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T cell receptor β-chain repertoire analysis of tumor-infiltrating lymphocytes in pancreatic cancer

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Pancreatic cancer is lethal due to lack of perceptible symptoms and effective treatment methods. Immunotherapy may provide promising therapeutic choices for malignant tumors like pancreatic cancer. Tumor-infiltrating lymphocytes (TIL) in tumor mesenchyme could recognize peptide antigens presented on the surface of tumor cells. The present study aimed to test the relationship between the T cell receptor (TCR) β repertoire of the tumor and peripheral blood, and also to investigate the intra-tumor spatial heterogeneity of the TCR β repertoire in pancreatic cancer. To the best of our knowledge, this is the first study to evaluate the clonal composition of TCR β repertoire in TIL across the spatial extent of pancreatic cancer. In this study, we studied 5 patients who were diagnosed with primary pancreatic cancer. Ultra-deep sequencing was used to assess the rearrangement of the TCR β-chain (TCR β) gene. HE staining and immunohistochemistry of CD3, CD4, CD8 and HLA class I were used to show histopathology and immune conditions macroscopically. TIL repertoire showed that different regions of the same tumor showed a greater number of repertoire overlaps between each other than between peripheral blood, which suggested that T cell clones in pancreatic cancer might be quite different from those in peripheral blood. In contrast, intra-tumoral TCR β repertoires were spatially homogeneous between different regions of a single tumor tissue. Based on these results, we speculated that the cellular adaptive immune response in pancreatic cancer was spatially homogeneous; this may pave the way for immunotherapy for the treatment of pancreatic cancer patients.

KEYWORDS

immunohistochemistry, intra-tumor spatial heterogeneity, pancreatic cancer, T cell receptor, ultra-deep sequencing

Can Cui, Xiuyun Tian, Jianhui Wu contributed equally to this work.

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1 | INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-associated deaths worldwide,[1] and its poor prognosis can be ascribed to its malignant biological behavior and few detectable symptoms in the early stages. A study on familial pancreatic cancer demonstrated that pancreatic cancer might show heterogeneity in numerous gene deletions and amplifications.[2] Pancreatic cancer has been proved to be genetically heterogeneous based on the therapeutic effects seen in primary tumors and during metastases.[3-5] Therefore, personalized treatment for pancreatic cancer is essential owing to its heterogeneity.

Immunotherapeutic methods such as adoptive cell therapy (ACT) and the use of immune checkpoint inhibitors (such as inhibitors of CTLA4, PD-1 and PD-L1) have been shown to produce durable responses in the treatment of some solid tumors.[6-9] Increased numbers of tumor-infiltrating lymphocytes and improved survival in several types of tumors, including pancreatic cancer, could be correlated.[10] In Diana et al. (2016), pancreatic cancer patients with higher numbers of $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating lymphocytes (TIL) showed significantly superior overall survival (OS).[11] Several other reports also demonstrated that high numbers of $CD8^+$ and low numbers of $Foxp3^+$ T cells correlated with better clinical outcomes in patients with resectable primary pancreatic cancer.[12-14] CD4/8(+/+) status is an independent favorable prognostic factor for pancreatic cancer patients.[15] This suggests that TIL directed against tumor antigens may play a significant role in targeting tumor cells. Moreover, whether the biopsy of a single tumor portion might represent the immune condition of the entire tumor tissue remains to be elucidated.

The clonal composition of TIL can be assessed by analyzing the TIL repertoires generated by somatic recombination of the T cell receptor (TCR) α- and β-chains. Rearrangement of the V (variable), D (diversity), and J (joining) segments of the TCR β chain generates the highly variable complementary determining region 3 (CDR3), which is critical for determination of the specificity of each T cell clone.[16] Therefore, ultra-deep sequencing of the TCR β CDR3 region might help in approximately assessing the clonal composition of TIL.

In addition, T cell infiltrates might be uniform or heterogeneous across different regions of the same tumor. The heterogeneity is probably driven by intra-tumoral competition and selection by the host's immune response to tumor therapeutics.[17,18] It is necessary to assess the clonal composition of TIL across the spatial extent of the entire tumor to examine whether different parts of the tumor have different types of clones. Meanwhile, several studies have investigated the heterogeneity of TCR repertoires in different regions of the same tumor, and the conclusions seem controversial in different types of tumors.[19-21] Thus, the clonal composition of TIL in different tumors may be distinct. In addition, Bai et al. (2015) reveal that both the tumor tissue and blood from pancreatic cancer patients show little similarity with that from healthy individuals, which implies that the immune condition is altered in pancreatic cancer.[22] Therefore, it is essential to assess the clonal composition of TIL in pancreatic cancer owing to its complexity.

