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Original Article

Infiltrating CD4/CD8 high T cells shows good prognostic impact in pancreatic cancer

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Abstract: Tumor infiltrating lymphocytes in a certain tumor microenvironment are associated with the prognosis of cancer patients. The function of $CD4^+$ and $CD8^+$ T cells in the microenvironment of pancreatic cancer remains largely unknown. This study aimed to investigate the prognostic value of both $CD4^+$ and $CD8^+$ TIL subsets and their combined role in pancreatic cancer. In this study, pancreatic cancer tissues and corresponding adjacent normal tissues were collected from 90 patients. The expression levels of CD4 and $CD8^+$ T cells in pancreatic cancer tissues were detected by immunohistochemistry method. The results showed that $CD4^+$ iTIL expression was significantly correlated with tumor stage. $CD8^+$ iTILs were significantly correlated with lymphatic vessel invasion and tumor stage; $CD8^+$ sTILs not only showed correlation with lymphatic vessel invasion and tumor stage, but also had correlation with pathologic differentiation; the survival time of high CD4 expression group was longer compared to the low CD4 expression group. $CD4^+$ T cells were capable of killing tumor cells and prolonging the survival time of patients either directly or indirectly. According to Cox regression analysis, it was indicated that pathological differentiation, lymphatic vessel invasion, tumor stage, $CD4^+$ and $CD8^+$ TILs were the principle risk factors of pancreatic cancer prognosis. Especially multivariate analysis showed that pathological differentiation and the combination of $CD4^+$ and $CD8^+$ TILs expression were independent predictors of pancreatic cancer survival. Expression levels of $CD4^+$ and $CD8^+$ TILs in pancreatic cancer may provide promising and useful markers for prognosis of pancreatic cancer.

Keywords: Pancreatic cancer, tumor infiltrating lymphocytes, marker

Introduction

The immune system contains both the innate and adaptive immune systems. The innate immune system mediates tumor-promoting inflammation that contributes to development and immune escape of the tumor, which is recognized as a hallmark of cancer [1]. There are various innate cells, such as macrophages, mast cells and neutrophils, which contribute to tumor angiogenesis and tumor infiltration and lead to a poor prognosis [2]. On contrary, abundant infiltrating lymphocytes often indicate a favorable prognosis [3]. Accumulated evidence has demonstrated that abundant tumor infiltrating lymphocytes (TILs) in the certain tumor microenvironment are correlated with the prognosis of cancer, which plays an important role in tumor immune responses [4-6].

Generally, it is thought that $CD8^+$ TILs are a favorable prognostic indicator of many types of cancer, including esophageal cancer, colorectal cancer, and non-small cell lung cancer [7, 8]. CD4 T cells play a key role in regulating the immune response via sending signals to other types of immune cells [4]. CD4 T cells are significant in resisting cancer cells. Naïve CD4 T cells can be differentiated into four types, including helper T cells (Th1, Th2, Th17) and regulatory T cells (Tregs), which play important and various roles in tumor immune microenvironment, tumor immune evasion, immune homeostasis, and anti-tumor immunity [6, 9, 10]. Pancreatic cancer patients exhibited Th17/Treg balance disorders with higher Treg and lower Th17 cells, which affect cytokine IL-10, IL-23, INF-γ, TGF-β, and IL-17 expression changes mainly through regulating transcription factors such as RORα, RORγt, FoxP3 and CTLA-4 [11].

