



Impact of the tumor immune microenvironment on the outcome of pancreatic cancer: a retrospective study based on clinical pathological analysis

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Background: The cancerous microenvironment, characterized by the infiltration of CD4⁺ and CD8⁺ T cells, play a critical role in regulating the progression of cancer and treating efficiency of immunotherapy. However, the distribution of these cells and their associated cytokines in the tumor microenvironment of pancreatic cancer (PC) are not yet fully understood. Our study aims to analyze the contents of CD4⁺IL-17⁺ and CD8⁺ T cells in PC and their relationship with the clinicopathological features and survival outcomes of patients.

Methods: PC tissues and adjacent tissues were retrospectively collected from 40 patients in our hospital. The expression of CD4, IL-17, and CD8 in histological samples was measured by immunohistochemistry. The correlation between CD4, IL-17, and CD8 expression and clinical characteristics was analyzed using Kaplan-Meier survival analysis. The risk factors affecting the outcome of PC were examined by the Cox proportional hazards model, then a nomogram predicting the survival of PC using these risk factors was established.

Results: The content of CD4⁺IL-17⁺ T cells in PC tissues was significantly higher than that in adjacent normal tissues, while the number of CD8⁺ T cells was significantly lower than that in adjacent normal tissues ($P < 0.01$). CD4⁺ T cells in PC tissues was significantly associated with TNM stage and lymph node metastasis ($P < 0.05$). IL-17 and CD8 were significantly associated with histological grade, TNM stage, local infiltration, and lymph node metastasis ($P < 0.05$). The median survival times (MSTs) of CD4 positive and negative patients were 13.2 and 21.4 months, respectively. The MSTs of IL-17 positive and negative patients were 10.4 and 24.8 months, respectively. The MSTs were 21.9 and 11.8 months for CD8 positive and negative patients, respectively ($P < 0.05$). The Cox regression model demonstrated that TNM staging, lymph node metastasis, and CD4⁺IL-17⁺ and CD8⁺ T cells affected PC prognosis ($P < 0.05$). The nomogram showed that the survival probability was reduced in patients with TNM stage III to IV, lymph node metastasis, high CD4⁺IL-17⁺ level, and low CD8⁺ expression.

Conclusions: CD4⁺IL-17⁺ and CD8⁺ T cells in PC tissues are associated with TNM staging, lymph node metastasis, and MST, and can be used as new prognostic indicators for PC.

Keywords: CD4⁺IL-17⁺ T cells; CD8⁺ T cells; pancreatic cancer; pathological features; survival outcome

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Introduction

Pancreatic cancer (PC) is one of the most aggressive tumors of the digestive system, with insidious and atypical clinical symptoms, rapid progression, and extremely high malignancy. The 5-year overall survival rate after diagnosis is less than 5% (1). Surgery is still the first choice for the treatment of PC, but residual tumor cells in the operation field and subclinical metastasis in the non-surgical area are likely to cause postoperative recurrence, and patients are not sensitive to radiotherapy and chemotherapy. Besides, the 5-year overall survival rate after surgery rarely exceeds 10–19%, so it is necessary to find new diagnosis and treatment methods (2).

Immunotherapy, following the radiotherapy and chemotherapy, represents another important anti-tumor method after surgery. It activates the immune system and kills cancer cells and tumor tissues by relying on autoimmune functions. In recent years, immunotherapy has achieved remarkable therapeutic effects in solid tumors such as melanoma and lung cancer (3). However, immunotherapy does not provide obvious benefit for PC when used as a single agent mainly due to the intrinsic low immunogenicity and the establishment of a powerful immunosuppressive tumor microenvironment (4). The formation of immunosuppressive microenvironment caused an escape of tumor cells from immune surveillance, limiting the activation and function of immune cells, eventually leading to an unimproved poor prognosis with a 5-year survival less than 8% (5). In pancreatic cancer, the immunosuppressive microenvironment consists of tumor-associated macrophages, myeloid-derived suppressor cells, CD4⁺/CD8⁺T lymphocytes, regulatory T cells (Treg), regulatory B cells and so on (6). To date, the antitumor immune response is known to be downregulated in the complicated pancreatic microenvironment, particular for the functionally exhausted T cells. CD4⁺ T cells play a central role in regulating tumor immune effects. CD4⁺IL-17⁺ T cells are a new type of effector CD4⁺ T cell discovered in recent years, which can promote the development of tumors by inducing angiogenesis and recruiting inflammatory cells (7). Relevant data show that CD4⁺IL-17⁺ T cells play a key role in autoimmune diseases and infectious diseases (8). Previous studies uncovered that IL17 recruited neutrophils, and excluded cytotoxic CD8⁺ T cells from tumors. IL-17 blockade increases immune checkpoint blockade (PD-1, CTLA4) sensitivity (9,10). CD8⁺ T cells is an important subset in tumor immune surveillance. Initial CD8⁺ T cells can differentiate into