To determine the antigen receptor repertoire of TIL in pancreatic cancer, we collected multiple tumor samples from different regions of the same tumor and peripheral blood from 5 primary pancreatic cancer patients at the same time. Then, we performed deep sequencing for the TCR β CDR3 region using these samples, to test the relationship between the TCR β repertoire of the tumor and peripheral blood, and also to investigate the intra-tumoral spatial heterogeneity of the TCR β repertoire in pancreatic cancer. We performed HE staining and immunohistochemistry of CD3, CD4, CD8 and HLA class I to evaluate the histopathology and immune condition of the tumor tissue. To the best of our knowledge, this is the first study to evaluate the TCR β clonal composition of TIL across the spatial extent of pancreatic cancer.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Tumor tissues and peripheral blood were obtained from 5 patients who were diagnosed with pancreatic cancer and received surgical resection at Peking University Cancer Hospital, Beijing, China. None of these 5 patients had received chemotherapy or radiotherapy before surgery. The tumor samples were collected immediately after resection, and we collected 4 or 5 samples from different regions to represent the spatial heterogeneity of the entire tumor. Each region was cut into 2 parts, 1 for DNA extraction and the other for immediate formalin fixation. Images of the tumor samples are shown in Figure S1. For each patient, the sample named "T1" was taken from the center of the entire tumor tissue, and the other samples were taken from the surrounding areas. Peripheral blood was drawn at the time of surgery into EDTA-coated tubes prior to administration of anesthesia. DNA was extracted from fresh tumor tissues using the EasyPure Genomic DNA Kit (Transgen Biotech, Beijing, China), and DNA from blood cells was isolated using the Whole Blood Genomic DNA Kit (BioTeke Corporation, Beijing, China) following the manufacturer's instructions. Moreover, ultra-deep TCR sequencing allows the analysis of the T cell repertoire using DNA extracted from samples without prior separation of the T cell population. Table 1 shows the details and number of samples from each patient. DNA from the 4 tumor samples from patient 1 was amplified using PCR and sequenced in duplicates (T1A and T1B: duplicate samples of T1; T2A and T2B: duplicate samples of T2; T3A and T3B: duplicate samples of T3; T4A and T4B: duplicate samples of T4). Patient 1A included T1A, T2A, T3A, T4A and peripheral blood of patient 1. Patient 1B included T1B, T2B, T3B, T4B and peripheral blood of patient 1. This study was approved by the Ethical Committee from Peking University Cancer Hospital, and written informed consent was provided by each patient prior to initiation. A diagram of our experimental program is shown in Figure 1.

2.2 | High-throughput sequencing

The TCR β CDR3 region was amplified using Multiplex PCR to obtain the region of the antigen receptor gene, and the TCR β CDR3 region was sequenced using Illumina NextSeq 500 (MyGenostics, Beijing, China). Sequencing reads were identified according to the definition established by the International ImMunoGeneTics (IMGT) collaboration.[23] Sequencing reads that did not match the canonical structure of CDR3 sequences were not used for subsequent analysis. A standard algorithm was used to identify which V, D and J segments contributed to each TCR β CDR3 sequence.[23]

2.3 | CDR3 sequence analysis

To evaluate the similarity between the TCR β repertoires of samples within the same tumor and between those of the tumor and peripheral blood, we used the metric of TCR β repertoire overlap.[19] For example, for 2 samples, *a* and *b*, we identified *n* as TCR β CDR3 sequences present in both samples, and *C* as the sequencing read counts for each sequence in each sample. Meanwhile, the TCR β repertoire overlap was defined as the sum of the sequencing reads of shared TCR β sequences in both samples divided by the sum of sequencing reads observed in these 2 samples, which ranged from 0 to 1. The formula used for calculation is:

TABLE 1 Sample information of 5 pancreatic cancer patients

Patient	Gender	Age	Size of tumor	Site of tumor	Differentiation and/or pathology	Stage of tumor	Tumor samples	Blood
Patient1	Female	66	5 * 2 * 2 cm	Neck of pancreas	Moderate differentiation, adenocarcinoma	T3N0M0	T1,T2, T3, T4	B
Patient2	Male	64	4 * 4 * 3 cm	Body and tail of pancreas	Moderate differentiation, adenocarcinoma	T3N1M0	T1,T2, T3, T4, T5	B
Patient3	Female	79	4.5 * 3.5 * 3 cm	Neck and body of pancreas	Moderate differentiation, adenocarcinoma	T3N0M0	T1,T2, T3, T4, T5	B
Patient4	Male	73	6 cm (the maximum diameter)	Head of pancreas	Adenosquamous carcinoma	T3N1M0	T1,T2, T3, T4, T5	B
Patient5	Male	56	3 * 3 * 2 cm	Head of pancreas	Moderate differentiation, adenocarcinoma	T3N1M0	T1,T2, T3, T4	B

