

# The role of IL-17, IFN- $\gamma$ , 4-1BBL and tumour-infiltrating lymphocytes in the occurrence, development and prognosis of pancreatic cancer

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**Abstract.** Immunotherapy has made progress in the treatment of tumours; however, in patients with pancreatic cancer, immunotherapy has not achieved effective results. The present study investigated changes in the immune microenvironment during tumour development and progression, and the relationship between the immune microenvironment and prognosis, to clarify the mechanism of immune escape in pancreatic cancer. A total of 40 patients with pancreatic cancer (including 22 with stage I-II disease and 18 with stage III-IV disease) and 20 patients with chronic pancreatitis were included in the present study. The expression of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL was assessed by immunohistochemistry, and the mRNA expression levels were detected by reverse transcription-quantitative PCR (RT-qPCR). The clinicopathological characteristics and prognoses of patients with pancreatic cancer were analysed to further explore the role of IL-17, IFN- $\gamma$ , 4-1BBL and tumour-infiltrating lymphocytes in pancreatic cancer. Notably, the expression levels of CD3, CD8, CD56, IFN- $\gamma$  and 4-1BBL in patients with stages I-II and III-IV cancer were lower than those in patients with chronic pancreatitis ( $P < 0.05$ ), especially in patients with stage III-IV cancer ( $P < 0.05$ ). In addition, the expression of IL-17 in patients with stages I-II and III-IV cancer was greater than in patients with chronic pancreatitis ( $P < 0.05$ ), especially

in patients with stage III-IV cancer ( $P < 0.05$ ). The RT-qPCR results regarding CD3, CD4, CD8, CD56, IFN- $\gamma$  and IL-17 were almost the same as those obtained from immunohistochemical analysis; however, the mRNA expression levels of 4-1BBL were not significantly different between stages I-II and III-IV. Furthermore, patients with pancreatic cancer with higher expression levels of CD3, CD8, CD56, IFN- $\gamma$  and 4-1BBL exhibited longer survival, whereas those with higher expression of IL-17 had a shorter survival time. The expression levels of CD3, CD8, CD56, cytokines IL-17 and IFN- $\gamma$ , and costimulatory molecule 4-1BBL were revealed to be related to the degree of differentiation, Tumour-Node-Metastasis staging and the prognosis of pancreatic cancer, and may serve as novel immunological indicators for evaluating the condition and treatment effectiveness in patients with pancreatic cancer.

## Introduction

Pancreatic cancer is a common malignancy of the digestive system with increasing global incidence and an overall 5-year survival rate of 5% (1). Pancreatic cancer is a highly malignant tumour, which is currently treated through surgery, chemotherapy and radiotherapy; however, there are no effective therapeutic methods (2). In recent years, immune surveillance, immune escape, immune tolerance, T-lymphocyte signalling, inflammatory mediators, cytokines and the downregulation of costimulatory molecules have been recognized as potential targets for therapy (3,4).

The analysis of tumour-infiltrating lymphocyte (TIL) subsets, which include CD3-, CD4- and CD8-positive cells, natural killer cells and myeloid cells, is a key indicator of cellular immune function, which is important for determining the recurrence, metastasis and prognosis of malignancies (5,6). Our previous research demonstrated that the infiltration of T lymphocytes is associated with the prognosis of patients with colorectal cancer (6).

Cytokines are messengers that lead to immune and inflammatory responses, and they are important for cell growth and differentiation (7). IL-2, IFN- $\alpha$  and IFN- $\gamma$  are secreted mainly by T helper (Th)1 cells, which can enhance the cytotoxic

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effects of killer effector cells and mediate antitumour cellular immune responses (8). IFN- $\gamma$  is an important cytokine that can change the surface composition of tumour cells, enhance the antigenicity of MHC-I and tumour-related antigens, induce the differentiation of tumour cells into normal cells, and activate cytotoxic cells [such as macrophages, natural killer (NK) cells and lymphokine-activated killer cells] to exert antitumour immune effects (9). Cytokines such as IL-4, IL-5, IL-6 and IL-10 are secreted mainly by Th2 cells, which stimulate B-lymphocyte proliferation and produce specific antibodies associated with humoral immunity (10), while also inhibiting cytokines secreted by Th1 cells. Under normal conditions in the body, Th1 and Th2 cells are in equilibrium, restricting the function of the other through their respective secreted cytokines; however, when the level of IL-4 in the immune microenvironment increases, it can bind to the IL-4R on the surface of undifferentiated Th cells. Once the IL-4R is activated, it will initiate intracellular signal transduction pathways to promote the transition of Th1 cells to Th2 cells, and immune suppression and tumour escape can occur. In addition, cytokines such as IL-17, IL-21, IL-22 and IL-6 are mainly secreted by Th17 cells (11). IL-17 enhances the expression of inflammatory cytokines, such as IL-1, IL-6 and IL-23, thus exacerbating the inflammatory response. In addition, IL-17 can stimulate cancer cells to secrete VEGF and indirectly promote blood vessel formation, promote tumour development by inhibiting CD8<sup>+</sup> cells and enhance the entry of myeloid-derived suppressor cells into tumour tissue (12). Increased IL-17 expression in patients with colon cancer and hepatocellular carcinoma has been reported to predict a poor prognosis (13,14).