cytotoxic CD8⁺ T cells in response to the stimulation of cancer cell antigens, which can kill antigen-specific cancer cells and inhibit tumorigenesis and development. Therefore, our focused the expression of CD4, IL-17 and CD8 in PC based on their pivotal roles in regulating immune function and affecting immunotherapy efficiency.

In spite that several studies have indicated the effects of CD4⁺ T cells, CD8⁺ T cells and regulatory T cells on prognosis of patients with PC, no consensus has been achieved with regarding to their contributions. Study by Wang *et al.* revealed that high CD4⁺ and high CD8⁺ T cells were correlated with good prognosis compared with low CD4⁺ and low CD8⁺ T cells (11). While, Kumai *et al.* showed that the frequency of CD4⁺ T cells in pancreatic cancer tissues did not affect prognosis after surgery, but prognosis was good in the group with a high frequency of CD8⁺ T cells and low frequency of regulatory T cells (12). MacNeil reported that high CD8 significantly predicted improved patient overall survival (13). Therefore, the distribution and function of these cells and their related cytokines in the tumor microenvironment of PC are not well characterized due to their variation across different sites of disease and their effects on malignant cell state and therapeutic response (14). In this study, samples were gathered from 40 patients with PC. The expression of CD4, IL-17, and CD8 were measured by immunohistochemical staining. We then explored the correlation between CD4⁺IL-17⁺ T cells and CD8⁺ T cells and the clinicopathological characteristics and outcomes of patients. We present the following article in accordance with the TRIPOD reporting checklist (available at <https://gs.amegroups.com/article/view/10.21037/gs-22-45/rc>).

Methods

Subject selection

The tumor tissue specimens of 40 patients with PC in the Third Xiangya Hospital from May 2016 to May 2019 were retrospectively analyzed. Normal pancreatic tissues adjacent to the tumor were collected as controls. The patients were 33 to 85 years old, with an average age of 61.22±10.58 years old. There were 21 males and 19 females. The inclusion criteria were as follows: (I) all were diagnosed with PC by postoperative histopathology; (II) tissue specimens were obtained by surgical resection; (III) no radiotherapy, chemotherapy, and immunotherapy were performed before the operation; (IV) no hormones and immunosuppressive

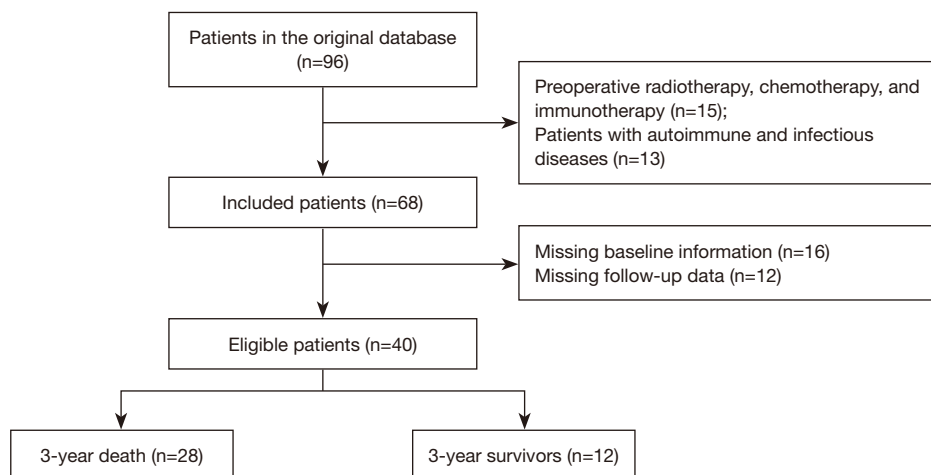


Figure 1 Flow diagram of patient selection in this study.

agents were previously taken; (V) the medical records and follow-up data were complete; (VI) the patients and their family members signed the informed consent. The exclusion criteria were as follows: (I) patients with autoimmune and infectious diseases; (II) patients with secondary PC; (III) patients with distant metastasis before surgery; (IV) patients with combined portal vein resection or combined resection of metastatic organs; (V) combined with other blood system diseases and solid tumors; (VI) patients with unclear pathological diagnosis after surgery. The flow chart in *Figure 1* depicts the patient selection process and reasons for exclusion. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics committee of the Third Xiangya Hospital [No. SYXK(Xiang)2017-0002] and informed consent was taken from all the patients.

