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Prognostic Value of Tissue-Resident Memory T Cells and Tumor Microenvironmental Features in Resected Pancreatic Adenocarcinoma

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Background: Pancreatic ductal adenocarcinoma differs from other solid tumors with its unique immunosuppressive microenvironment and non-immunogenic feature. There are not many studies in the literature investigating the effect of these features on prognosis.

Aims: To investigate the prognostic value of tissue-resident memory T cells, tumor microenvironment features, and tumor-associated immune cells in resected pancreatic ductal adenocarcinomas.

Study Design: Retrospective cross-sectional study.

Methods: Of 138 patients diagnosed with pancreatic ductal adenocarcinoma between 2011 and 2018, 81 were included in the study. Specimens from operated patients were reassessed separately as peritumoral and intratumoral areas for tissue resident memory cells and tumor microenvironmental elements (tumor infiltrating lymphocytes, tumor stroma, CD204+ macrophages, PDL1+ immune cells). Disease-free survival and overall survival were defined from the date of operation to the date of recurrence and the date of first diagnosis to the date of death, respectively. If the patient was alive, the last visit date was taken into account.

Results: The median age at diagnosis was 63 (range: 40-78). The median follow-up period was 18.9 months (range: 1.4-80.4 months). Median overall survival was 23.7 months (1.4-80.4 months) and median disease-free survival was 10.8 months (1.4-74.4 months).

Patients with higher intra-tumoral tissue-resident memory cell counts had a longer survival trend than those having lower values (25.6 months vs. 18 months, respectively, P = .84). According to microenvironmental evaluations, lower stromal score (defined as stroma having less desmoplasia and rich in cells) and presence of peritumoral Crohn's-like inflammatory response were associated with higher survival (29.2 months vs. 19.7 months for low vs. high stromal scores, respectively, P = .16 and 30.2 months vs. 18.1 months for the presence of Crohn's-like inflammatory response P = .13). Decreased survival was observed in tumors with increased CD204+ tumor-associated macrophages which were immunosuppressive elements of the microenvironment (12 months vs. 26.3 months for intra-tumoral assessment, P = .29).

Conclusion: Tissue-resident memory T cells and other microenvironmental features may be prognostic in resectable pancreatic ductal adenocarcinomas. Further studies with larger cohorts are needed for validation.

The manuscript was presented in 4th National Immunotherapy and Oncology Congress (October 29-November 1, 2020, Turkey) as an oral presentation and was chosen as the best presentation.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has an increasing incidence. Its prognosis is still poor with a 5-year survival rate being less than 10%. Only 20% are in the resectable stage at the time of diagnosis.[1]

Unlike other solid tumors, immunotherapies have not achieved expected success in PDAC. Studies aiming to increase the immunogenicity of pancreatic cancer are a topic of interest.

One of the most important reasons why pancreatic cancer is often unresponsive to standard treatments is the complex tumor microenvironment (TME), which has unique characteristics. Pancreatic microenvironment is hypoxic and has poor vascular perfusion.[2] It acts as a physical and biological barrier and blocks penetration of chemotherapy, immunotherapeutic agents, and immune cells into the tumor. Therefore, TME blocks immune response with its immunosuppressive feature.

As in the concept of "cold tumor" defined in the literature for non-immunogenic tumors such as the pancreatic carcinomas, despite these inflammatory cells being concentrated around the tumor, there is a low number of tumor-infiltrating lymphocytes (TILs) in the tumor center.[3] The reason for reduced T cell infiltration into the tumor might be lack of tumor antigenicity, defected antigen-presenting cells, cytokines responsible for T cell inactivation, or lack of T cell penetration into the tumor bed due to tumor stroma known as biological barrier.[4,5] This definition is the main idea that requires evaluating tumor separately as intratumoral and peritumoral areas.

One of the most critical components of TME is CD8+ memory T cells which can be classified into 3 groups: 1) circulating effector memory (TEM), 2) central memory (TCM), and 3) non-circulating tissue-resident memory (TRM) cells.[6] TRM T cells permanently reside in the tissue even if there is no antigenic stimulation. Many transcription factors and adhesion molecules are involved in the interaction between TRM and tumor cells. Most-commonly known marker expressing on the surface of TRM cells was CD103. CD103 is the αE subunit of the heterodimeric αEβ7 integrin molecule and plays an important role in adhesion, migration, and signal transmission. CD103 is also a receptor for E-cadherin which is also an important adhesion molecule. Increased expression of E-cadherin and other adhesion molecules enhance the mobilization of cytotoxic granules and improve the cytotoxic effects of T cells.[7] Reduced expression of E-cadherin in pancreatic cancer has been shown to be associated with advanced stage and higher histological grade.[8] Epithelial cells and various immune cells can express CD103. The majority of these immune cells are natural killer cells, natural killer-like T cells, mast cells, dendritic cells, and especially CD8+ and CD4+ T cells.[9] Among these, CD103 expressing CD8+ T cells are closely linked to tumor immune response.[10]