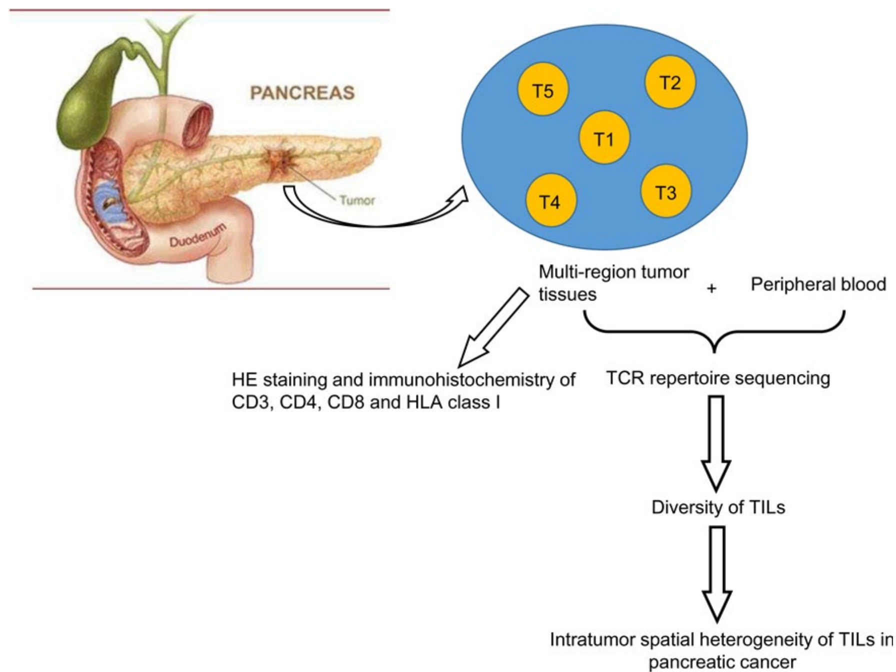

FIGURE 1 Diagram showing our experimental program. Four or five different regions of 1 single tumor were collected immediately after surgical resection. Peripheral blood was drawn at the time of surgery into EDTA-coated tubes prior to anesthesia. DNA from both tumor tissue and peripheral blood was extracted for T cell receptor (TCR) repertoire sequencing. Ultra-deep TCR sequencing allows the analysis of the T cell repertoire directly from DNA extracted from samples without prior separation of the T cell population. We divided each tumor tissue into 2 parts, 1 for DNA extraction and the other for immediate formalin fixation. These samples were used for HE staining and immunohistochemistry of CD3, CD4, CD8 and HLA class I

$$\text{TCR overlap} = \frac{\sum_{i=1}^{n} C_i(a) + C_i(b)}{\sum C(a) + \sum C(b)}.$$

To convert the overlap metric into a distance metric, we defined it as follows:

$$\frac{1}{\text{TCR overlap}} - 1$$

The distance metric ranged from 0 to infinity. For clustering analysis, the TCR β sequence distance was used to construct a neighbor-joining tree using the MEGA7 software.[24,25]

To quantify the diversity of T cell clones in the samples, we used the concept of clonality, which could provide diversity measurement as a function of the distribution of clone frequencies in each sample. Clonality was calculated as 1 − (Shannon's entropy)/log2 (number of productive unique sequences). The clonality score of a maximally diverse population and a completely monoclonal population would be 0 and 1, respectively.[26,27]

During analysis of the sequencing results, the sequences that contained stop codons or frameshifts in the CDR3 region were unlikely to be functional; therefore, they were not used for subsequent analysis. In addition, the human T cell repertoire can be highly complex. Thus, the total number of unique T cell clones could be much higher than the sequencing depth used in the present study,[28] and this could result in the failure to detect low-frequency T cell clones, which might lead to an overestimation of the heterogeneity of the T cell repertoire between samples.[29] Therefore, to avoid the impact of low-frequency clone sampling bias, we only analyzed the top 100 T cell clones which were detected at the highest frequency in all samples. Furthermore, to verify the results for the top 100 T cell clones, we also evaluated the top 50 T cell clones detected at the highest frequency in all samples, and the results of the 2 types of analysis were similar (shown in Figures S2-S5).

2.4 | Immunohistochemistry

The 4-μm thick tissue sections were baked at 72°C for 1 hour, and then dewaxed in xylene and rehydrated in graded alcohol concentrations. Hydrogen peroxide (3%) was used to block the activity of endogenous peroxidase for 15 minutes. Antigen retrieval was performed in a pressure cooker after 3 PBS washes. Upon cooling to room temperature, sections were blocked using corresponding blocking solution for 1 hour at 37°C. Then, the sections were incubated with the primary antibody at suitable dilutions overnight at 4°C. The following day, the ready-to-use EnVision regent (EnVision detection system peroxidase/DAB, rabbit/mouse; Dako, Glostrup, Denmark) was used to bind the primary antibody. 3.3′-Diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) was used to visualize the reaction. Then, the sections were counterstained with hematoxylin and dehydration was performed in the sequence of graded alcohol concentrations and xylene. The detailed experimental methods and information regarding the primary antibody are shown in Table S1.

2.5 | Statistical analysis

Statistical analyses were performed using the SPSS software, version 13.0 (SPSS, Chicago, IL, USA). Unpaired *t* test and nonparametric tests were used depending on the category of data. A 2-sided *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | High-throughput sequencing of T cells

We used Multiplex PCR to amplify the TCR β CDR3 region and then used high-throughput sequencing to examine the sequence of the TCR β CDR3 region. As shown in Table 2, we detected approximately 2.09 million sequence reads on average, distributed among an average of 15 646 unique TCR β CDR3 rearrangements in tumor tissue samples from 5 patients. The average unique TCR β reads in tumor tissue samples were less than those in blood samples (15 646 ± 5984 vs 23 838 ± 7944, unpaired *t* test, and *P* = .012). The average fraction of the top 100 TCR β sequence was 50.2% in tumor samples and 33.7% in peripheral blood samples, respectively (unpaired *t* test, *P* < .001). The results of clonality which reflected the diversity of T cell clones showed that the TCR β repertoires of tumor tissues had fewer clonotypes than in their matched peripheral blood (average clonality was .55 in tumor tissues and average clonality was .49 in peripheral blood; *P* = .035 using the nonparametric test). These results showed that TCR β repertoires in pancreatic tumor tissues were more oligoclonal.