4-1 BBL is a member of the tumour necrosis factor (TNF) family and is a type II membrane protein, which is mostly expressed on activated antigen-presenting cells. 4-1 BBL acts primarily in the late stage of the immune response and mainly regulates the proliferation of T cells (especially CD8-positive cells) after activation (15,16). It has been reported that the transfection of the 4-1BBL gene into antigen-presenting cells can significantly enhance its co-stimulatory effect on T cells (17). Previous studies have shown that the expression of the costimulatory molecule 4-1BBL is closely related to the occurrence and metastasis of prostate cancer, gastric cancer, glioma and laryngeal cancer (18-21), and its low expression confirms the existence of immune escape in tumours, indicating a poor prognosis.

The present study used immunohistochemistry and reverse transcription-quantitative PCR (RT-qPCR) to determine the expression of TILs, cytokines and costimulatory molecules in chronic pancreatitis and pancreatic cancer tissues. Subsequently, the study analysed the relationships of TILs, cytokines and costimulatory molecules with clinicopathological characteristics and prognosis, explored the significance of immune function in the occurrence and development of pancreatic cancer, and provided a theoretical basis for the immunotherapeutic treatment of pancreatic cancer.

## Materials and methods

**Patient characteristics.** A total of 60 paraffin-embedded tissue samples (including 20 samples from patients with chronic

pancreatitis and 40 samples from patients with pancreatic cancer) were obtained from patients who had been treated at The First Affiliated Hospital of Soochow University (Suzhou, China) between November 2006 and December 2016, and their data were accessed. Prior to the collection of these tissue samples, the patients had provided their informed consent for their tissues to be used in scientific research. None of the patients had previously undergone radiotherapy, chemotherapy or immunotherapy. Postoperative pathology confirmed chronic pancreatitis or pancreatic cancer, and Tumour-Node-Metastasis classification and differentiation grading for pancreatic cancer were performed according to the criteria described by the Union for International Cancer Control (22,23). The present study was a retrospective analysis approved by the research Ethics Committee of The First Affiliated Hospital of Soochow University (approval no. 2023-410).

**Immunohistochemical staining for CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL.** Tissue sections (4  $\mu$ m) were prepared from paraffin-embedded specimens. Following deparaffinization with xylene (three times; 5 min each) and rehydration with anhydrous ethanol, and 95, 75 and 50% ethanol (5 min each), the slides were heated to 100°C in 10 mmol/l sodium citrate buffer (pH 6) for 15 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections at 25°C with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. The sections were subsequently blocked with 10% normal horse serum (Wuhan Boster Biological Technology, Ltd.) for 10 min at 25°C, and were then incubated with the following anti-human antibodies at room temperature for 2 h in moisture chambers in the dark: Monoclonal mouse IgG against CD3 (cat. no. sc-20047), polyclonal rabbit IgG against CD4 (cat. no. sc-7219) and CD8 (cat. no. sc-7188), monoclonal mouse IgG against IFN- $\gamma$  (cat. no. sc-373727) and 4-1BBL (cat. no. sc-398933) (all from Santa Cruz Biotechnology, Inc.; dilution, 1:100), monoclonal mouse IgG against CD56 (cat. no. ab9272; Abcam; dilution, 1:500) and polyclonal rabbit IgG against IL-17 (cat. no. ab79056; Abcam; dilution, 1:100). The sections were subsequently washed with PBS and incubated for 1 h in moisture chambers in the dark at room temperature with polyclonal goat anti-mouse/rabbit IgG biotinylated secondary antibodies (cat. no. K5007; Dako; Agilent Technologies, Inc.; dilution, 1:2,000). Finally, the sections were developed with 3,3'-diaminobenzidine tetrahydrochloride hydrate and counterstained with haematoxylin for 5 min at room temperature. A total of five randomly selected fields were assessed using a BX53 light microscope (Olympus Corporation), and areas of necrosis were avoided. A paraffin-embedded section of human tonsillar tissue, provided by the Pathology Department of the First Affiliated Hospital of Soochow University was used as a positive control, and the volunteer who provided this tissue provided written informed consent for it to be used in subsequent scientific research. PBS was used instead of primary antibody as a negative control.

**Scoring system for immunohistochemistry.** The expression levels of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL were scored using a semi-quantitative system (24). PBS was used instead of primary antibody as a negative control, and tonsillar tissue was used as a positive control, with a double-blind

Table I. Primer pairs used for reverse transcription-quantitative PCR.