The prognostic value of TRM cells on the survival of cancer is the subject of current studies. It has been shown that an increased number of TRM cells is associated with increased survival in tumors such as lung cancer,[11] breast cancer,[12] melanoma,[13] ovarian cancer,[14] colorectal cancers,[15] and bladder cancer.[16]

Tissue-resident memory T cells frequently express programmed cell death protein-1 (PD-1). There are studies that showed anti PD-1/PDL-1 treatments increase the activation of TRM cells and improve tumor-associated cytotoxicity in the literature. It is also associated with prolonged survival which has been reported in malign melanoma before.[17,18]

Tumor-associated macrophages (TAM) are important cells of the immune escape. Different subtypes of TAMs have tumor suppressor (M1) and tumor promoter (M2) functions.[19] CD204, CD206, and CD163 are M2-TAM markers and have been associated with poor prognosis in many cancers.[20] CD204+ TAMs play an important role in immunosuppression of tumor microenvironment.[21] Cytokines and chemokines released from macrophages prevent T cell proliferation and activation. There may be PDL-1 expression on their surface as in T lymphocytes.

In this study, our aim is to investigate the prognostic value of TRM cells and tumor microenvironment characteristics and tumor-associated immune cells in resected PDACs.

MATERIAL AND METHODS

Study Design and Patients Selection

Files of 138 patients diagnosed with PDAC who were followed up in our oncology clinic between July 2011 and December 2018 were retrospectively reviewed. Fifty-seven patients with ampullary and duodenal carcinomas had stage IV tumors that were unresectable and did not have any tumor specimen available for immunohistochemistry, as well as patients with incomplete data in their files were excluded from the study.

A total of 81 patients over the age of 18 that were operated and diagnosed with PDAC and whose paraffin blocks were evaluated at the Department of Pathology were included in the study.

Characteristics of TME were evaluated. All data and observations were recorded. Demographic data, laboratory results, clinical follow-up parameters, treatment responses, and survival information available in the medical oncology files of the patients were recorded. By analyzing the relationship between oncological and pathological data obtained, the markers that could be predictive and prognostic in the clinic were determined.

The current status of the patients without outpatient follow-ups in recent years were contacted by phone, and information was obtained verbally. Ethical approval was taken with a number of 09.2019.483.

Specimen Selection

All hematoxylin- and eosin-stained tumor slides belonging to 81 cases included in the study were retrieved from the pathology archives and have been re-evaluated. Two tumor samples were selected from each case, 1 principal and 1 substitute. These samples were carefully chosen to reflect both the center and periphery of the tumor and not to contain necrosis and autolysis.

Pathological tumor stage (T stage) and lymph node stage (N stage) of all cases were re-determined according to the "American Joint Committee on Cancer" (AJCC) 2017 TNM staging system.

Immunohistochemical Evaluation

The entire immunohistochemical staining process, including deparaffinization and antigen retrieval procedures on sections prepared from formalin-fixed-paraffin-embedded tissues, were performed in a fully automated immunohistochemistry stainer (Ventana BenchMark Ultra, Ventana Medical Systems, Tucson, Ariz). A detection kit (ultraViewTM Universal DAB Detection Kit, Catalog number 760-500, Ventana Medical Systems), which is suitable for the device, containing hydrogen peroxide substrate and DAB chromogen, without biotin, based on HRP multimer was used. CD103 (Leica, EP206 clone, UK), CD204 (Atlas antibodies, polyclonal, Netherlands), PDL-1 (Cell Signaling, E1L3N (R) clone, Netherlands) antibodies were used.

Olympus BX53 model microscope was used for all evaluations of the applied antibodies. Cell counting procedures were also performed using a 40 HPF (high-power field) objective of the same microscope.

Evaluation of CD103

In the specimens, there were lymphocytes stained positive for CD103 in the duodenal intraepithelial areas, peritumoral pancreatitis areas, inside the tumor, and at the periphery of the tumor. A total of 6 areas was selected for each case for each antibody; the 3 most densely stained areas (hot spots) consisting of intra-tumor lymphocytes and the 3 most intensely stained areas (hot spots) consisting of lymphocytes in the peritumoral stroma were determined by the expert pathologist. After the hot spot selection was completed, positively stained lymphocytes at 40 HPF were counted for each focus (Figure 1a,b).

If concentration of intraepithelial lymphocytes (lymphocytes within the tumor cell) were met, the lymphocyte/tumor ratio was determined by the number of lymphocytes per 100 tumor cells. After counting the stromal lymphocytes in 3 separate foci within the tumor and around the tumor, the arithmetic mean of each was calculated.