However, the function of $CD4^+$ and $CD8^+$ T cells in the microenvironment of pancreatic cancer

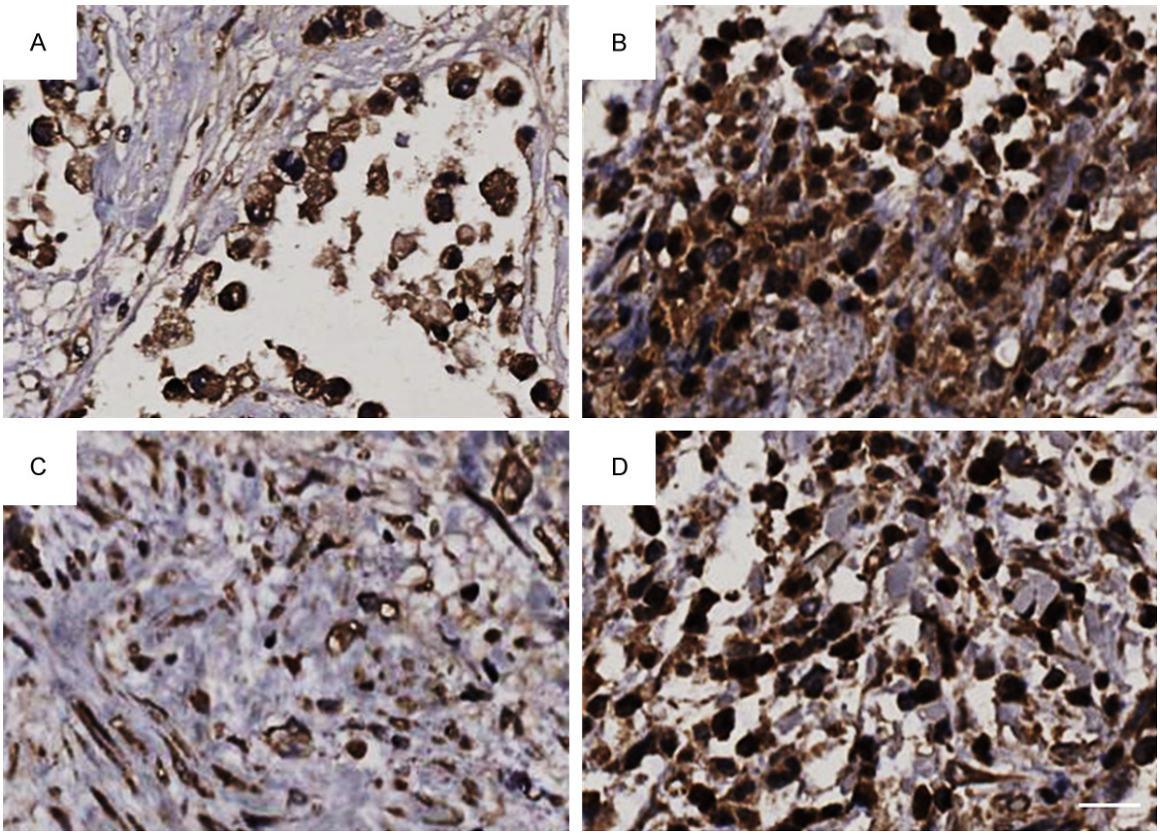

Figure 1. Immunohistochemical staining of TILs in pancreatic intratumoral cancer. A. Low $CD4^+$ iTILs; B. High $CD4^+$ iTILs; C. Low $CD8^+$ iTILs; D. High $CD8^+$ iTILs. Bar: 50 μm.

remains largely unknown. In this study, expression levels of CD4 and $CD8^+$ T cells in pancreatic cancer tissues were detected by immunohistochemistry method. This study aimed to investigate the prognostic value of both $CD4^+$ and $CD8^+$ TIL subsets and their combined role in pancreatic cancer.

Material and methods

Patients

Pancreatic cancer tissues and corresponding adjacent normal tissues were harvested from 90 cases (57 males and 33 females) in the Inner Mongolia Medical College Affiliated Hospital. Pancreatic cancer samples were completely removed and confirmed pathologically. The adjacent normal tissues were resected 3 cm from the pancreatic cancer tissues. Informed consent was obtained from all patients prior to surgery. Pancreatic cancer was diagnosed and staged according to the 7th edition Staging Manual of American Joint Committee on Cancer. The pathological types of pancreatic cancer consist of duct adenocarcinoma, mucinous adenocarcinoma, mucinous cystadenocarcinoma and adenosquamous carcinoma. Complete follow-up history was available for at least 5 years. Overall survival referred to the interval from operation to death. The protocol was approved by the Ethics Committee of Inner Mongolia Medical College Affiliated Hospital.