Gene name (GenBank no.)	Sequence	Product size, bp
CD3 (NM_000732.4)	F: 5'-GGGAGTCTTCTGCTTTGCTG-3' R: 5'-TTGTTCCGAGCCCAAGTTTC-3'	153
CD4 (NM_000616.4)	F: 5'-GTGAACCTGGTGGTGATG-3' R: 5'-GAGACCTTTGCCTCCTTG-3'	122
CD8 (NM_001768.7)	F: 5'-ATGGCCTTACCAGTGACCG-3' R: 5'-AGGTTCCAGGTCCGATCCAG-3'	104
CD56 (NM_000615.6)	F: 5'-CCAACCATCATCTGGAAACA-3' R: 5'-CTGCCCTCACAGCGATAAGT-3'	137
IFN- $\gamma$ (NM_000619)	F: 5'-GAGTGTGGAGACCATCAAGGA-3' R: 5'-GTATTGCTTTGCGTTGGACA-3'	128
IL-17 (U32659.1)	F: 5'-AACGATGACTCCTGGGAAGA-3' R: 5'-CTCAGAATTTGGGCATCCTG-3'	115
4-1BBL (NM_003811.3)	F: 5'-GCCTGGGCGTCCATCTTC-3' R: 5'-AGTCCGGCTGGGATTTTCG-3'	112
$\beta$ -actin (NM_001101.3)	F: 5'-CACTGTGCCCATCTACGAGG-3' R: 5'-AATGTCACGCACGATTTCC-3'	154

F, forward; R, reverse.

reading by two pathologists with generally consistent results. The staining intensity was scored as 0 (achromatic), 1 (light yellow), 2 (brownish yellow) or 3 (brown). In addition, the percentage of positive cells was scored as 0 (<5%), 1 (5-24%), 2 (25-49%), 3 (50-74%) or 4 (>75%). The two scores were added together and the samples were assigned to one of four levels as follows: (-), score 0-1; (+), score 2; (++) , score 3-4; or (+++), score  $\geq$ 5. (-) and (+) were defined as negative expression, (++) as weak expression and (+++) as strong expression.

**RT-qPCR.** Total RNA from formalin-fixed paraffin-embedded (FFPE) tissue sections was purified using an RNeasy FFPE Kit (cat. no. 73504; Qiagen, Inc.); all of the reagents used for RNA extraction were obtained from this kit. Firstly, the paraffin was removed from freshly cut FFPE tissue sections by treatment with a deparaffinization solution (Qiagen, Inc.). The samples were then incubated in optimized lysis buffer to release RNA from the sections. A short incubation at 80°C partially reversed the formalin cross-linking of the released nucleic acids; this was followed by deoxyribonuclease treatment, which was optimized to eliminate all genomic DNA. The lysate was then mixed with Buffer RBC. Ethanol was added to provide appropriate binding conditions for RNA and the samples were applied to the provided RNeasy MinElute spin columns. The RNA was then eluted in a minimum of 14  $\mu$ l RNase-free water. cDNA was subsequently synthesized from total RNA using RevertAid™ First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. mRNA levels were quantified with qPCR using a FastStart Universal SYBR Green Master (Rox) kit (cat. no. 4913914001; Sigma-Aldrich KGaA). The qPCR cycling conditions were as follows: One cycle of initial template degeneration at 95°C for 1 min; followed by 45 cycles of template degeneration at 95°C for 20 sec, annealing at 58°C

for 30 sec and extension at 68°C for 45 sec.  $\beta$ -actin was used as an internal reference. Three independent experiments were performed to analyse relative target gene expression. The expression levels of RNA were quantified using Cq values and were normalized to  $\beta$ -actin using the  $2^{-\Delta\Delta Cq}$  method (25). All primers were supplied by Sangon Biotech Co., Ltd., as shown in Table I. The success rate of mRNA extraction was determined by calculating the ratio of the number of samples from which mRNA was successfully extracted to the total number of samples.

**Statistical analysis.** All statistical analyses were carried out using SPSS (v25; IBM Corporation). The data are presented as the means  $\pm$  standard deviation. The  $\chi^2$  test or Fisher's exact test were employed to assess the association between expression and patient characteristics. For comparing the immunohistochemical staining scores among different groups, the Kruskal-Wallis test followed by Dunn's test was utilized. The mRNA expression levels in various groups were assessed by one-way ANOVA followed by the Tukey's honest significant difference post hoc test. The survival of each group was depicted as a Kaplan-Meier curve, and differences were compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17, and 4-1BBL in chronic pancreatitis, and stages I-II and III-IV pancreatic cancer.** Immunohistochemical staining revealed that CD3, CD4, CD8 and CD56 were located mainly in the cell membrane and appeared yellow or brown, and IFN- $\gamma$ , IL-17 and 4-1BBL were located in the cell membrane or cytoplasm (Fig. 1). The expression of CD3, CD4, CD8, CD56, IFN- $\gamma$  and