CD 103 epithelial staining was not detected in tumor cells or in areas with pancreatitis.

Evaluation of CD204 (MRS-1 Macrophage Scavenger Receptor-1)

Membranous surface of the macrophages in the tumoral and peri-tumoral stromal areas was evaluated for CD204 staining. Since staining intensity was the same in almost all of the cases, only the extent of staining was evaluated regardless of the staining intensity (1: minimal, 2: moderate, 3: maximum) (Figure 2a-c).

Evaluation of PDL-1

Staining of plasma cells for PDL-1 was accepted as the internal control. Staining of placenta and tonsil tissues taken on the same slide was used as external control. Cytoplasmic and membranous staining in tumor cells and TILs were evaluated and recorded separately. While membranous staining above 1% in tumor cells was accepted as positive, all other staining was accepted as negative.

Combined positive score (CPS) was also calculated per case according to the Collage of American Pathologists' June 2021 guidelines defined for gastrointestinal organs.[22]

CPS = PD-L1 positive cells (tumor cells, lymphocytes, and macrophages) ÷ Total of PD-L1 positive + PD-L1 negative tumor cells ×100.

Evaluation of Stromal Response (Desmoplasia)

Hematoxylin-eosin sections prepared from each case were examined, and tumor stroma was evaluated in terms of cellularity and extracellular material accumulation. Depending on whether they were rich or poor in cells such as inflammatory cells,

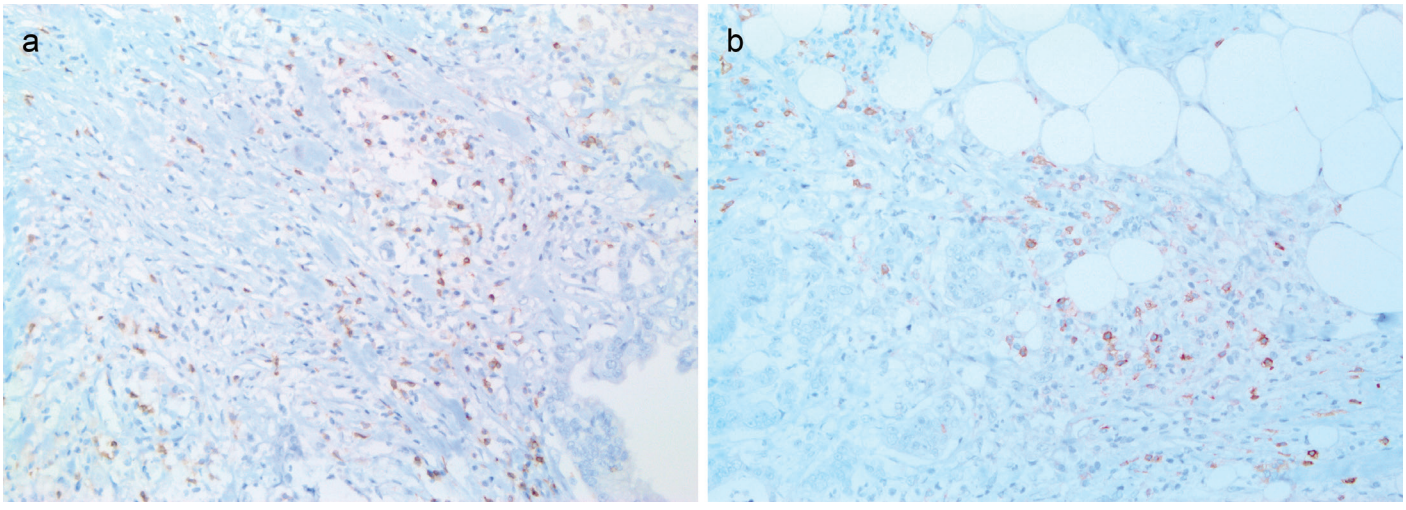

FIG. 1 a,b. Immunostaining for CD103 antibody, (a) CD103 staining of 1 hot spot in tumor area, (b) CD103 staining of 1 hot spot in peritumoral area. (Immunohistochemistry CD103 antibody, x200 and x400).