Immunohistochemistry

Immunohitochemistry was performed on 4 μm thick paraffin-embedded sections. Briefly, the sections were deparaffinized and then hydrated. The sections were washed with PBS. Then the sections were immersed in 0.01 mol/L citrate buffer solution (pH 6.0), and placed in microwave for 10 minutes. Peroxidase was quenched by 3% H_2O_2 in phosphate-buffered saline (PBS) for 15 minutes. The sections were incubated at 4°C overnight with the primary antibody following PBS washing. After washed by PBS for three times, the sections were treated with the Envision detection system (EnVision™ Detection System, Peroxidase/DAB+,

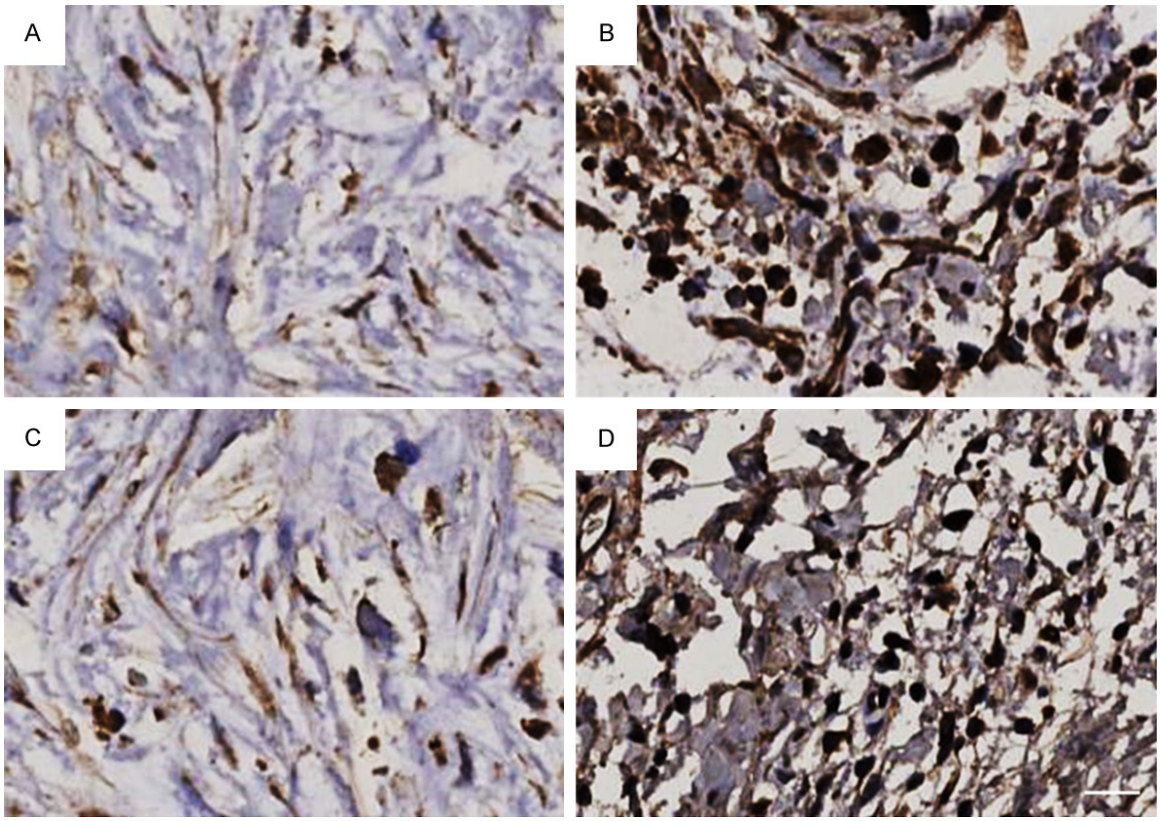

Figure 2. Immunohistochemical staining of TILs in peritumoral cancer. A. Low $CD4^+$ iTILs; B. High $CD4^+$ iTILs; C. Low $CD8^+$ iTILs; D. High $CD8^+$ iTILs. Bar: 50 μm.