3.2 | Spatial homogeneity of T cell clones within a single tumor

We used the TCR β repertoire overlap and the distance metric to evaluate the heterogeneity or homogeneity among different tumor regions, and between the tumor and peripheral blood samples.

However, the noise inherently found in sampling randomly from a diverse population might cause random sampling error even if we only analyze the top 100 T cell clones. Thus, to evaluate the interference of sequencing error and sampling noise caused by sequencing, we performed duplicate PCR reactions in 4 tumor samples of patient 1. Using replicate PCR reactions, the only factor that influenced the TCR β overlap metric was technical error and sampling noise.[19]

As shown in Figure 2, the TCR β repertoire overlap illustrated that different regions of the same tumor showed much more repertoire overlaps with each other than with peripheral blood. Moreover, dendrograms clearly show that peripheral blood was quite distant from tumor samples (shown in Figure 3). These results indicated that different tumor regions shared more TCR β sequences with each other than with corresponding peripheral blood.

We also compared the average TCR β overlap of duplicate samples (Duplicate T), tumor tissues (T and T) and tumor tissues vs peripheral blood (T and B), as shown in Figure 4; we found that the average TCR β overlaps of "T and T" were not significantly lower than

TCR β overlaps of “Duplicate T” (unpaired *t* test, *P* = .104), whereas there were significant differences in the average TCR β overlaps of “T and T” vs “T and B” (nonparametric test, $P < .001$), as well as in the average TCR β overlaps of “Duplicate T” vs “T and B” (unpaired *t* test, *P* = .001). These results demonstrated that the T cell response within pancreatic cancer was homogeneous across the spatial extent in 1 single tumor.

To further evaluate the degree of spatial heterogeneity of the T cell response in pancreatic cancer, we classified sequences into ubiquitous and heterogeneous sequences. Heat maps were constructed to show the frequencies of each ubiquitous and heterogeneous clone in each sample (shown in Figure 5). Heat maps for regional abundance of the top 100 T cell clones in all samples showed that T cell clones in different regions of a single tumor were spatially homogeneous, especially in clones with high frequencies.

3.3 | HE staining and immunohistochemistry of CD3, CD4, CD8 and HLA class I

Figures 6 and S6-S9 showed HE staining and representative immunohistochemical staining images of CD3, CD4, CD8 and HLA

TABLE 2 T cell receptor (TCR) β CDR3 sequencing metrics in 5 patients

Patient	Region[a]	Productive TCR β reads	Unique TCR β reads	Highest frequency clone (%)	Top 100 clones (%)	Clonality
Patient 1	T1A	1 525 728	11 186	8.68	48.47	.54
	T1B	1 799 768	12 818	7.64	46.38	.54
	T2A	1 779 081	13 835	10.51	48.29	.54
	T2B	1 951 744	14 045	9.98	48.33	.54
	T3A	2 357 465	18 481	12.96	48.42	.54
	T3B	2 525 659	21 760	11.60	45.58	.53
	T4A	1 804 027	13 281	8.97	44.68	.53
	T4B	2 382 890	16 738	7.58	41.48	.52
	B	2 551 761	28 421	3.54	28.84	.46
Patient 2	T1	1 467 014	8886	4.22	56.00	.56
	T2	1 818 896	14 242	4.51	47.04	.53
	T3	654 888	4947	7.63	61.22	.57
	T4	1 243 241	6960	3.92	54.49	.56
	T5	935 497	5755	8.66	63.73	.59
	B	1 556 359	11 729	34.63	53.44	.61
Patient 3	T1	1 438 610	13 834	6.01	47.76	.52
	T2	2 145 798	24 214	6.41	38.33	.49
	T3	2 232 971	14 612	7.44	52.46	.56
	T4	1 986 334	16 015	2.77	44.26	.51
	T5	2 052 436	15 550	6.41	50.22	.54
	B	2 643 033	32 898	12.07	36.64	.49
Patient 4	T1	3 101 702	21 685	7.84	58.18	.58
	T2	3 127 847	32 022	4.17	40.37	.50
	T3	3 385 193	19 232	9.04	56.33	.59
	T4	3 400 284	20 930	10.97	60.96	.60
	T5	2 857 367	23 608	7.87	54.46	.56
	B	2 070 463	23 945	1.58	20.61	.44
Patient 5	T1	2 106 881	14 340	4.41	47.92	.54
	T2	1 823 809	17 671	5.12	45.51	.52
	T3	2 365 188	11 934	9.55	59.11	.59
	T4	2 241 304	13 855	3.93	44.81	.54
	B	1 555 349	22 197	4.61	29.10	.45

[a]T1A and T1B: duplicate samples of T1; T2A and T2B: duplicate samples of T2; T3A and T3B: duplicate samples of T3; T4A and T4B: duplicate samples of T4.