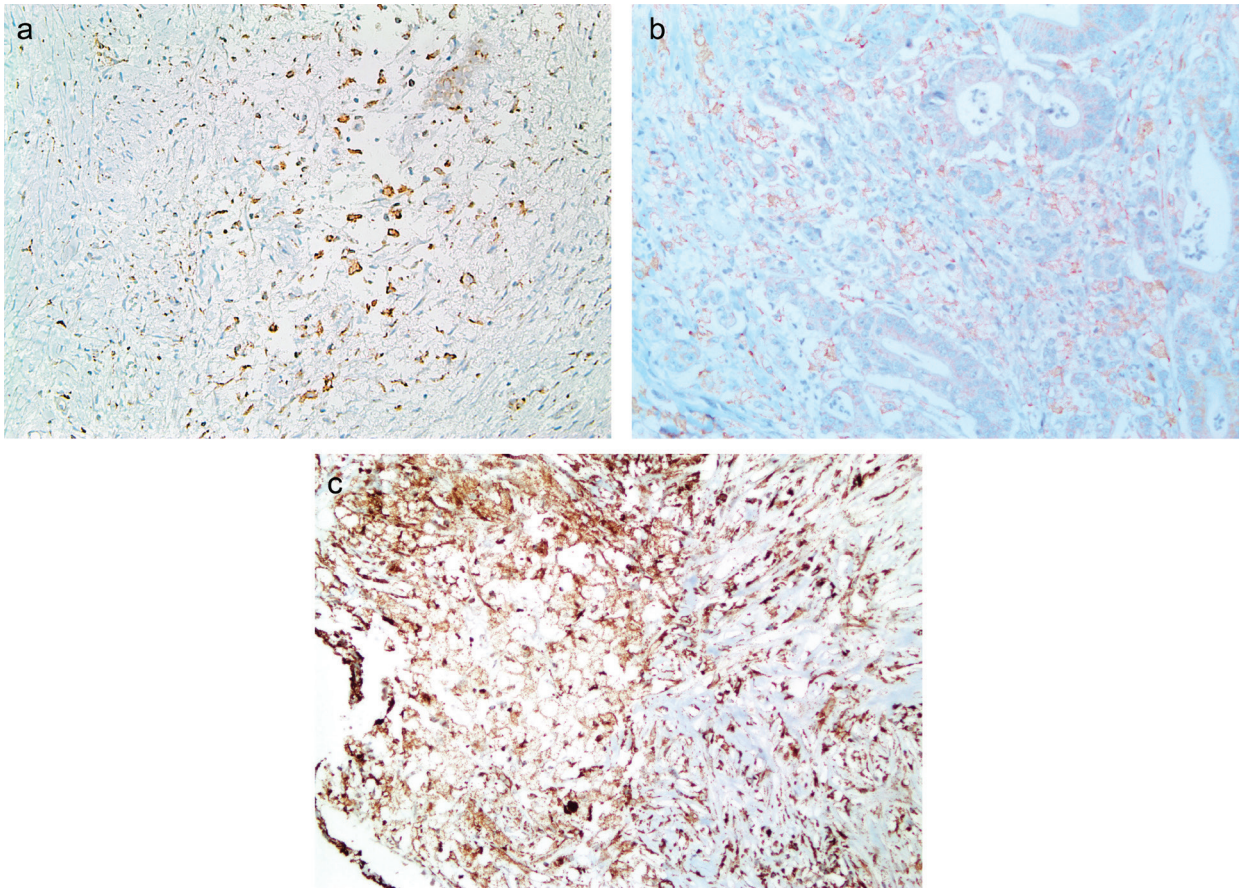

FIG. 2 a-c. Immunostaining of macropahaes for CD204 in tumor area, (a) minimal-score 1, (b) moderate-score 2, (c) maximum-score 3 (Immunohistochemistry CD103 antibody, x400).

myofibroblasts and fibroblasts stromal response was scored as 1-3. Score 1: stroma is rich in cells and less extracellular matrix; score 2: stroma has equal cell quantity and extracellular matrix ratio; score 3: stroma is poor of cells but has abundant extracellular matrix (Figure 3a-c).

Presence of peritumoral Crohn's-like inflammatory response (CLR) was also noted (Figure 4).

Statistical Analysis

Regularly distributed data were expressed using mean standard deviation, and non-uniformly distributed data were expressed using median values. Chi-square test was used for the comparison of categorical data. Comparison of the continuous data was made by determining the distribution pattern using Kolmogorov-Smirnov and Shapiro-Wilk tests.

Disease-free survival time (DFS) was defined from the date of the operation to the date of recurrence or to the date of the last visit (months) if the patient was still alive.

Overall survival time (OS) was defined as the time from the date of initial diagnosis to the date of death or the date of the last visit (months) if the patient was still alive.

During the statistical analysis, median values calculated as cut-off values for TRM counts were accepted which was consistent with the literature.

CD204+ TAMs groups were combined into two groups according to their intensity (score 1: minimal and intermediate intensity and score 2: maximum intensity).

Kaplan-Meier method was used for survival analysis and log-rank test was used for univariate analysis. In order to determine prognostic factors for survival, a Cox regression model was created with variables that were statistically significant or nearly significant ($P < .1$) in univariate analysis. Since statistically significant results could not be obtained in univariate analysis, multivariate analysis was not performed.