Table 1. Relationship between $CD4^+$ iTILs and clinicopathological factors

Clinical pathological factors		$CD4^+$ iTILs		*P* value
		Low	High	
Gender	Male	30	27	0.351
	Female	14 (31.8%)	19 (41.3%)	
Age	≤ 60 years	19	23	0.517
	> 60 years	25 (58.8%)	23 (50%)	
Pathologic differentiation	High	27	32	0.413
	Low	17 (38.6%)	14 (30.4%)	
Tumor size	≤ 4 cm	24	27	0.691
	> 4 cm	20 (45.5%)	19 (41.3%)	
Nerve invasion	Negative	24	29	0.413
	Positive	20 (45.5%)	17 (37.0%)	
Lymphatic vessel invasion	Negative	27	30	0.705
	Positive	17 (38.6%)	16 (34.8%)	
Stage (I + II vs II + IV)	I	13	27	0.005
	II + IV	31 (70.5%)	19 (41.3%)	

Rabbit/Mouse, Dako, Denmark). Finally, the sections were counterstained with hematoxylin (Hematoxylin, Sigma-Aldrich, Germany) to visualize the nuclei. Images were obtained with LEICA AMIL (LEICA, Germany). CD4 (clone L26 and CD8 (clone 1A5) antibodies from Ventana Medical (Tucson, Ariz, USA) were employed in this study.

Evaluation of staining

A couple of pathologists who were blinded to the scores of the other markers and the patient outcomes independently scored the samples. TILs were classified into two parts: iTILs and pTILs. The former was defined as TILs within intratumoral tissues, and the latter as TILs within peritumoral tissues (**Figure 1**). According to reference [12], the area of tumor and stromal components was determined in a 0.4×0.4 mm microscopic grid under 200× magnification, and the number of immunoreactive cells was counted in the specified area. The number of

Table 2. Relationship between $CD4^+$ pTILs and clinicopathological factors

Clinical pathological factors		$CD4^+$ pTILs		*P* value
		Low	High	
Gender	Male	23	34	0.929
	Female	13 (36.1%)	20 (37.0%)	
Age	≤ 60 years	18	24	0.605
	> 60 years	18 (50.0%)	30 (55.6%)	
Pathologic differentiation	High	26	33	0.277
	Low	10 (27.8%)	21 (38.9%)	
Tumor size	≤ 4 cm	22	29	0.487
	> 4 cm	14 (38.9%)	25 (46.3%)	
Nerve invasion	Negative	19	34	0.336
	Positive	17 (47.2%)	20 (37.0%)	
Lymphatic vessel invasion	Negative	23	34	0.929
	Positive	13 (36.1%)	20 (37.0%)	
Stage (I vs II + IV)	I	14	26	0.386
	II + IV	22 (61.1%)	28 (51.9%)	

Table 3. Relationship between $CD8^+$ iTILs and clinicopathological factors

Clinicopathological factors		$CD8^+$ iTILs		*P* value
		Low	High	
Gender	Male	29	28	0.620
	Female	15 (34.1%)	18 (39.1%)	
Age	≤ 60 years	21	21	0.844
	> 60 years	23 (52.3%)	25 (54.3%)	
Pathologic differentiation	High	27	32	0.413
	Low	17 (38.6%)	14 (30.4%)	
Tumor size	≤ 4 cm	26	25	0.650
	> 4 cm	18 (40.9%)	21 (45.7%)	
Nerve invasion	Negative	24	29	0.413
	Positive	20 (45.5%)	17 (37.0%)	
Lymphatic vessel invasion	Negative	22	35	0.010
	Positive	22 (50.0%)	11 (23.9%)	
Stage (I vs II + IV)	I	13	27	0.005
	II + IV	31 (70.5%)	19 (41.3%)	

$CD4^+$ cells and $CD8^+$ cells was counted in the same region. For the definition of high and low TILs, the median TIL value as the cut-off was employed. The expression levels of the combined CD4 and CD8 were scored individually in the same tumor.