Immunohistochemistry (IHC) staining

To determine the infiltrated CD4⁺, IL-17⁺ and CD8⁺ T cells in PC cancer and adjacent tissues, IHC analysis was performed. The surgically resected samples were immediately fixed with 10% neutral formaldehyde and routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The 4 μm thick sections were routinely deparaffinized and hydrated. The sections were first blocked by H₂O₂ and then incubated with normal serum for 10 minutes, and then incubated with the primary antibody at 4 °C overnight. After rewarming at 37 °C, the secondary antibody was added to sections and incubated

for 1 hour. Finally, the tissue sections were color-developed by diaminobenzidine (DAB), stained with hematoxylin, dehydrated, cleared, and sealed. Meanwhile, samples with known positive staining were used as positive controls, and phosphate buffer saline (PBS) was used as the negative control.

Scoring of staining results

Two senior pathologists reviewed and observed the slices by the double-blind method. Ten high power fields were examined in every section, and 100 tumor cells were counted in each high power field. Positive expression of CD4 and CD8 was shown by brownish-yellow particles located in the cell membrane, while positive expression of IL-17 was shown by brown particles located in the cytoplasm. The judgment criteria of staining intensity were as follows: sepia: 3 points, brown: 2 points, faint yellow: 1 point, and colorlessness: 0 point. According to the percentage of stained cells, >75% was 3 points, 51–75% was 2 points, 11–50% was 1 point, and ≤10% was 0 point. The product of the staining intensity score and staining range score was calculated, and 0–2 was negative while more than 3 was positive.

Statistical analysis

Data in this study are represented by $\bar{x} \pm s$. Count data are shown as n (%) and compared using the independent sample *t*-test. Group comparisons were conducted by the χ^2 test. Kaplan-Meier survival curves were plotted for survival analysis. Survival rates were measured by the log-rank test.

Table 1 The expression of CD4, IL-17, and CD8 in PC tissues

Tissue	CD4, n (%)		IL-17, n (%)		CD8, n (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
PC	32 (80.00)	8 (20.00)	26 (65.00)	14 (35.00)	16 (40.00)	24 (60.00)
Adjacent	18 (45.00)	22 (55.00)	15 (37.50)	25 (62.50)	33 (82.50)	7 (17.50)
χ^2	10.453		6.054		15.221	
P	0.001		0.014		<0.001	

PC, pancreatic cancer.

Table 2 Cell counts of CD4⁺IL-17⁺ and CD8⁺ T cells in PC tissues

Group	Cases	CD4 ⁺ IL-17 ⁺	CD8 ⁺
PC	40	5.32±1.90	24.25±3.05
Adjacent	40	3.35±1.27	43.11±4.62
<i>t</i>		5.452	21.546
P		<0.001	<0.001

PC, pancreatic cancer.

The Cox proportional risk model was applied to investigate the risk factors influencing the outcome of PC patients, and the nomogram for predicting survival was established. $P < 0.05$ indicates statistical significance. Data were analyzed using the SPSS20.0 software package and R 3.4.1 software.

Results

The expression of CD4, IL-17, and CD8 in PC

The results of immunohistochemical staining showed that CD4 was mainly distributed in the cell membrane in PC tissues, with brownish-yellow particles. There were 32 cases (80.00%) with CD4 positive expression out of the 40 PC tissues, and 18 cases (45.00%) had CD4 positive expression in adjacent tissues. IL-17 was distributed in the cytoplasm of PC tissues as brown granules. IL-17 was positively expressed in 26 of the 40 PC tissues (65.00%) and 15 of the corresponding adjacent tissues (37.50%). CD8 was distributed in the cell membrane in PC tissues, showing brownish-yellow particles. CD8 was expressed in 16 of the 40 PC tissues (40.00%) and 33 of the corresponding adjacent tissues (82.50%). The positive expression of CD4, IL-17, and CD8 in PC and adjacent samples had significant differences ($P < 0.05$, *Table 1*).