Based on findings that reported better prognosis with high intra-tumoral TRM cells in lung, breast, colorectal cancers, we hypothesized that having high (more than median value) intra-tumoral TRM cells will predict better DFS in curatively resected pancreatic adenocarcinoma at 18 months. Our patient population was largely treated with gemcitabine in adjuvant setting. Disease-free survival rate of standard treatment arm (gemcitabine alone) in PRODIGE 24-ACCORD trial was about

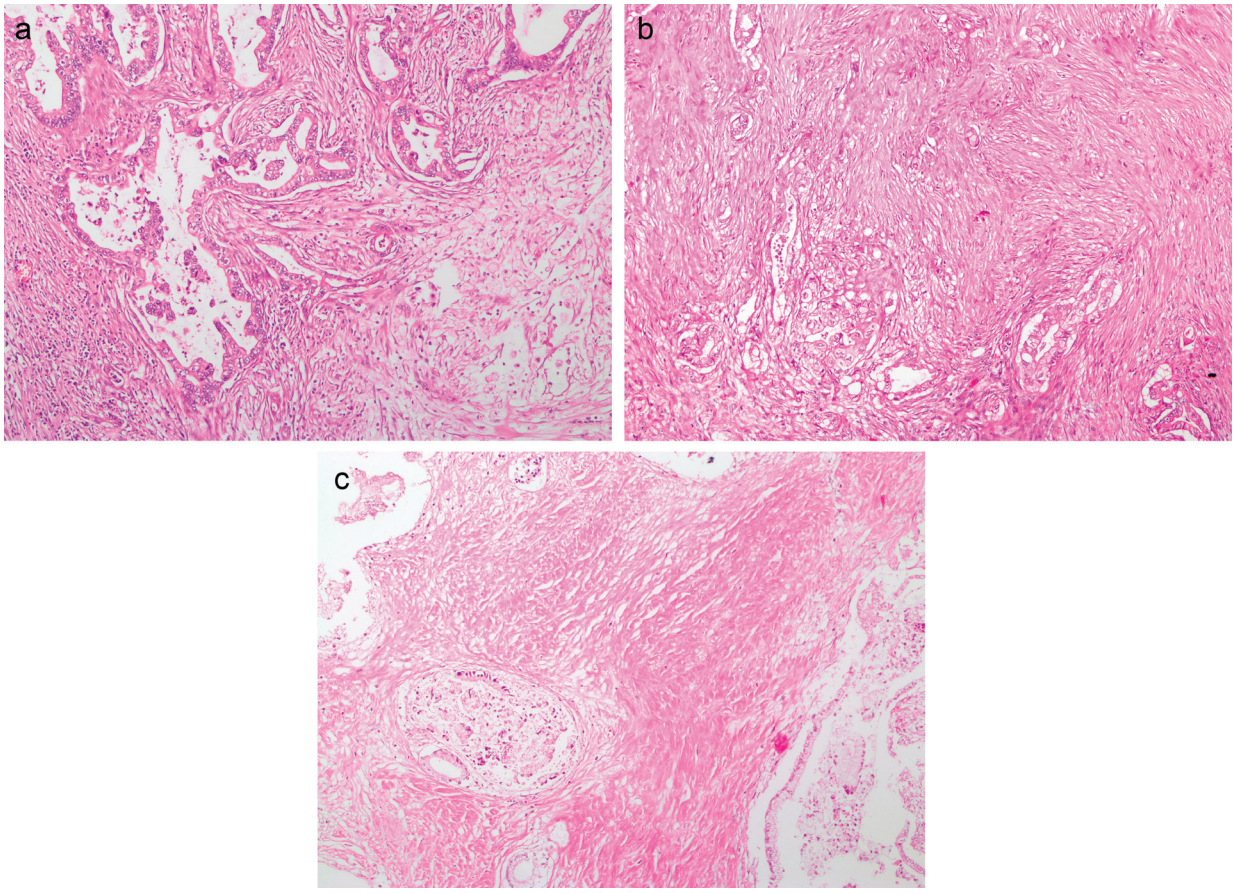

FIG. 3 a-c. Stromal response. (a) Score 1: stroma rich in cells and less extracellular matrix; (b) Score 2: stroma with equal cell quantity and extracellular matrix ratio; (c) Score 3: the stroma with poor of cells but with abundant extracellular matrix (H&E staining, x200).

40%.[23] We hypothesized that an absolute 20% improvement will be observed in a patient with high intratumoral TRM cells. We calculated that this study must include complete data of 75 patients in each group to have 80% power with a 5% type I error level to detect a minimum clinically significant difference of 20% improvement in DFS, when the expected DFS at 18 months is 40% in the control group. Confidence interval for statistical significance was accepted as 95% and a two-sided *P* value < .05. The data were evaluated with the software "SPSS 22.0".