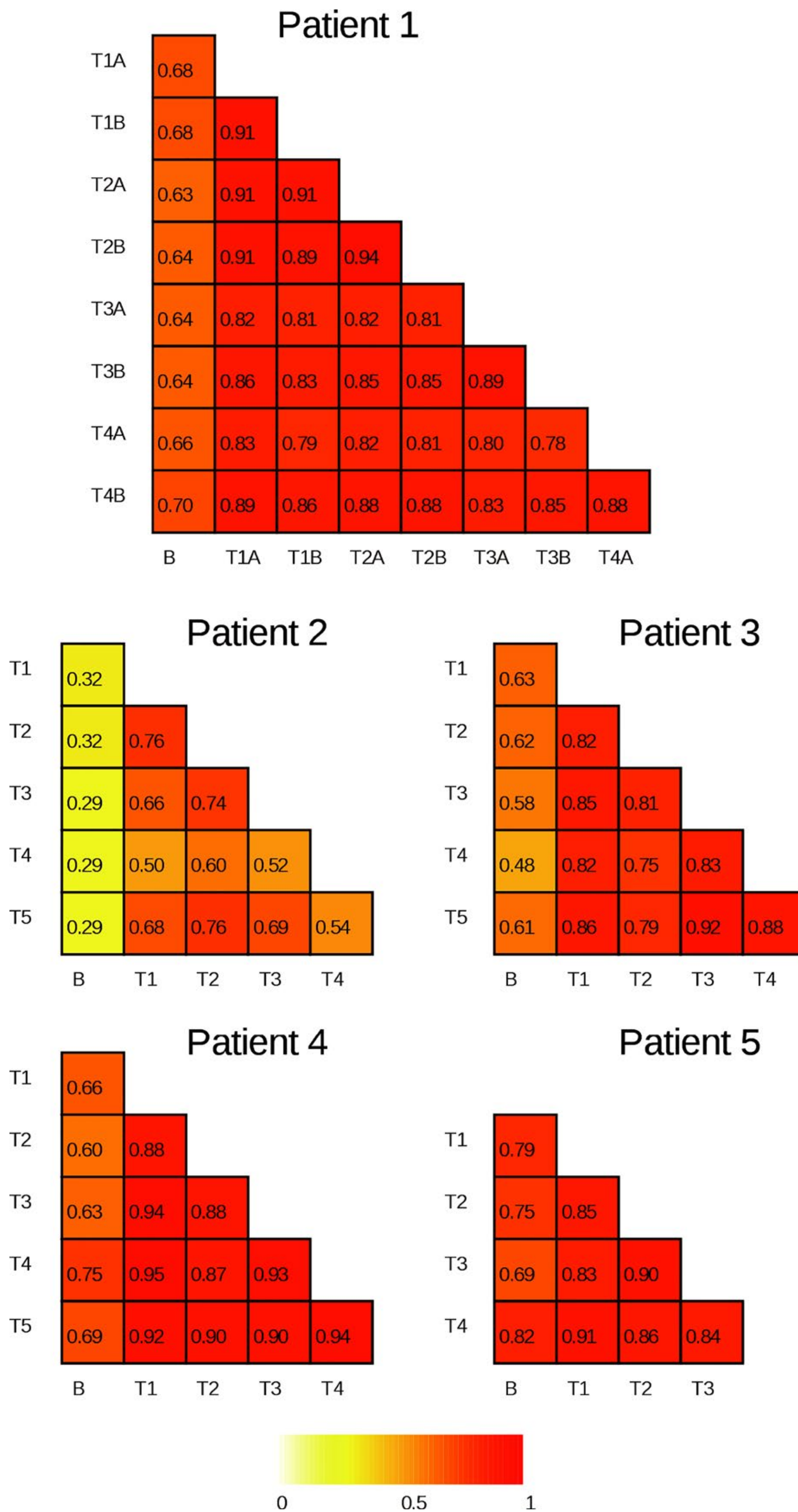

FIGURE 2 Comparison of the T cell receptor (TCR) β repertoire overlaps between different samples of each patient. To evaluate the similarity between TCR β repertoires of samples taken from the same tumor and between those of samples taken from tumors and peripheral blood, we used the metric of the TCR β repertoire overlap. For each patient, pairwise overlaps were calculated among all samples, including 4 duplicate samples from patient 1. Different regions in the same tumor shared a greater repertoire overlap with each other than with peripheral blood

class I of corresponding tumor tissues. CD3, CD4 and CD8 expression were localized mainly on the plasma membrane of T cells in the mesenchyme of the tumor tissue. Staining of CD3, CD4 and CD8 could roughly represent the quantity of T cells in the mesenchyme of tumor tissue. CD3, CD4 and CD8 expression were mostly seen scattered throughout the tumor while