Cell counts of CD4⁺IL-17⁺ and CD8⁺ T cells in PC tissues

The percentage of CD4⁺IL-17⁺ T cells in PC samples was significantly higher than that in normal adjacent samples, and the number of CD8⁺ T cells was markedly lower than that in normal samples, with significant differences ($P < 0.01$, *Table 2*).

Correlation between the expression of CD4, IL-17, and CD8 and clinicopathological features in PC tissues

The expression level of CD4 in PC tissues was strongly associated with TNM stage and lymph node metastasis ($P < 0.05$). Furthermore, the expression of IL-17 and CD8 were significantly associated with histological grade, TNM stage, lymph node metastasis, and local infiltration ($P < 0.05$, *Table 3*).

The relationship between the expression of CD4, IL-17, CD8 and the clinicopathological characteristics and prognosis of patients

The 40 patients in this study were followed up for 2–40 months, and the average survival time was 16.85±8.21 months. The median survival times (MSTs) of patients with TNM stages I–II and III–IV were 12.5 and 23.0 months, respectively. The MSTs were 12.3 and 20.8 months for patients who had or did not have lymph node metastasis, respectively. The MSTs were 13.2 and 21.4 months in CD4 positive and negative patients, respectively. The MSTs were 10.4 and 24.8 months in IL-17 positive and negative patients, respectively. The MSTs for CD8 positive and negative patients were 21.9 and 11.8 months, respectively. The differences between groups were statistically significant ($P < 0.05$), as shown in *Table 4* and *Figure 2*.

Table 3 Correlation between levels of CD4, IL-17 and CD8 and clinicopathological features in PC tissues

Clinicopathological features	Subtypes	Cases	CD4, n (%)			IL-17, n (%)			CD8, n (%)		
			Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
Gender	Male	21	17 (80.95)	4 (19.05)	0.874	14 (66.67)	7 (33.33)	0.816	9 (42.86)	12 (57.14)	0.698
	Female	19	15 (78.95)	4 (21.05)		12 (63.16)	7 (36.84)		7 (36.84)	12 (63.16)	
Age (years)	≤60	17	14 (82.35)	3 (17.65)	0.749	11 (64.71)	6 (35.29)	0.973	8 (47.06)	9 (52.94)	0.433
	>60	23	18 (78.26)	5 (21.74)		15 (65.22)	8 (34.78)		8 (34.78)	15 (65.22)	
Tumor size (cm)	≤2	18	13 (72.22)	5 (27.78)	0.266	13 (72.22)	5 (27.78)	0.088	10 (55.56)	8 (44.44)	0.069
	>2	22	19 (86.36)	3 (13.64)		10 (45.45)	12 (54.55)		6 (27.27)	16 (72.73)	
Tumor site	Head of pancreas	27	21 (77.78)	6 (22.22)	0.613	18 (66.67)	9 (33.33)	0.750	12 (44.44)	15 (55.56)	0.408
	Body tail	13	11 (84.62)	2 (15.38)		8 (61.54)	5 (38.46)		4 (30.77)	9 (69.23)	
Histological grade	Poorly differentiated	9	7 (77.78)	2 (22.22)	0.395	7 (77.78)	2 (22.22)	0.006*	6 (66.67)	3 (33.33)	0.047*
	Moderately differentiated	18	16 (88.89)	2 (11.11)		7 (38.89)	11 (61.11)		8 (44.44)	10 (55.56)	
	High differentiated	13	9 (69.23)	4 (30.77)		12 (92.31)	1 (7.69)		2 (15.38)	11 (84.62)	
TNM stage	I-II stage	22	15 (68.18)	7 (31.82)	0.039*	10 (45.45)	12 (54.55)	0.004*	13 (59.09)	9 (40.91)	0.006*
	III-IV stage	18	17 (94.44)	1 (5.56)		16 (88.89)	2 (11.11)		3 (16.67)	15 (83.33)	
Lymph node metastasis	Yes	21	14 (66.67)	7 (33.33)	0.027*	17 (80.95)	4 (19.05)	0.026*	4 (19.05)	17 (80.95)	0.004*
	No	19	18 (84.74)	1 (5.26)		9 (47.37)	10 (52.63)		12 (63.16)	7 (36.84)	
Local infiltration	Yes	23	18 (78.26)	5 (21.74)	0.749	20 (86.96)	3 (13.04)	0.001*	5 (21.74)	18 (78.26)	0.006*
	No	17	14 (82.35)	3 (17.65)		6 (35.29)	11 (64.71)		11 (64.71)	6 (35.29)	

*, P<0.05 represents significant difference. PC, pancreatic cancer; TNM, tumor node metastasis.