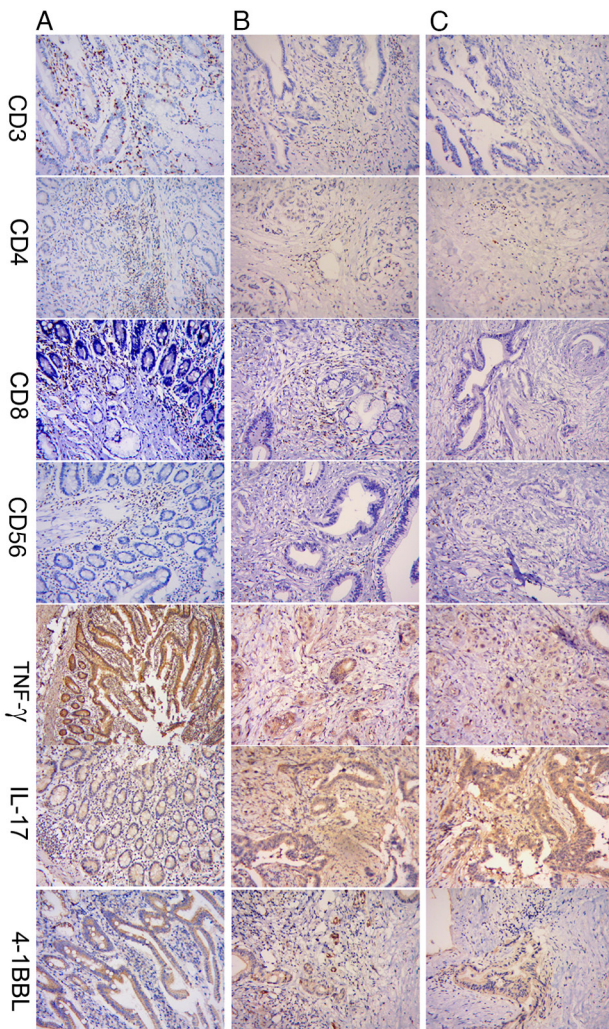


Figure 1. Immunohistochemical staining images of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL in each group (magnification, x200). (A) Chronic pancreatitis; (B) stage I-II pancreatic cancer; (C) stage III-IV pancreatic cancer.

4-1BBL were gradually decreased in samples from patients with stages I-II and III-IV pancreatic cancer compared with those from patients with chronic pancreatitis. By contrast, the expression of IL-17 was gradually increased in samples from patients with stages I-II and III-IV pancreatic cancer compared with those from patients with chronic pancreatitis (Fig. 1).

The expression levels of CD3, CD4, CD8, CD56, IFN- $\gamma$  and 4-1BBL were significantly lower in pancreatic cancer tissues than those in chronic pancreatitis tissues ( $P < 0.05$ ; Fig. 2). By contrast, the expression levels of IL-17 were significantly increased in patients with pancreatic cancer than in those with chronic pancreatitis ( $P < 0.05$ ). Furthermore, the expression levels of CD3, CD8, CD56, IFN- $\gamma$  and 4-1BBL was significantly lower in stage III-IV than in stage I-II ( $P < 0.05$ ), and the expression of IL-17 was significantly greater in stage III-IV than in stage I-II ( $P < 0.05$ ). However, no significant difference was observed between stages I-II and III-IV with respect to CD4 staining (Fig. 2).

*CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17, and 4-1BBL mRNA expression levels in chronic pancreatitis, and stages I-II and III-IV pancreatic cancer.* A total of 50 blocks were randomly

selected from the 60 paraffin-embedded samples and were used for mRNA extraction, including 16 chronic pancreatitis samples, 17 stage I-II samples and 17 stage III-IV samples. The total extraction rate of mRNA from the wax blocks of pancreatic tissue was  $\sim 74\%$  (37/50), and the extraction rates of the chronic pancreatitis, stage I-II and stage III-IV tissue samples were 10/16 (62.5%), 13/17 (76.5%), and 14/17 (82.4%), respectively. Similar to the immunohistochemistry results, CD3, CD4, CD8, CD56 and IFN- $\gamma$  mRNA expression levels were significantly lower in pancreatic cancer tissues than those in chronic pancreatitis tissues ( $P < 0.05$ ; Fig. 3). By contrast, the mRNA expression levels of IL-17 were significantly increased in tissues from patients with pancreatic cancer than in those from patients with chronic pancreatitis ( $P < 0.05$ ; Fig. 3). The mRNA expression levels of CD3, CD8, CD56 and IFN- $\gamma$  were significantly lower in samples from patients with stage III-IV pancreatic cancer than in those from patients with stage I-II pancreatic cancer ( $P < 0.05$ ), whereas the opposite was shown regarding IL-17. The mRNA expression levels of CD4 were not significantly different between stages I-II and III-IV. In contrast to the immunohistochemistry results, the mRNA expression levels of 4-1BBL were not significant different among the chronic pancreatitis, stage I-II and stage III-IV groups ( $P > 0.05$ ; Fig. 3).

*Association between CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL expression and clinicopathological data.* The infiltration of CD3-, CD8- and CD56-positive cells was related to the differentiation and stage of pancreatic cancer, with greater infiltration detected in patients with highly differentiated cancer ( $P < 0.05$ ) and in patients with stage I-II cancer ( $P < 0.05$ ), independent of the patient age, sex and tumour site ( $P > 0.05$ ) (Table II). The infiltration of CD4-positive cells was not related to age, sex, tumour site differentiation or stage ( $P > 0.05$ ) (Table II). Furthermore, the expression levels of IFN- $\gamma$  and 4-1BBL were greater in the patients with highly differentiated cancer ( $P < 0.05$ ) and in patients with stage I-II cancer ( $P < 0.05$ ); however, the expression levels of IL-17 were lower in patients with highly differentiated cancer ( $P < 0.05$ ) and in patients with stage I-II cancer ( $P < 0.05$ ) (Table III).