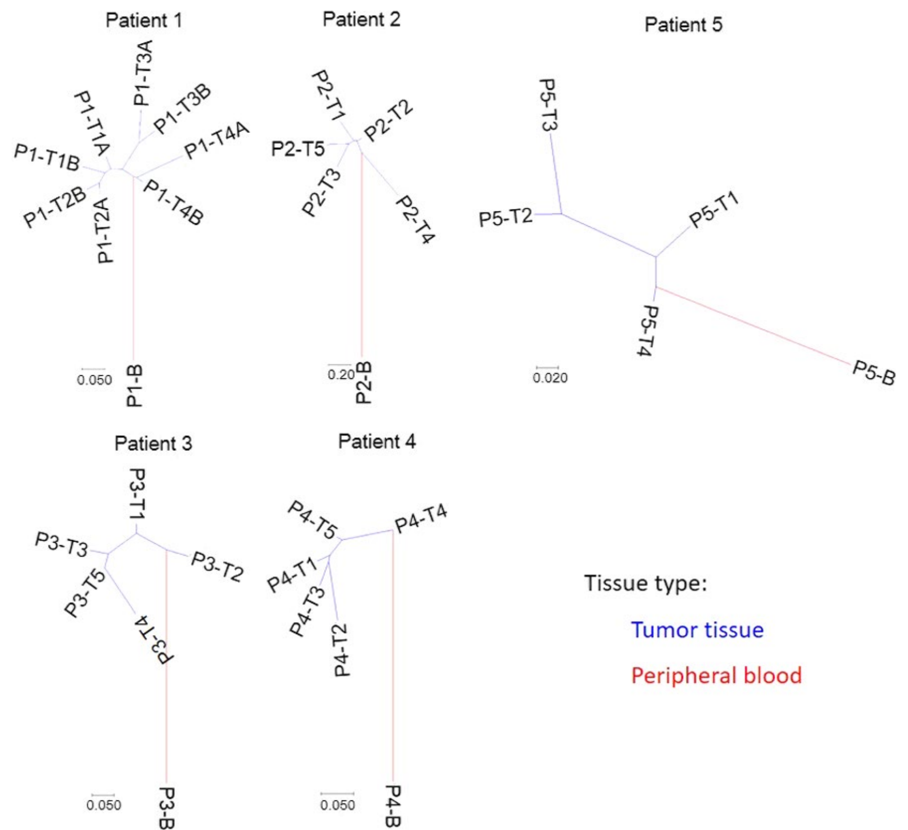

FIGURE 3 Clustering analysis of the T cell receptor (TCR) β repertoire overlaps of all patients. We converted the overlap metric into a distance metric and constructed neighbor-joining trees. Trees were color-coded by tissue types: tumor tissue (blue) and peripheral blood (red). The relatively larger distance between tumor tissue and blood samples indicated that different tumor regions shared more TCR β sequences with each other than with peripheral blood

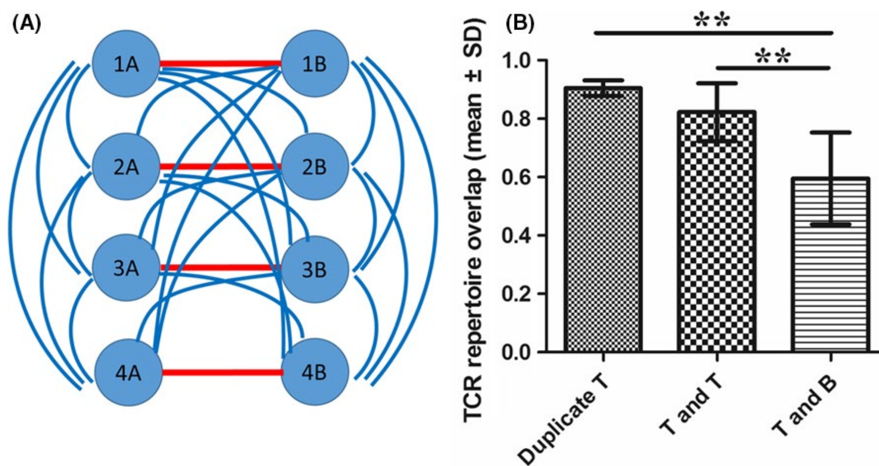

FIGURE 4 T cell receptor (TCR) β repertoire overlaps (mean ± SD) of all samples. A, The overlaps within-tissue sample comparison (red lines) should be influenced by technical error and TCR β repertoire sampling error, while overlaps between-tissue comparison should be affected by technical error, sampling error and spatial heterogeneity. B, We compared the average TCR β overlap of duplicate samples (Duplicate T), tumor tissues (T and T) and tumor tissues vs peripheral blood (T and B) according to the results shown in Figure 2. “Duplicate T” included 4 overlaps of T1A vs T1B, T2A vs T2B, T3A vs T3B, T4A vs T4B. “T and T” included all paired comparison overlaps among tumor samples from all patients except comparisons of T1A vs T1B, T2A vs T2B, T3A vs T3B, T4A vs T4B. “T and B” included all paired comparison overlaps of tumor and blood samples of the 5 patients. ** $P < .05$

occasionally in clusters. HLA class I staining was seen in the tumor cell membrane and cytoplasm. The antibody for HLA class I reacts with the heavy chains of human HLA class I A, B and C. Each tumor tissue showed moderate or strong expression of HLA class I.