Table 4 The relationship between the expression of CD4, IL-17, and CD8 and the clinicopathological characteristics and prognosis of patients

Factors	P	HR	95% CI
TNM stage I-II vs. III-IV	0.004	3.276	1.578–5.422
Lymph node metastasis (yes vs. no)	0.003	2.318	1.458–4.112
CD4 positive vs. negative	<0.001	2.359	2.116–5.201
IL-17 positive vs. negative	<0.001	3.185	2.156–5.871
CD8 positive vs. negative	<0.001	2.323	1.027–6.278

HR, hazard ratio; 95% CI, 95% confidence interval; TNM, tumor node metastasis.

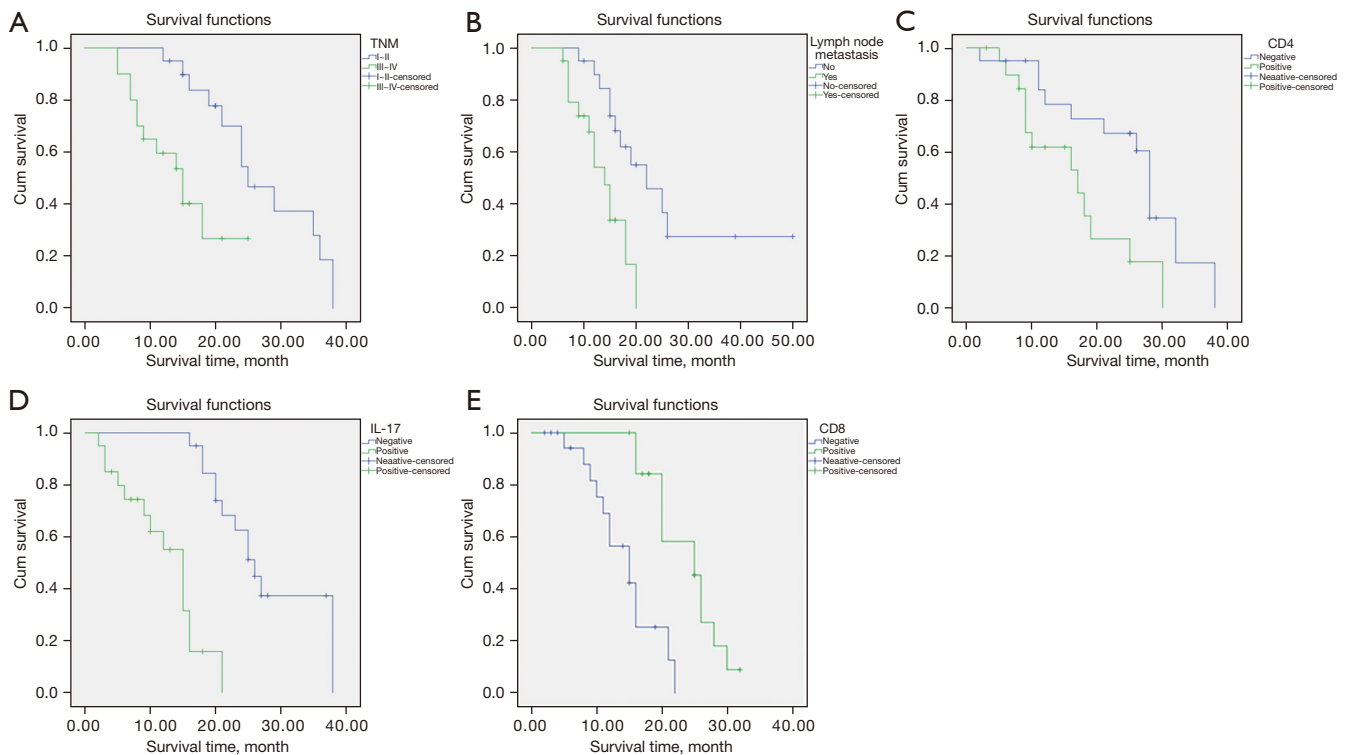


Figure 2 The relationship between the expression of CD4, IL-17, and CD8 and the prognosis of patients. (A) Survival curve of PC patients with different TNM stages; (B) survival curve of PC patients with lymph node metastasis; (C) survival curve of PC patients with different CD4⁺T expression; (D) survival curve of PC patients with different IL-17 expression; (E) survival curve of patients with different CD8⁺T expression. PC, pancreatic cancer; TNM, tumor node metastasis.

