreatic cancer in the future.

The peritumoral CD8 P/T ratio in our patients decreased as the survival time increased. This relationship was analyzed independently within patient subsets (T1 and T2 tumor stages, N0 and N1 nodal stages, with and without PD-L1 positivity, and with and without peripancreatic fatty tissue invasion), and consistent results were observed. Notably, CD3 P counts increased with advancing tumor stages. Additionally, PD-L1-positive patients with an increased CD3 P/T ratio exhibited better survival outcomes. Notably, the CD3 P/T ratio was low in patients with poorly differentiated tumors and in those with longer survival.

Our study had certain limitations. First, this was a retrospective and single-center study, and the disease-free survival (DFS) of the patients could not be included in the statistical analysis because of insufficient data. Furthermore, owing to the limited proportion of PD-1-positive patients, statistical analyses could not be performed and lacked long-term results. In addition, manual and automatic measurements were not compatible with all CD3 and CD8 measurement parameters, and sufficient data were lacking.

Our findings support the prognostic importance of the peritumoral CD3 and CD8 levels. To obtain more statistically significant data elucidating the association between peritumoral CD3 and CD8, and PD-1 and PD-L1, further studies with larger patient groups are needed. Concurrent analysis of TILs distribution and TME, in addition to the accepted criteria, is warranted for more accurate prognosis prediction in pancreatic cancer. To expedite data generation, the main goal should be to develop digital pathology examination software, as in our study, and to obtain sufficient data to elucidate the relationship between the TME and tumors.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

Concept: K.C.; F.V.A.; M.G.G., design: M.G.G.; K.C.; A.G., definition of intellectual content: M.G.G.; F.V.A; M.T., literature search: K.C.; M.G.G.; M.O.G. , data acquisition: K.C.; A.G.; F.V.A. , data analysis: M.G.G.; K.C. ; A.G., statistical analysis: K.C.; M.T.; M.O.G., manuscript preparation: K.C.; A.G.; M.O.G. , manuscript editing: M.G.G.; K.C.;A.G., and manuscript review M.G.G.; K.C.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
This study was approved by the Health Sciences University Haydarpaşa Numune Training and Research Hospital Clinical Research Ethics Committee (approval no. HNEAH KAEK 2021/220 dated 06.09.2021).

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