4 | DISCUSSION

Immunotherapy is a novel method used in fundamental and clinical pancreatic cancer research. Some progress has been made, such as the development of adoptive T cell therapy.[30,31] T cells can

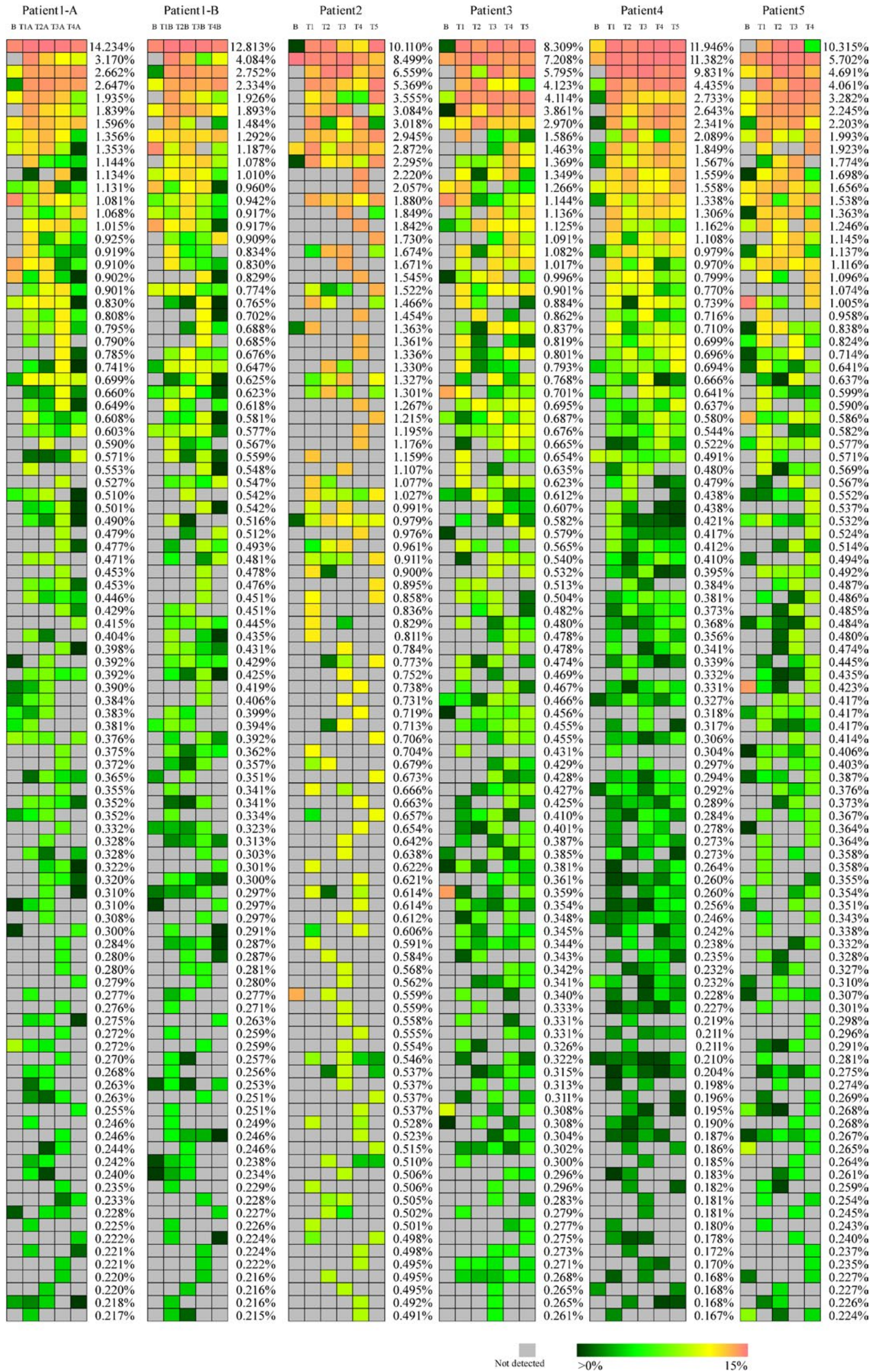

FIGURE 5 Heat maps showing regional abundance of top 100 T cell clones of all samples. Heat maps revealed the regional frequencies of the top 100 T cell clones. The colors of each grid that represent different tumor regions of 1 patient are similar to each other. Results show that the T cell clones in different regions of a single tumor are spatially homogeneous, especially in clones with high frequencies

infiltrate solid tumors, and the adaptive immune system is mediated by TIL in order to respond to solid tumors.[10] Different kinds of TIL which might direct the behavior of tumor cells may influence clinical outcomes.[10-15]

In this study, to investigate the heterogeneity or homogeneity of TIL across different regions of the same tumor in pancreatic cancer, we evaluated the characteristics of TIL in primary pancreatic cancer using ultra-deep sequencing for the rearranged TCR β gene. We found that different regions of the same tumor showed more repertoire overlaps with each other than with peripheral blood, which suggested that TCR β repertoires in TIL of tumor tissues were quite different from circulating T cells. Some studies have reported similar results,[22,32,33] and in the present study, we have further confirmed the difference between TIL of tumor tissues and circulating T cells in pancreatic cancer. This suggested that T cell clones within pancreatic cancer predominantly react to tumor antigens and that T cells in tumor tissue do not circulate throughout to peripheral blood.