Statistics

The statistical package IBM SPSS version 18 was employed for data analysis. The Chi-square test was used to compare the data between different groups. Prognostic factors were examined by univariate and multivariate analyses. Kaplan-Meier method and the COX regression model were used to analyze survival and prognostic factors, respectively. *P* value < 0.05 was considered significant.

Results

As showed in **Figures 1** and **2**, $CD4^+$ iTILs and $CD8^+$ iTILs were stained in pancreatic intratumoral cancer and peritumoral cancer, respectively. The relationship between TILs and clinicopathological factors is summarized in **Table 1**. There was significant correlation between $CD4^+$ iTILs and tumor stage (P = 0.005). However, there was no significant correlation between $CD4^+$ pTILs and these clinical pathological factors (**Table 2**). $CD8^+$ iTILs were significantly correlated with lymphatic vessel invasion and tumor stage (P = 0.010 and 0.005, respectively) (**Table 3**); $CD8^+$ pTILs were not only correlated with lymphatic vessel invasion and tumor stage (P = 0.033 and P < 0.001, respectively), but also correlated with pathologic differentiation (P = 0.009) (**Table 4**).

The Kaplan-Meier survival curve was used to evaluate the overall survival of patients. The median survival of patients in high $CD4^+$ TILs expression group (median, 27 months; 95% CI, 24.30-29.70 months) was significantly shorter than that in low $CD4^+$ TILs expression group (median 17 months; 95% CI 12.14-21.86 months) (**Figure 3**). Similarly, the median survival time in high CD8 TILs expression group (median 28 months; 95% CI 22.62-33.39 months) was significantly longer than that in low CD8 TILs expression group (median 18 months; 95% CI 13.77-22.23 months) (P < 0.001) (**Figure 4**). The median survival time in

Table 4. Relationship between $CD8^+$ pTILs and clinicopathological factors

Clinicopathological factors		$CD8^+$ pTILs		*P* value
		Low	High	
Gender	Male	32	25	0.070
	Female	12 (27.3%)	21 (45.7%)	
Age	≤ 60 years	20	22	0.822
	> 60 years	24 (54.5%)	24 (52.2%)	
Pathologic differentiation	High	23	36	0.009
	Low	21 (41.7%)	10 (21.7%)	
Tumor size	≤ 4 cm	24	27	0.691
	> 4 cm	20 (45.5%)	19 (41.3%)	
Nerve invasion	Negative	24	29	0.413
	Positive	20 (45.5%)	17 (37.0%)	
Lymphatic vessel invasion	Negative	23	34	0.033
	Positive	21 (47.7%)	12 (26.1%)	
Stage (I vs II + IV)	I	11	29	< 0.001
	II + IV	33 (75.0%)	17 (37.0%)	