*Association between CD3, CD4, CD8, CD56, IFN- $\gamma$  and 4-1BBL expression and the prognosis of patients with pancreatic cancer.* The median survival times of patients with strongly positive CD3, CD8, CD56, IFN- $\gamma$  and 4-1BBL expression were 18.7, 22.5, 20.3, 20.1 and 18.3 months, respectively, longer than those with weakly positive and negative expression ( $P < 0.05$ ; Fig. 4). The median survival time of those with strong positive CD4 expression was 17.3 months, which was longer than those with weak positive and negative CD4 expression, but the difference was not statistically significant ( $P > 0.05$ ; Fig. 4). The median survival time of those with strong positive IL-17 expression was 9.8 months, which was shorter than that of those with weak positive and negative expression ( $P < 0.05$ ; Fig. 4).

## Discussion

The immune system has contradictory and complex functions in the development of pancreatic cancer, and the role of immune

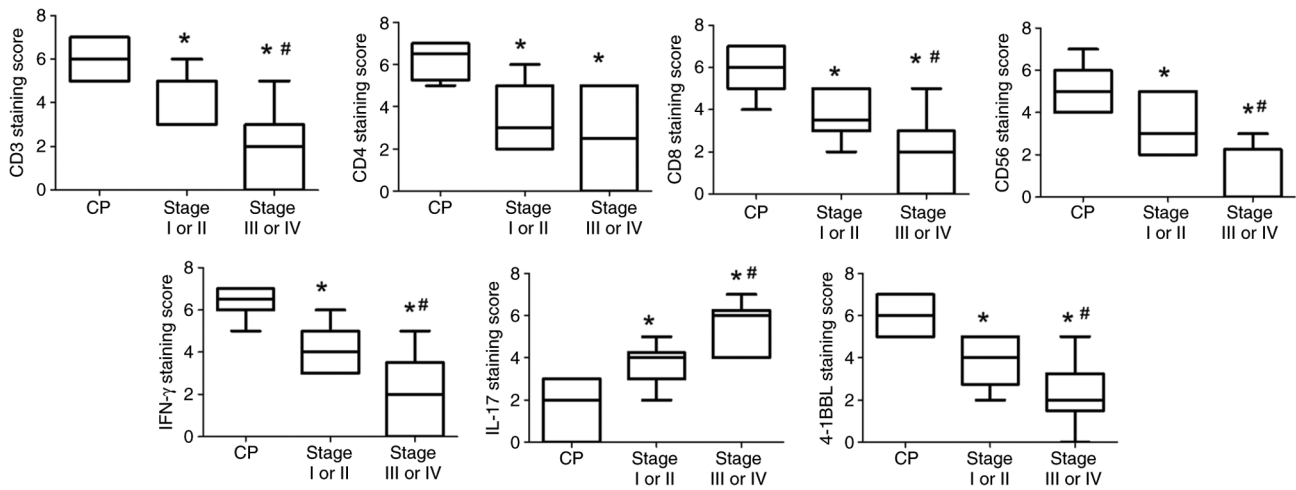


Figure 2. Immunohistochemical staining scores of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL in each group. \*P<0.05 vs. CP; #P<0.05 vs. stage I-II. CP, chronic pancreatitis.

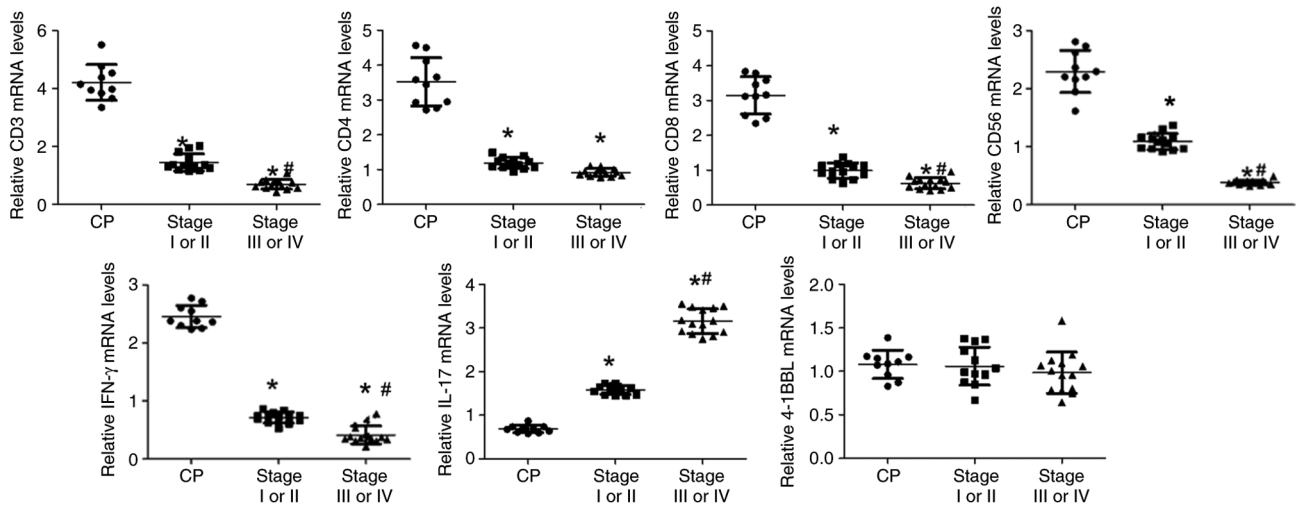


Figure 3. mRNA expression levels of CD3, CD4, CD8, CD56, IFN- $\gamma$ , IL-17 and 4-1BBL in each group. \*P<0.05 vs. CP; #P<0.05 vs. stage I-II. CP, chronic pancreatitis.