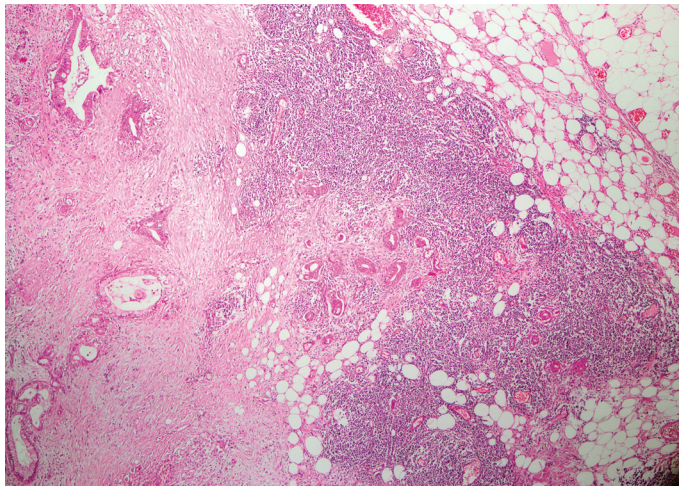

FIG. 4. Peritumoral Crohns'-like lymphoid response (H&E staining, x100).

RESULTS

A total of 81 patients diagnosed with PDAC were included in the study, with a male predominance (56.8%). Median age at diagnosis was 63 (range: 40-78) years.

Histology of all tumors were adenocarcinoma. Most of the tumors (80.2%) were located in the head of the pancreas. Descriptive characteristics are given in Table 1. Five patients did not receive adjuvant chemotherapy (CT) due to poor performance status, delayed wound healing, or patient-related reasons. Forty-two (51.9%) patients received adjuvant radiotherapy (RT) in addition to adjuvant CT.

Survival Analysis

The median follow-up period was 18.9 months (range 1.4-80.4 months). In the final analysis, 68 (84%) patients had relapsed

TABLE 1. Descriptive Characteristics

Descriptives	Patients, n (%)
Gender	
Male	46 (56.8)
Female	35 (43.2)
Diagnostic age	
<60 years	36 (44.4)
≥60 years	45 (55.6)
ECOG PS	
PS 0-1	75 (92.6)
PS 2-3	6 (7.4)
Location of tumor	
Head	65 (80.2)
Corpus	5 (6.2)
Tail	11 (13.6)
Grade groups	
Grade <3	64 (79)
Grade ≥3	17 (21)
T stage	
T1-2	54 (66.7)
T3-4	27 (33.3)
N stage	
Positive	19 (23.5)
Negative	62 (76.5)
Stage groups	
Stage I	12 (14.8)
Stage II	41 (50.6)
Stage III	28 (34.6)
PNI	
No	6 (7.4)
Yes	75 (92.6)
LVI	
No	11 (13.6)
Yes	70 (86.4)
Surgical margin	
Negative	38 (46.9)
Positive	43 (53.1)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; PNI, perineural invasion; LVI, lyphovascular invasion.

and 56 (69.1%) patients had died. Median OS was 23.7 months (1.4-80.4 months) and median DFS was 10.8 months (1.4-74.4 months).

According to pathological evaluation, patients with above-median intra-tumoral TRM cells had longer survival trend than those having below-median values (25.6 months vs. 18 months, respectively, P = .84) (Table 2).

As we presented in Table 3, lower stromal score (defined as having less desmoplasia and more cells in stroma) and/or presence of peritumoral CLR were associated with a higher survival trend in microenvironment evaluation (29.7 months vs. 19.7 months for lower vs. higher stroma score, P = .16 and 30.2 months vs. 18.1 months for positive vs. negative CLR, P = .13). However, decreased survival trend was seen in tumors with increased CD204+ TAMs which were immunosuppressive elements of the microenvironment (26.3 months vs. 12 months for lower CD204+ TAMs vs. higher respectively, P = .29). Membranous PDL1 positivity in tumor cells was detected in only 1 case. However, when we evaluated 'combined positive score' to detect PDL-1 positivity, we determined 29 (35.8%) PDL-1 positive cases. Patients with positive PDL-1 combined score did not reveal any significant correlation with the survival but also had a higher survival trend (30.6 months vs. 17 months, P = .57).

Kaplan-Meier survival curves according to pathological evaluations are presented in Figure 5a-e.

Multivariate analysis was not performed because there was no statistically significant variable in univariate analysis.

DISCUSSION

Understanding the immunogenicity of pancreatic carcinoma is the main idea of this study. Here we investigated the prognostic value of TRM and TME which has previously been shown to be valuable in different types of immunogenic solid tumors, with insufficient data for pancreatic carcinoma in the literature. Despite inflammatory cells being concentrated around the tumor, there is a low number of TILs in the tumor center in pancreatic carcinoma which is also known as a cold tumor.[3]

Tissue-resident memory T cells which are known to be closely linked to tumor immune response were detected to be higher in peritumoral stroma than the center of tumor in this study. On the other hand, it has been shown in the literature that an excess number of TRM in the tumor center is associated with increased survival.[24,25] We determined a higher survival trend in the group of patients with higher intra-tumoral TRM cells (25.6 months intratumoral vs. 18 months peritumoral, respectively, P = .84).