Multivariate analysis of factors affecting the prognosis of PC by the Cox regression model

Cox regression model analysis results showed that TNM staging, lymph node metastasis, and CD4⁺IL-17⁺ and CD8⁺ expression have a significant effect on the outcome of PC patients ($P < 0.05$, Table 5). The nomogram results demonstrated that the survival of PC patients with TNM stage III–IV, lymph node metastasis, high CD4⁺IL-17⁺

expression, and low CD8⁺ expression was further reduced, as shown in Figure 3. The representative images for expression of CD4, IL-17 and CD8 in patients with 3-year survival and death outcomes were shown in Figure 4.

Discussion

PC has the biological characteristics of high invasiveness and metastatic potential. Currently, there is no effective

Table 5 Multivariate analysis of factors influencing the outcome of PC by the Cox regression model

Factors	β	SE	Wald	P	OR	95% CI
Age	0.735	0.391	3.531	0.060	2.086	0.969–4.490
Tumor size	0.691	0.417	2.750	0.097	1.996	0.882–4.517
Tumor site	0.117	0.474	0.061	0.805	1.124	0.444–2.844
Histological grade	0.595	0.459	1.682	0.195	1.813	0.738–4.457
TNM stage	2.395	1.080	4.919	0.027	10.964	1.321–91.002
Lymph node metastasis	2.201	1.076	4.187	0.041	9.030	1.097–74.332
Local infiltration	0.774	0.439	3.103	0.078	2.168	0.916–5.129
CD4 ⁺ IL-17 ⁺	0.259	0.118	4.789	0.029	1.295	1.027–1.633
CD8 ⁺ T	-0.286	0.069	17.176	<0.001	0.751	0.656–0.860

PC, pancreatic cancer; β , regression coefficient; SE, standard error; Wald, test statistic; OR, odds ratio; 95% CI, 95% confidence interval; TNM, tumor node metastasis.