cells in the pancreatic cancer microenvironment varies. The occurrence and development of chronic pancreatitis are related to immunity. An immunohistochemical study revealed that CD4- and CD8-positive T lymphocytes, macrophages and mast cells were the main infiltrates in chronic pancreatitis; however, CD56-positive NK lymphocytes and B lymphocytes were less abundant (5). The present study analysed the changes in CD3-, CD4-, CD8- and CD56-positive cells in chronic pancreatitis and pancreatic cancer tissues at different stages via immunohistochemistry and RT-qPCR, and revealed that when inflammation developed in the pancreas, the number of CD3-, CD4- and CD8-positive cells increased through autoimmune regulation, thus killing target cells and carrying out immune functions. However, the decrease in the number of these cells in pancreatic cancer tissues indicated that the cellular immune function of patients with malignant tumours may be inhibited, and that the ability of these patients to identify and kill mutant cells decreases, resulting in an imbalance in the immune state of the body, and the growth of tumour cells and

even metastasis. The present study also revealed that CD3-, CD4-, CD8-, CD56-positive cells and IFN- $\gamma$  expression were present in highly differentiated pancreatic cancer. In addition to CD4-positive cells, CD3-, CD8-positive cells, NK cells, and IFN- $\gamma$  were also associated with clinical stage; the higher the stage, the lower the expression. Moreover, it was revealed that high expression was associated with a good prognosis during the follow-up process, similar to the findings of previous studies (26,27). It is possible that T lymphocytes prevent the infiltration of tumour cells into deep tissue, preventing their invasion into the lymphatic tract and metastasis, thus delaying the development of the tumour and prolonging the life of the patient.

Some studies have shown that IFN- $\gamma$  can directly inhibit fibroblast activation and proliferation, and inhibit or block the occurrence and development of pancreatic fibrosis (28,29). The present study revealed that IFN- $\gamma$  expression was increased in chronic pancreatitis compared with in pancreatic cancer, suggesting that IFN- $\gamma$  may have an antifibrotic effect on chronic

Table II. Relationship between clinicopathological parameters and CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD56<sup>+</sup> T-cell infiltration in patients with pancreatic cancer.

Characteristic	n	CD3 <sup>+</sup> T cells			CD4 <sup>+</sup> T cells			CD8 <sup>+</sup> T cells			CD56 <sup>+</sup> T cells		
		Strong	Negative/ weak	P-value	Strong	Negative/ weak	P-value	Strong	Negative/ weak	P-value	Strong	Negative/ weak	P-value
Age, years													
≤60	17	10	7	0.481 <sup>a</sup>	8	9	0.822 <sup>a</sup>	4	13	0.730 <sup>b</sup>	4	13	0.505 <sup>b</sup>
>60	23	16	7		10	13		7	16		8	15	
Sex													
Male	26	18	8	0.445 <sup>a</sup>	11	15	0.641 <sup>a</sup>	8	18	0.715 <sup>b</sup>	9	17	0.484 <sup>b</sup>
Female	14	8	6		7	7		3	11		3	11	
Tumour site													
Pancreatic head	24	17	7	0.343 <sup>a</sup>	11	13	0.897 <sup>a</sup>	9	15	0.148 <sup>b</sup>	6	18	0.398 <sup>a</sup>
Nonpancreatic head	16	9	7		7	9		2	14		6	10	
Differentiation													
High	15	13	2	0.040 <sup>b</sup>	9	6	0.140 <sup>a</sup>	10	5	<0.001 <sup>b</sup>	9	6	0.003 <sup>b</sup>
Moderate/Low	25	13	12		9	16		1	24		3	22	
Stage													
I and II	22	18	4	0.014 <sup>a</sup>	10	12	0.949 <sup>a</sup>	10	12	0.011 <sup>b</sup>	10	12	0.035 <sup>b</sup>
III and IV	18	8	10		8	10		1	17		2	16	

<sup>a</sup> $\chi^2$  test or <sup>b</sup>Fisher's exact test was used to evaluate the significance of the differences among the covariates.