We investigated the clone types of these TIL. We found that intra-tumoral TCR β repertoires of different regions in 1 single tumor were spatially homogeneous, which was similar with findings of studies in ovarian cancer.[19] Moreover, there were some ubiquitous T cell clones in different tumor regions, and T cell clones in different regions of a single tumor were spatially homogeneous, especially in the clones with high frequencies, which meant that these ubiquitous T cell clones were activated by common neoantigens. Some other studies show that tumors are heterogeneous not only in cancer cells but also in TCR β repertoires. In addition, the heterogeneity may affect several aspects of the disease, such as response to therapy.[17,18,21,34]

Therefore, as the intra-tumoral TCR β repertoires of the TIL population in pancreatic cancer were found to be homogeneous in a single tumor, a single biopsy of pancreatic cancer could provide sufficient information about the immune conditions of the entire tumor tissue. Furthermore, isolation of TIL specific for antigens of a random region of the tumor might facilitate effective corresponding adoptive cell therapy for patients. A study by Xueli Bai et al[22] shows the heterogeneity of TCR repertoires among different pancreatic cancer individuals; thus, even though TCR β repertoires of different regions in a single tumor are spatially homogeneous, personalized immunotherapy is necessary. In addition, using immune staining we also found that CD3, CD4 and CD8 expression were mostly scattered throughout the tumor tissue while occasionally in clusters in the mesenchyme of tumor tissue. This lymphocyte distribution feature was similar with some other diseases.[35-38]

There are also aspects to be improved in the present study. In our work, we only chose the TCR β chain to represent all characteristics of TCR and have not considered the TCR α chain. Some other studies have demonstrated that TIL in pancreatic cancer are complicated. TIL in pancreatic cancer have unrestricted TCR Vα gene usage[39] and contain considerable numbers of TCR $\gamma\delta^+$ T cells.[40] Different types of TIL quantification might act as predictive biomarkers for response to immunotherapy.[41,42] Therefore, even if TCR share common TCR β chains, there might be some other differences that affect the response to immunotherapy; extensive analysis might provide comprehensive results. In addition, results might be more reliable and precise if $CD4^+$ and $CD8^+$ T cells can be sorted using flow cytometry and then sequenced, respectively. We also need to expand the sample size to further validate our results.

In summary, to the best of our knowledge, this is the first study to evaluate the clonal composition of TIL across the spatial extent of pancreatic cancer using high-throughput TIL repertoires, and we

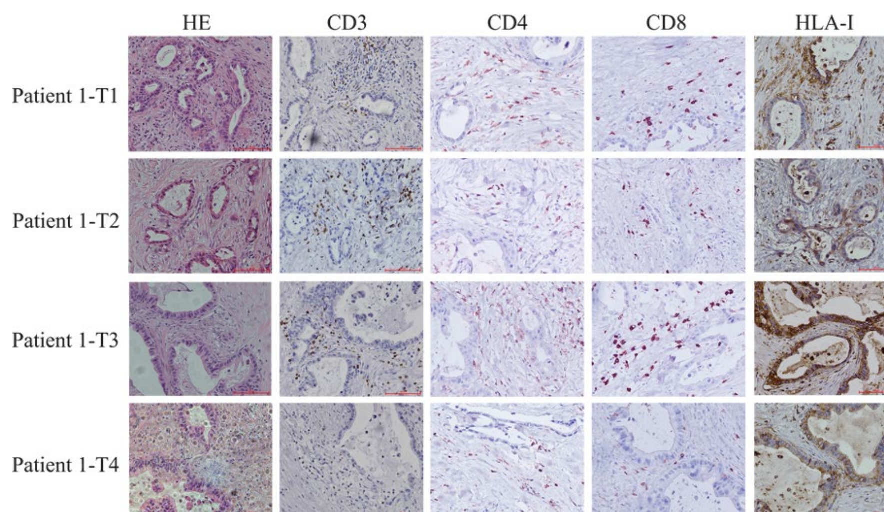

FIGURE 6 The HE staining and representative immunohistochemical staining images of CD3, CD4, CD8 and HLA class I in patient 1 (magnification: ×200). CD3, CD4 and CD8 expression were localized mainly on the plasma membrane of T cells in the mesenchyme of the tumor tissue. Staining of CD3, CD4 and CD8 could roughly represent the quantity of T cells in the mesenchyme of tumor tissue. CD3, CD4 and CD8 expression were mostly seen scattered throughout the tumor while occasionally in clusters. HLA class I staining was seen in the tumor cell membrane and cytoplasm. Each tumor tissue showed moderate or strong expression of HLA class I

have demonstrated that ultra-deep sequencing of the TCR gene allowed analysis of the TCR gene sequence using DNA directly extracted from pancreatic cancer tissues without prior separation of the T cell population. We found that the immune response in pancreatic cancer was distinct from the circulating immune repertoire, and that the intra-tumoral microenvironment was characterized by internally homogenous T cell repertoires. Therefore, a single biopsy of pancreatic cancer could provide sufficient information about the entire tumor. In future studies, we will also compare TCR repertoires in liver metastases or lymph node metastases with primary pancreatic cancer tissue, which could provide references for immunotherapy of advanced pancreatic cancer. A study referring to the TIL of pancreatic cancer has shown that pancreatic cancer is not an immunologically "cold" tumor;[43] thus, if a method is developed to isolate TIL specific for antigens of a random region of the tumor, the corresponding adoptive cell therapy may be efficient for patients, which might provide a novel method for immunotherapy to treat pancreatic malignancies.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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