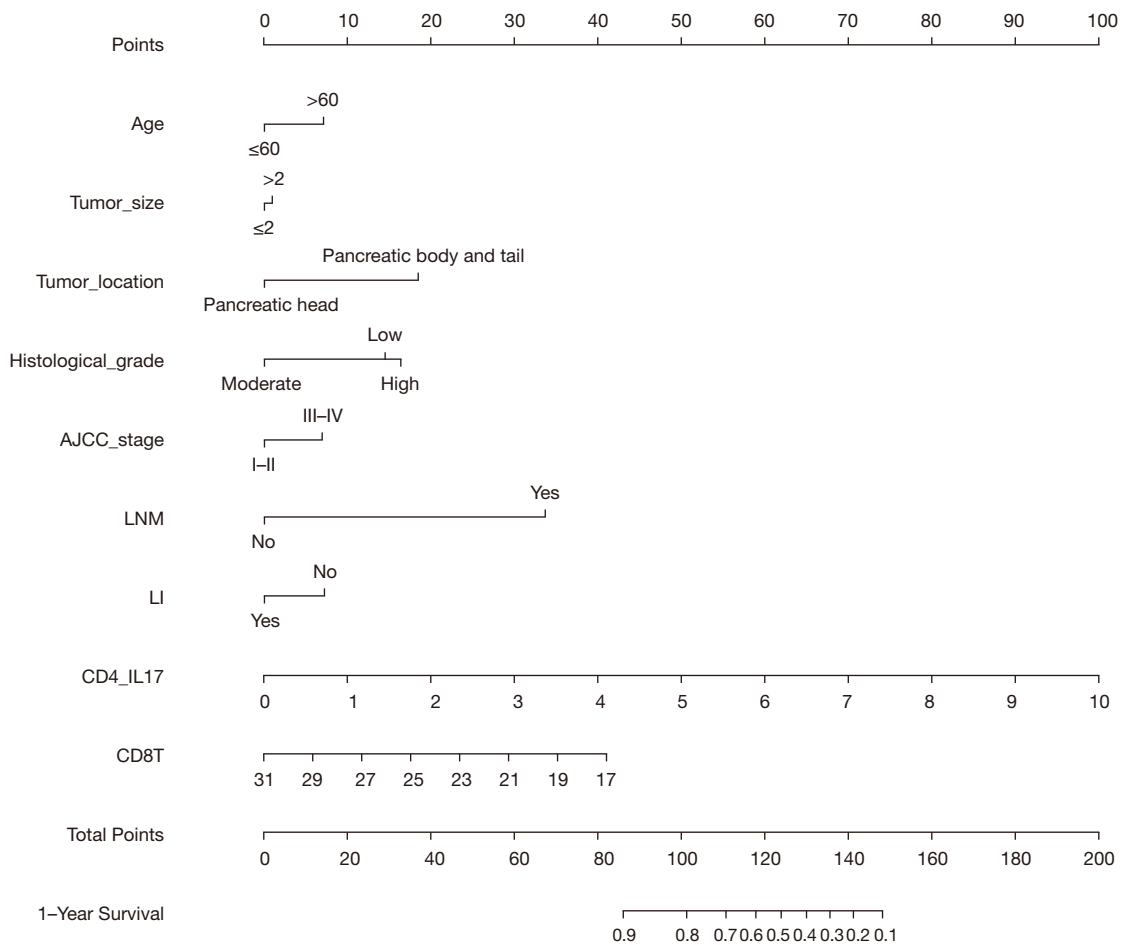


Figure 3 Nomogram of prognostic factors for predicting the survival of PC patients. PC, pancreatic cancer.

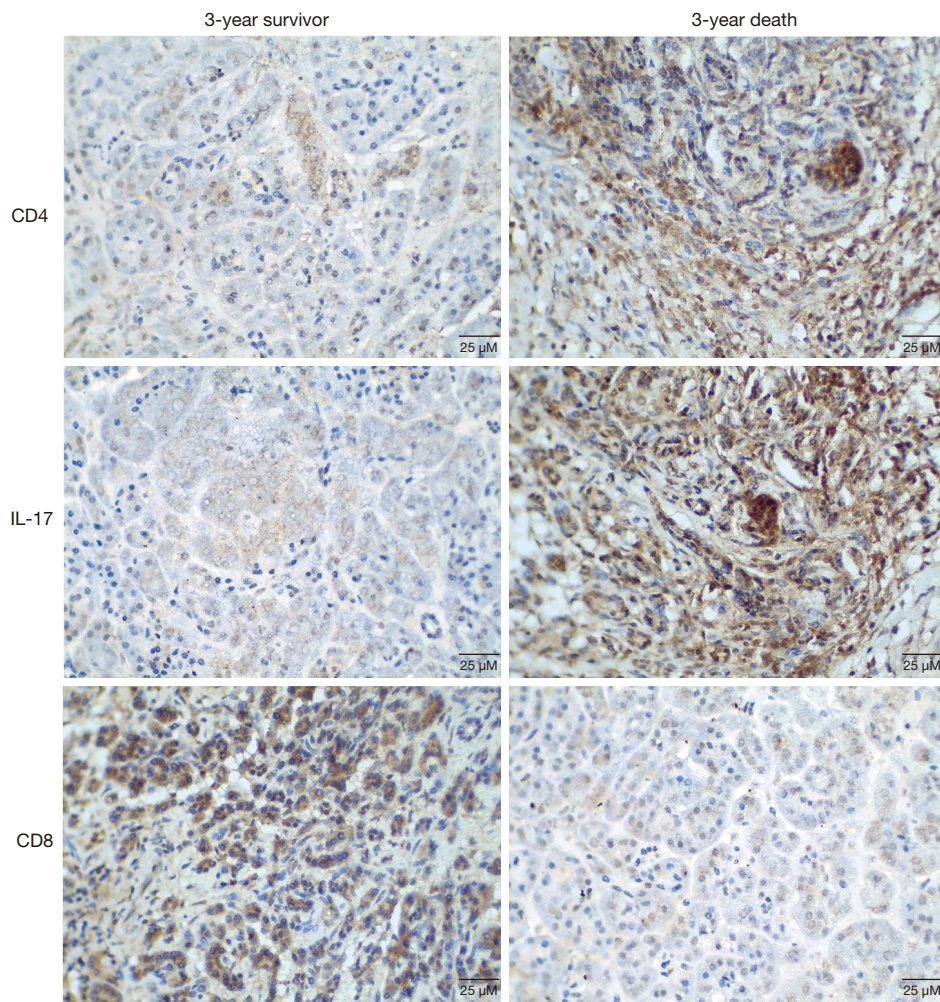


Figure 4 Representative immunohistological staining of CD4, IL-17 and CD8 expression in patients with 3-year survival and death.

early diagnosis method. The surgical resection rate is between 10% to 20% (15,16). Most patients still have early recurrence and metastasis, and the MST is only about 18 months (17). Therefore, it is particularly important to analyze the pathogenesis of PC, and to evaluate the prognosis of patients and find therapeutic targets (18). According to relevant data, the poor prognosis and high mortality of PC are closely related to the tumor microenvironment (19,20). The microenvironment of normal tissues can effectively inhibit tumor growth and is an important barrier against tumors. Malignant solid tumor tissue can induce a state of low pH, hypoxia, and nutrient deficiency in the microenvironment by recruiting fibroblasts and regulating anti-tumor immune cells, and this is strongly linked to the tumor-suppressing effect of immune cells and the sensitivity to chemotherapeutics.