**Table III. Relationship between clinicopathological parameters and IL-17, IFN- $\gamma$  and 4-1BBL expression in patients with pancreatic cancer.**

Characteristic	n	IFN- $\gamma$			IL-17			4-1BBL		
		Strong	Negative/ weak	P-value	Strong	Negative/ weak	P-value	Strong	Negative/ weak	P-value
Age, years										
≤60	17	9	8	0.151 <sup>a</sup>	6	11	0.973 <sup>a</sup>	10	7	0.131 <sup>a</sup>
>60	23	7	16		8	15		8	15	
Sex				0.104 <sup>a</sup>			0.730 <sup>b</sup>			0.842 <sup>a</sup>
Male	26	8	18		10	16		12	14	
Female	14	8	6		4	10		6	8	
Tumour site				0.792 <sup>a</sup>			0.787 <sup>a</sup>			0.243 <sup>a</sup>
Pancreatic head	24	10	14		8	16		9	15	
Nonpancreatic head	16	6	10		6	10		9	7	
Differentiation				0.001 <sup>a</sup>			0.005 <sup>b</sup>			0.033 <sup>a</sup>
High	15	11	4		1	14		10	5	
Moderate/Low	25	5	20		13	12		8	17	
Stage				0.038 <sup>a</sup>			0.014 <sup>a</sup>			0.009 <sup>a</sup>
I and II	22	12	10		4	18		14	8	
III and IV	18	4	14		10	8		4	14	

<sup>a</sup> $\chi^2$  test or <sup>b</sup>Fisher's exact test was used to evaluate the significance of the differences among the covariates.

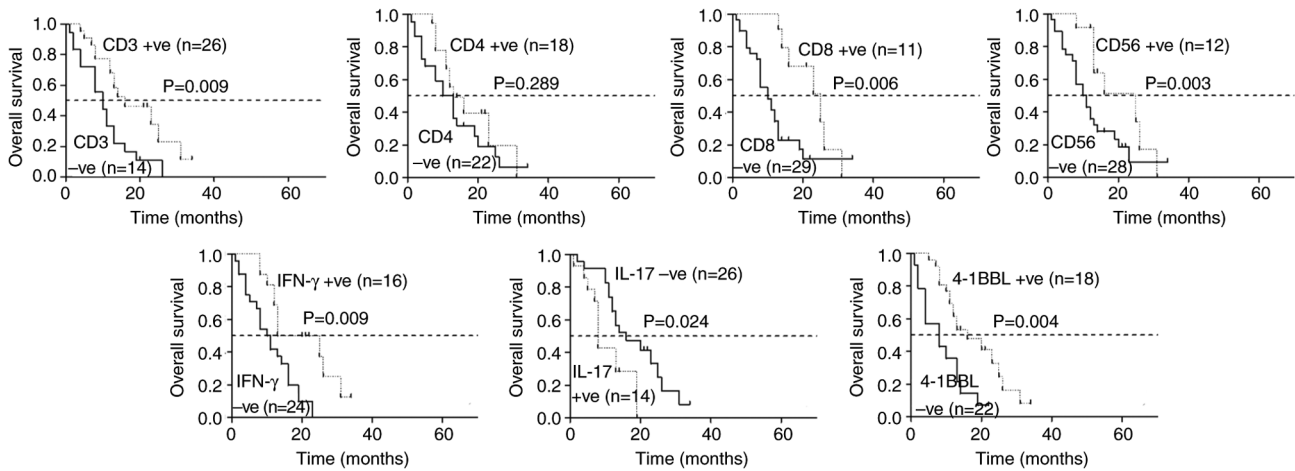


Figure 4. Association between survival time and CD3<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD56<sup>+</sup> T cells, IFN- $\gamma$ , IL-17 and 4-1BBL in patients with pancreatic cancer. Kaplan-Meier curves for overall survival in patients with pancreatic cancer. The P-values were determined using log-rank test. -ve, negative/weak expression; +ve, strong expression.

pancreatitis. However, in pancreatic cancer, IFN- $\gamma$  levels may decrease due to the depletion of body substances in the anti-tumour process, suggesting that IFN- $\gamma$  is immunosuppressive in patients with tumours (30).

Previous studies have shown that IL-17 is expressed in various tumour tissues (12,13); however, its role in tumours is controversial. On the one hand, IL-17 can selectively enhance the production of proangiogenic chemokines, such as CXCL1, CXCL5, CXCL6 and CXCL8, in tumour cells and endothelial cells (31). By contrast, IL-17 has been confirmed to inhibit certain hematopoietic tumours, such as mast cell tumours and plasma cytomas (32). Most studies have confirmed that IL-17 mainly affects tumour growth by stimulating cancer cells to secrete VEGF and indirectly promoting blood vessel formation (11,33). The results of the present study showed that the expression levels of IL-17 were greater in pancreatic cancer tissues than those in chronic pancreatitis tissues. The present study also revealed that IL-17 was related to tumour differentiation and Tumour-Node-Metastasis (TNM) stage (22); lower differentiation and higher TNM stage indicated higher expression levels. In the initial stage of pancreatic cancer, Th17 cells that produce IL-17 may serve a proinflammatory role and inhibit tumour growth. With the development of pancreatic cancer, the immune system is disrupted and inflammation cannot be alleviated; at this time, Th17 cells may participate in the protumour effects or block the antitumour effects (34), eventually leading to the occurrence of pancreatic cancer. During clinical follow-up, the strong positive expression of IL-17 was associated with a poor prognosis in patients with pancreatic cancer and the median survival time of patients with strong positive IL-17 expression was 9.8 months, which was significantly shorter than that of patients with weak positive or negative expression. These findings suggested that IL-17 may have an important role in predicting the prognosis of patients with pancreatic cancer.