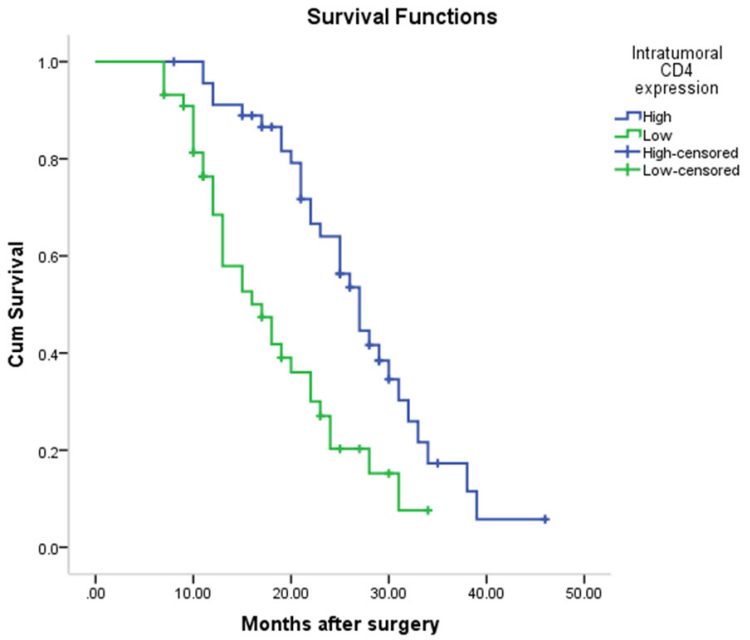

Figure 3. The median survival time in high $CD4^+$ TILs expression group (median 27 months; 95% CI 24.299-29.701 months) was significantly shorter than that in low $CD4^+$ TILs expression group (median 17 months; 95% CI 12.138-21.862 months) (P = 0.001).

the combination of CD4 high CD8 high TILs expression group (median 28 months; 95% CI 23.41-32.60 months) was shorter than that in the combination of low CD4 low CD8 low TILs expression group (median 15 months; 95% CI 11.08-18.92 months) (P < 0.05) (**Figure 5**).

The results of Cox regression analysis are shown in **Tables 5** and **6**. Univariate analysis demonstrated that the main prognostic factors of pancreatic cancer are pathological differentiation, lymphatic vessel invasion, tumor stage, $CD4^+$ and $CD8^+$ TILs in pancreatic cancer tissues (**Table 5**). Moreover, multivariate analysis clearly showed that pathological differentiation, and $CD4^+$ and $CD8^+$ TILs expression were independent prognostic factors for overall survival in pancreatic cancer (**Table 6**). The age, sex and tumor size were not correlated with the prognosis of pancreatic cancer.

Discussion

Cellular immunity is significant for the immune system, which plays a major role in killing cancer and preventing inflammation. T cells are the key in cellular immunity [13]. Increasing evidence suggests that tumor-specific tumor-infiltrating T cells (TILs) but not circulating T cells predict clinical outcomes of cancer patients [14, 15]. A model including three intratumoral infiltrating immune markers ($CD15^+$, $CD206^+$ and $CD117^+$) and a SMAD4 mutation can be used to predict recurrence and survival in patients after surgery for pancreatic ductal adenocarcinoma [16]. Prognostic significance of CD3, CD8 and CD20 positive lymphocytes and survival have a correction in pancreatic ductal adenocarcinoma [17]. The involvement of both $CD4^+$ and $CD8^+$ T cells is required for an effective antitumor immune response [18, 19]. $CD8^+$ T cells have long been deemed as the ideal option for adoptive cell therapy due to their ability to lyse tumor cells directly. Although $CD8^+$ T cells are important in antitumor immunity, it has been