Therefore, these findings support the point that peritumoral TRMs are not as effective as intra-tumoral TRMs. We believe that the factor affecting survival might not be the TILs count alone but it might also be the penetration of lymphocytes into tumor. Most probably tumor tissue might be blocking the penetration of effective immune cells like TRMs through barriers.

In accordance with the hypothesis of the inactivation of lymphocytes outside the tumor, we evaluated CLR in our study. Crohn's-like inflammatory response indicates an active lymphoid reaction and an increased adaptive immune response to the tumor. It has been previously described as a host response to tumor in colon cancer, and increased CLR was associated with prolonged survival for colon cancer.[26,27] This has not been previously reported for pancreatic cancer in the literature. Active lymphocytes might be the other important factors that reflect immune response and improve survival regardless of TILs count substantially in our study. Patients with higher CLR scores showed a higher survival trend (30.2 months vs. 18.1 months, $P = .13$).

Similarly, desmoplastic stroma which has abundant extracellular matrix and lower inflammatory cells reflects a poor immune response to cancer.[28] In this study, the patients who have high stroma score showed better OS numerically (29.2 months vs. 19.2 months, $P = .16$).

CD204+ TAMs, which are another valuable element of TME, contribute to immunosuppressive microenvironment. Previous studies have shown that higher CD204+ TAMs in pancreatic cancer are associated with a poor prognosis.[20,29] In our study, a shorter survival trend correlated with an increased total number of CD204+ TAMs (either intra-tumoral or peri-tumoral) which is consistent with the literature.

Finally, variable results between 1 and 60% have been reported for PDL1 expression in pancreatic cancers in the literature.[30,31] Prognostic value of PDL1 either in tumor cells or tumor-associated inflammatory cells has not been proven yet. We detected only 1 tumor positive for PDL1 (1.2%) and 37% in tumor-associated inflammatory cells in our study, which was also reported by

TABLE 2. Univariate Analysis According to Tissue Resident Memory Cells

Categorical Variables	n	OS (months)	95% CI	*P*	DFS (months)	95% CI	*P*
TRM cells – intratumoral							
Groups according to median							
≤8	42	18	2-39	.84	8.0	3.6-12.4	.80
>8	39	25.6	19.0-32.2		12.6	8.7-16.6	
TRM cells – peritumoral							
Groups according to median							
≤43	40	29.2	23.18-35.29	.198	13.4	7.2-19.5	.15
>43	41	17	10.19-23.84		7.8	4.6-11.0	

OS, overall survival; DFS disease-free survival; TRM, tissue-resident memory; CI, confidence interval.

TABLE 3. Univariate Analysis According to Microenvironment Features

Categorical Variables	n	OS (months)	95% CI	*P*	DFS (months)	95% CI	*P*
Stromal score							
1	35	29.2	22.8-35.5	.16	10.8	7.8-13.8	.83
2	46	19.7	13.6-25.9		8.2	4.3-15.8	
Crohn's-like peritumoral response							
No	58	18.1	7.7-28.6	.13	10.1	5.8-14.4	.15
Yes	23	30.2	21.5-38.9		13.4	3.3-23.4	
CD204+ TAMs score - intratumoral							
1	68	26.3	15.7-36.9	.29	11.4	8.3-14.6	.65
2	11	12	4.2-25.5		6.5	4.4-8.6	
Missing	2						
CD204+ TAMs score - peritumoral							
1	63	23.7	12.6-34.7	.27	12.6	9.5-15.7	.41
2	16	22.4	4.6-46.9		6.5	4.1-8.9	
Missing	2						
PDL-1 combined positive score							
Positive	29	30.6	16.6-44.5	.57	11.4	4.5-18.3	.69
Negative	52	17	6.4-27.5		10.4	4.7-16.1	

OS, overall survival; DFS, disease free survival; CI, confidence interval; TAM, tumor-associated macrophage; PDL-1, programmed cell death protein-1.