4-1BB/4-1BBL-mediated costimulatory signals are involved in immunomodulatory processes in autoimmune diseases, tumours, viral infections and other diseases (16-20). Chronic pancreatitis is an immunological process characterized by increased expression of 4-1BBL. However, studies

on tumours have shown that there is an antitumour immune response during tumour growth and tumour cell surface expression of 4-1 BBL may participate in antitumour immunity (16,35). Our previous animal experiments revealed that 4-1BBL can promote bone marrow-derived dendritic cell (antigen-presenting cell) maturation leading to the secretion of IL-6 and IL-12 cytokines, which may enhance immunity (36,37). Although antitumour immunity and tumour growth coexist, the low expression levels of costimulatory molecules such as 4-1BBL may not be enough to fully activate the immune system, preventing tumour removal. In the present study, 4-1BBL expression was significantly lower in patients with pancreatic cancer compared with in patients with chronic pancreatitis, confirming the low degree of local immunity in patients with pancreatic cancer. The immunohistochemical results revealed differences between stages I-II and III-IV, indicating that a further decline in immune function occurred with the progression of the tumour. RT-qPCR revealed little difference among the three groups, possibly due to the detection of 4-1BBL expression in the tumour cell stroma by immunohistochemistry, which mainly involved various activated immune cells such as T cells, B cells and antigen-presenting cells (dendritic cells, macrophages). However, 4-1BBL is also expressed in tumour cells, and qPCR can detect mRNA expression levels in all tissues; thus, the results of qPCR may be inconsistent with those of immunohistochemistry (38).

The present study used qPCR of paraffin-embedded pancreatic tissue to detect TILs, cytokines and costimulatory molecule gene expression. The results revealed that the mRNA extraction rate of chronic pancreatitis (62.5%) was slightly lower than that of pancreatic cancer (76.5% and 82.4%), which may be due to two aspects: i) Chronic pancreatitis mainly involves fibrosis of the pancreatic parenchyma, leading to a reduction in the glands and the interstitium. ii) Some chronic pancreatitis tissues have high fat content, resulting in reduced expression of the corresponding target genes. Therefore, attention should be paid to addressing the issue concerning the accuracy, uniformity, quantity and appropriate method of obtaining pancreatic tissues for RNA extraction.

Chronic pancreatitis remains one of the highest risk factors for pancreatic cancer. Initially, the immune response is strong in chronic pancreatitis and the immune system can actively recognize tumour-specific antigens, leading to the activation of the innate and adaptive immune responses that eliminate transformed cells. However, with increasing age, environmental factors or certain genetic syndromes, such as mutations in the K-RAS gene, evade immune surveillance, leading to the occurrence of pancreatic cancer (39). Multiple mechanisms are immediately involved in pancreatic cancer progression. Extracellular vesicles derived from the pancreas can inhibit the immune response and are associated with the immune escape via the suppression of NK cells, antigen-presenting cells and cytotoxic T cells, the induction and activation of immunosuppressive cells, the secretion of cytokines, such as TNF- $\beta$ , or the induction of fibroblasts, which secrete large amounts of fibrin and proteoglycans to form the extracellular matrix and a protective physical barrier for tumour cells (40). As cancer progresses, the immune response is further suppressed in stages III-IV, as shown in the present study.

The lack of a normal control without pancreatic disease is a limitation of the present study. The study focused on the changes in these indicators during the process from benign lesions to cancerous transformation and metastasis, examining the trends of these indicators in chronic pancreatitis, stage I-II pancreatic cancer and stage III-IV pancreatic cancer. The present study reviewed the relevant literature and revealed that a number of studies, including animal experiments, clinical trials using tissues adjacent to pancreatic cancer as negative controls or studies comparing pancreatic benign diseases, such as pancreatic cysts, with pancreatic cancer, arrived at conclusions consistent with those of the present study (41-43). This provides additional support for the validity of the current research findings. In addition, as the present study assessed only 40 pancreatic cancer specimens, a multivariate analysis was not performed, since a requirement of multivariate analyses is that the sample size should be 10-15 times the number of independent variables. Therefore, only a univariate survival analysis was applied in the present study, which is also a limitation.

In conclusion, the changes in CD3-, CD8- and CD56-positive cells, the cytokines IL-17 and IFN- $\gamma$ , and the costimulatory molecule 4-1BBL in patients with pancreatic cancer were closely related to the degree of differentiation, TNM staging and prognosis. Notably, only the high expression of IL-17 was revealed to be an adverse prognostic factor of pancreatic cancer, all of the others indicated a good prognosis. These findings suggested that the suppression of antitumour immunity, the decrease in the infiltration density of TILs and the downregulation of costimulatory molecules may lead to the transformation from chronic pancreatitis to pancreatic cancer; however, the underlying mechanism requires further study.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

YYL, KZ, JXY and TTX conceived and designed the study, and contributed to data collection, data analysis, article editing and interpretation of the results. YYL drafted the initial manuscript. XDC and JKZ participated in the design of the research methodology and supervised the study. YXC and YJG contributed to data collection and carried out a part of the data analysis. YYL, JXY, TTX and KZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

### Ethics approval and consent to participate

The present study is a retrospective analysis and the data are anonymous; it was approved by the research ethics committee of the First Affiliated Hospital of Soochow University (approval no. 2023-410).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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