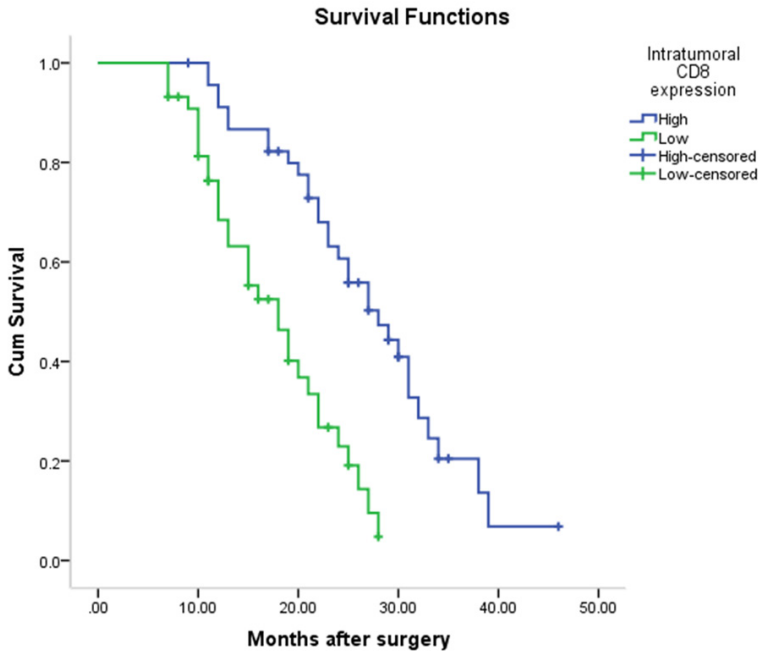

Figure 4. The median survival time in high CD8 TILs expression group (median 28 months; 95% CI 22.615-33.385 months) was significantly longer than that in low CD8 TILs expression group (median 18 months; 95% CI 13.770-22.230 months) (P < 0.001).

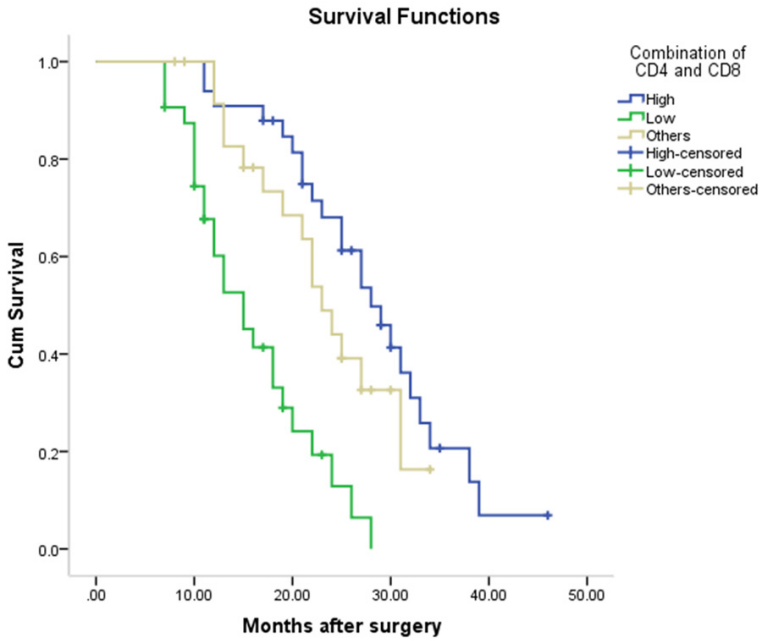

Figure 5. The median survival time in the combination of CD4 high CD8 high TILs expression group (median 28 months; 95% CI 23.407-32.593 months) was shorter than that in the combination of low CD4 low CD8 low TILs expression group (median 15 months; 95% CI 11.084-18.916 months) (P < 0.001).

shown that they cannot always protect the host for tumor relapse [20]. One reason is that $CD8^+$ T cells-mediated recognition of tumors is impaired [21]. Although only few studies investigated the role of $CD4^+$ iTILs in a variety of cancers as a prognostic factor, the role of $CD4^+$ T cells in anti-tumor immunity has been well investigated in both animal models and cancer patients [22].

In our current study, it was found that $CD4^+$ iTILs expression was significantly correlated with tumor stage. There was significant correlation between $CD8^+$ iTILs and lymphatic vessel invasion and tumor stage; $CD8^+$ sTILs were not only correlated with lymphatic vessel invasion and tumor stage, but also correlated with pathologic differentiation. The results showed that survival in high CD4 expression group was longer compared to the low group. It was suggested that $CD4^+$ T cells can directly or indirectly kill tumor cells and prolong the survival of patients. Cox regression analysis demonstrated that pathological differentiation, lymphatic vessel invasion, tumor stage, $CD4^+$ and $CD8^+$ iTILs were the main risk factors for pancreatic cancer prognosis. Especially multivariate analysis showed that pathological differentiation, and the $CD4^+$ and $CD8^+$ iTILs expression were independent predictors of survival of pancreatic cancer patients. Tumor-infiltrating CD4(+)T(high)/CD8(+)T(high)/%Treg(low) and %M1(high)/M2(low) are independent prognosticators useful for evaluating the immune microenvironment of PDC [23].