Tumor immunotherapy can eliminate tumors by activating and strengthening the immune system. The immune response regulated by T cells has a significant effect on tumor immunotherapy.

Tumor-infiltrating lymphocytes are one of the major participants in the tumor microenvironment. These infiltrating cells and the secreted cytokines constitute the main components of the tumor immune microenvironment and play a role in the immune regulation of tumors (21). There are many types of immune cells in the microenvironment of PC, and CD4⁺ T and CD8⁺ T cell are main representative immune subsets of the microenvironment serving as a principal component for effective antitumor immune response. Meanwhile, the proportion of these cells can gradually become unbalanced in the process of tumor progression, making regulatory T

cells (Tregs) with immunosuppressive function and tumor-associated macrophages more active and abundant by polarizing into two functionally contrasting subtypes, namely classical activated M1 macrophages and alternatively activated M2 macrophages (22). However, anti-tumor immune cells cannot play their role due to apoptosis, which is part of the immunosuppressive microenvironment of PC and becomes an important reason for treatment failure (23). After initial CD4⁺ T cells are stimulated by antigens, they differentiate into 3 types of effector CD4⁺ T cells: Th1, Th2, and Tregs.

In recent years, researchers have discovered CD4⁺IL-17⁺ T cells, a new type of effector CD4⁺ T cell, in encephalomyelitis and collagen-induced arthritis mouse models. Through clinical trials, it has been confirmed that CD4⁺IL-17⁺ T cells and their effector IL-17 have a significant influence on autoimmune diseases and infectious diseases (24,25). Besides, some researchers have discovered that the percentage of CD4⁺IL-17⁺ T cells in ovarian cancer tissues was higher than that in normal samples, and had a strong relationship with clinical staging (26). In addition, the proportion of CD4⁺IL-17⁺ T cells in different cancerous states of cervical cancer showed a gradually increasing trend, and was significantly correlated with clinical stage, vascular invasion, and other clinicopathological characteristics (27). CD8⁺ T cells have a significant impact on tumor immunity and play an important role in tumor immune surveillance. Initial CD8⁺ T cells can differentiate into cytotoxic CD8⁺ T cells under the stimulation of cancer cell antigens, which can kill antigen-specific cancer cells and inhibit tumorigenesis and development (28,29). Similar to previous reports, this study showed that the content of CD4⁺IL-17⁺ T cells in PC tissue was higher while the content of CD8⁺ T cells was lower than that in normal samples. This demonstrated that CD4⁺IL-17⁺ T cells may exhibit tumor-promoting effects in the microenvironment of PC, while CD8⁺ T cells may show tumor-inhibiting effects. In addition, Cox regression model analysis demonstrated that the TNM stage, lymph node metastasis, and CD4⁺IL-17⁺ and CD8⁺ T cell numbers were influencing factors for the prognosis of PC patients. These factors may become indicators for evaluating the clinical outcome of PC patients. The establishment of the nomogram model showed that the survival probability of patients with TNM stage III-IV, lymph node metastasis, high CD4⁺IL-17⁺ expression, and low CD8⁺ expression was further reduced.

In conclusion, CD4⁺IL-17⁺ and CD8⁺ T cells in PC tissue are associated with TNM staging, lymph node metastasis, and the MST. They can be used as new

predictors of the prognosis of PC patients. However, this study regarding the tumor immune microenvironment lacks comprehensive data, and the study is limited by relatively small case numbers. In our subsequent trials and studies, a more convincing theoretical and experimental basis on the immune response is needed to investigate its impact on the outcome of PC.

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at <https://gs.amegroups.com/article/view/10.21037/gc-22-45/rc>

Data Sharing Statement: Available at <https://gs.amegroups.com/article/view/10.21037/gc-22-45/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://gs.amegroups.com/article/view/10.21037/gc-22-45/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics committee of the Third Xiangya Hospital [No. SYXK(Xiang)2017-0002] and informed consent was taken from all the patients.

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