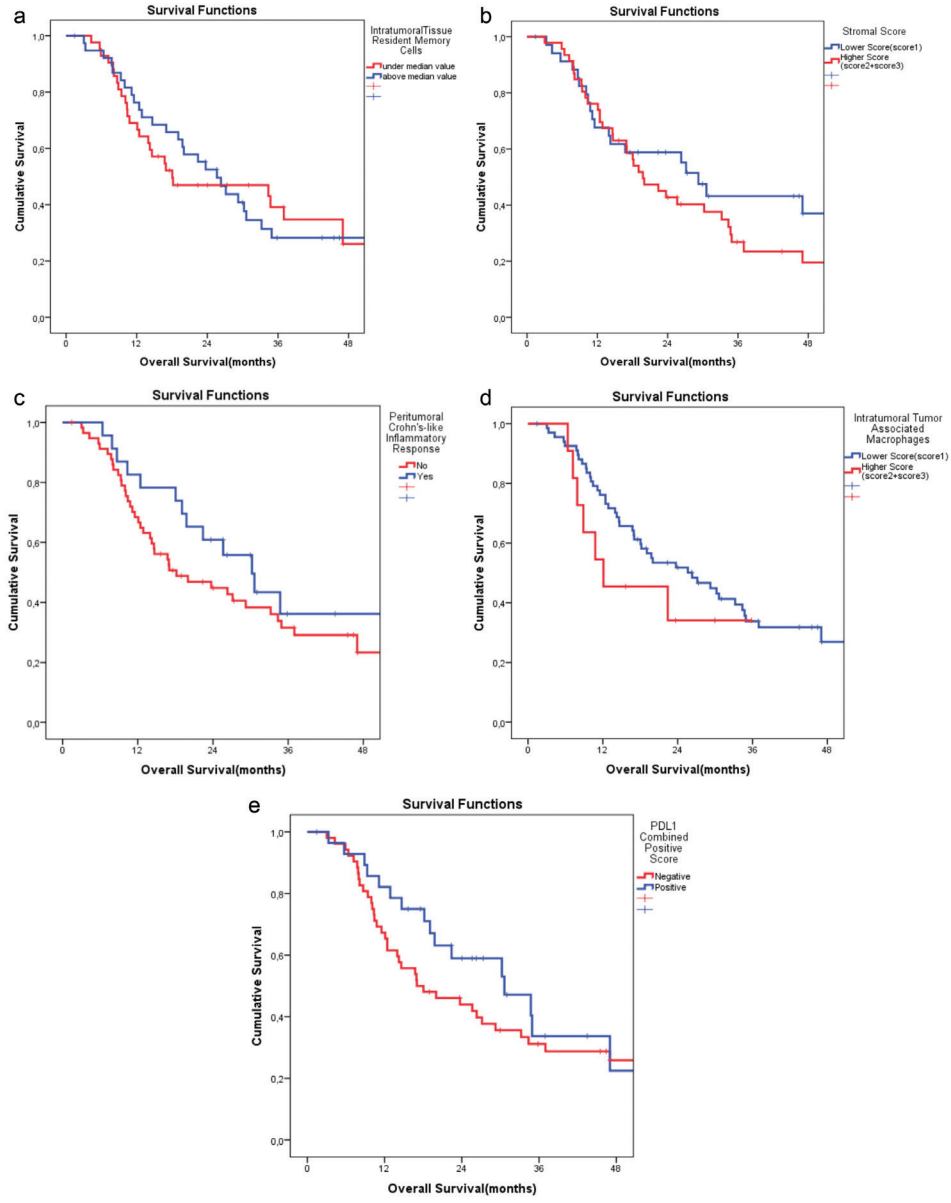

FIG. 5 a-e. Kaplan-Meier survival curves according to tumor characteristics. (a) Intratumoral tissue-resident memory T cells, (b) stromal score, (c) peritumoral Crohn's-like inflammatory response, (d) intratumoral tumor-associated macrophages, (e) PDL1 combined positive score.

Herbst et al.[31] They reported higher PDL1 positivity in tumor-associated inflammatory cells than in tumor cells. The importance of 'PDL-1 combined score' for gastrointestinal tumors has been emphasized in the updated College of American Pathologists (CAP) Protocols in June 2021, but it was also controversial for gastrointestinal tumors in the literature.[32-35] Twenty-nine patients for whom we detected a positive combine score for PDL-1 had higher survival numerically (30.6 months vs. 17 months, $P = .57$). Our statistically insignificant results might also be related to small sample.

In conclusion, despite our statistically insignificant results, the positive survival trend suggests that TRM, TAM, CLR, and stromal cellularity/matrix ratio may have prognostic significance in pancreatic cancer. The small number of sample is the restrictive factor for this study. Further studies are needed to improve our results and to investigate other mechanisms underlying PDAC immunogenicity.

Ethics Committee Approval: Ethics/Institutional Review Board approval of Research-School of Medicine Marmara University, İstanbul, Turkey (ethical approval no: 09.2019.483).

Patient Consent for Publication: Written informed consent was obtained from all patients.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Concept – T.B., N.B., P.B.; Design – T.B., N.B., P.B.; Supervision – N.B., P.B., F.Y.; Data Collection and/or Processing – T.B., E.A., F.E.Ö., N.C.D., R.A., T.A.T., O.E.; Analysis and/or Interpretation – M.A.Ö.; Literature Review – T.B.; Writing – T.B.; Critical Review – F.Y.

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

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