Kaplan-Meier analysis showed that high CD4 and CD8 TILs expression is associated with

Table 5. Univariate Cox regression analysis of the effect of TILs on survival

Variables	Patients (n)	Median survival, 95% CI (months)	Log-rank P
Gender			
Male	57	24 (19.465-28.535)	0.625
Female	33	22 (20.179-23.821)	
Age (yeas)			
≤ 60	42	24 (19.990-28.010)	0.591
> 60	48	21 (16.935-25.065)	
Pathologic differentiation			
High	59	24 (20.545-27.455)	0.016
Low	25	18 (11.503-24.497	
Tumor size			
≤ 4 cm	51	23 (15.533-27.467)	0.733
> 4 cm	39	22 (18.202-25.798)	
Nerve invasion			
Negative	53	23 (20.104-25.896)	0.858
Positive	37	21 (13.997-28.003)	
Lymphatic vessel invasion			
Negative	57	27 (20.330-33.670)	0.011
Positive	33	20 (14.920-25.080)	
Stage			
I	40	29 (24.093-33.907)	< 0.001
II + IV	50	19 (14.527-23.473)	
$CD4^+$			
Low	44	17 (12.138-21.862)	0.001
High	46	27 (24.299-29.701)	
$CD8^+$			
Low	44	18 (13.770-22.230)	
High	46	28 (22.615-33.385)	< 0.001
Combination of $CD4^+$ and $CD8^+$			
Both Low	29	15 (11.084-18.916)	< 0.001
Both High	31	28 (23.407-32.595)	
Others	30	23 (19.727-26.273)	

Table 6. Multivariate Cox regression analysis of the effect of TILs on survival

Variable	Hazard ratio (95% CI)	P
Pathologic differentiation		
High	0.405 (0.380-1.471)	0.405
Low	1.000	-
Lymphatic vessel invasion		
Negative	1.740 (1.034-2.927)	0.037
Positive	1.000	
Stage		
I	2.779 (1.631-4.736)	< 0.001
II + IV	1.000	-
Combination of $CD4^+$ and $CD8^+$		
Both Low	1.000	-
Both High	3.264 (1.678-6.348)	0.001
Others	0.646 (0.329-1.629)	0.205

better prognosis in pancreatic cancer. Our study also revealed that high CD4 and high CD8 iTILs and sTILs were correlated with good prognosis compared with low CD4 and low CD8 iTILs. The median survival in the high CD4 and high CD8 iTILs expression group was longer compared to low CD4 and low CD8 iTILs expression group. Our main finding was that the combination of $CD4^+$ and $CD8^+$ iTILs expression was a strong and independent prognostic factor in this group. Patients with low $CD4^+$ and low $CD8^+$ iTILs expression seemed to have poor prognosis. Our results indicated that CD4 and CD8 iILs may also play an important role in antitumor immune response and that the collaborative interaction between high CD8 and high CD4 iTILs was critical for cancer suppression. In pancreatic adenocarcinoma, the presence of $CD4^+$ iTILs together with $CD8^+$ iTILs serves as a good indicator of the patient's outcome after surgical treatment [24].

Conclusions

Expression of $CD4^+$ and $CD8^+$ iTILs in pancreatic cancer may provide promising and useful markers for prognosis of pancreatic cancer. Determination of CD4 and CD8 iTILs expression in pancreatic cancer tissues may aid to understand the local immune response in tumor microenvironment, which provides a basis for postoperative antitumor immunity therapy.

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Disclosure of conflict of interest

None.

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