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Original research

Mass cytometry-based peripheral blood analysis as a novel tool for early detection of solid tumours: a multicentre study

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ABSTRACT

Objective Early detection of a tumour remains an unmet medical need, and approaches with high sensitivity and specificity are urgently required. Mass cytometry time-of-flight (CyTOF) is a powerful technique to profile immune cells and could be applied to tumour detection. We attempted to establish diagnostic models for hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC).

Design We performed CyTOF analysis for 2348 participants from 15 centres, including 1131 participants with hepatic diseases, 584 participants with pancreatic diseases and 633 healthy volunteers. Diagnostic models were constructed through random forest algorithm and validated in subgroups.

Results We determined the disturbance of systemic immunity caused by HCC and PDAC, and calculated a peripheral blood immune score (PBIScore) based on the constructed model. The PBIScore exhibited good performance in detecting HCC and PDAC, with both sensitivity and specificity being around 80% in the validation cohorts. We further established an integrated PBIScore (iPBIScore) by combining PBIScore and alpha-fetoprotein or carbohydrate antigen 19-9. The iPBIScore for HCC had an area under the curve (AUC) of 0.99, 0.97 and 0.96 in training, internal validation and external validation cohorts, respectively. Similarly, the iPBIScore for PDAC showed an AUC of 0.99, 0.98 and 0.97 in the training, internal validation and external validation cohorts, respectively. In early-stage and tumour-marker-negative patients, our iPBIScore-based models also showed an AUC of 0.95–0.96 and 0.81–0.92, respectively.

Conclusion Our study proved that the alterations of peripheral immune cell subsets could assist tumour detection, and provide a ready-to-use detection model for HCC and PDAC.

INTRODUCTION

Malignant tumours, such as hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC), are characterised by insidious onset,

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Early detection of cancer helps patients receive timely treatment and improves their prognosis.
- ⇒ Tumour-burdened mouse models demonstrated the relationship between existence of tumour and systemic dysfunction of the immune macroenvironment.
- ⇒ Cytometry time-of flight (CyTOF) as a novel technology, could profile the composition and number of immune cells, reflecting immunity in higher-dimensional plane.

WHAT THIS STUDY ADDS

- ⇒ Our study determined the alterations of peripheral immune cell subsets caused by hepatocellular carcinoma (HCC) or pancreatic ductal adenocarcinoma (PDAC) in humans through CyTOF analysis for peripheral blood.
- ⇒ Constructed models based on selected cell markers and subsets that obtained from CyTOF and random forest algorithm exhibited excellent performance in detecting patients with malignancies.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ CyTOF serves as potential tool that assist in detection of HCC and PDAC, which also indicated that CyTOF may develop as novel tool method used in multicancer screening for its higher dimension of description for system immune.

leading to a delay in diagnosis and treatment. Routine serological tests currently used in the clinic are limited by their low sensitivity or low specificity. For instance, alpha-fetoprotein (AFP) has a sensitivity of less than 50%, and acute liver disease and pregnancy are often accompanied by elevated AFP.^{1–3} Similarly, approximately 20% of patients with PDAC are negative for carcinoma antigen 19-9 (CA19-9),⁴ while benign obstruction of the bile

duct and impaired renal function frequently cause an increased level of CA19-9.⁵⁻⁷ Thus, novel tools for tumour screening and early-stage detection are urgently needed.

The emerging technique of liquid biopsy, which is mainly related to detection of circulating tumour DNA (ctDNA), cell-free RNAs (eg, messenger RNAs, microRNAs), circulating tumour cells (CTCs) and exosomes,⁸ attempts to replace traditional serological screening methods or tissue biopsy. In HCC, for example, a serum metabolic panel, 5-hydroxymethylcytosines and ctDNA, exhibited good performance in detection of HCC in patients, with an area under the curve (AUC) ranging from 0.8 to 0.9.⁹⁻¹¹ These strategies have demonstrated their strengths and potential in the field of tumour detection. However, there are currently no well-accepted liquid biopsy strategies for tumour detection.

Over the past decade, the development of immunotherapy has changed the decision-making of cancer treatment, with success in many types of cancer.¹²⁻¹⁴ The rapid development of immunotherapy has deepened our understanding of the relationship between cancer and the immune system. A recent study using mouse models discovered tumour-induced systemic dysfunction of the immune macroenvironment, which could be restored by tumour resection.¹⁵ In addition, individual immunity is coordinated across tissues, and the local antitumour immune response depends on continuous communication with the peripheral blood.^{16,17} For example, a considerable proportion of regulatory T cells (Tregs) within tumours are derived from naturally occurring thymic Tregs.¹⁸ The frequency and status of immune cells change dynamically during tumour progression.¹⁵ Moreover, the number of regulatory B cells increased in the peripheral blood of patients with gastric cancer and lung cancer, and the expression of the inhibitory receptor of natural killer (NK) cells increased in the peripheral blood of patients with breast cancer.¹⁹⁻²¹ Increasing evidence supports the view that the occurrence and development of tumours are accompanied by systemic immune disturbance and alternations of peripheral immune cells.^{15,22-24} Therefore, immune profiling of the circulation might help to detect tumours; however, no such successful attempt has been made to date.

Mass cytometry by time-of-flight (CyTOF) is a newly developed technology that can profile the composition and number of immune cells.²⁵⁻²⁷ To test the value of peripheral blood immune cells in tumour detection, we launched a large-volume, multi-centre study and used CyTOF to obtain the comprehensive information of immune cell composition, phenotype and function in peripheral blood. The aim of this study was to establish diagnostic models for HCC and PDAC using CyTOF, and to try to understand the logic underlying the models for their better clinical application.

MATERIALS AND METHODS

Participant cohorts

This was a multicentre, prospective study that recruited a total of 2348 participants from 15 centres across China from October 2019 to July 2022. The training cohort and internal validation cohort were from the leading centre (The First Affiliated Hospital, Zhejiang University School of Medicine). Using an age-stratified random sampling method, we selected 900 and 500 of them to construct the training cohort of hepatic group and pancreatic group, respectively. The remaining participants from the leading centre were used for internal validation. The external validation cohort included participants from the other 14 centres in four provinces (online supplemental table S1). For the internal and external validation cohorts, all participants' information and

diagnoses were blinded for the experimenters and analysers. Diagnoses of the included participants were confirmed using ultrasound, CT or MRI. In particular, HCC was determined by pathology or clinical diagnosis, and PDAC was determined by pathology according to clinical guidelines. The diagnosis of patients who received surgical resection was further confirmed by histopathology. Hepatic benign diseases included, but were not limited to, haemangiomas (164 cases), cysts (66 cases) and focal nodular hyperplasia (71 cases). Pancreatic benign diseases included, but were not limited to, neuroendocrine tumours (28 cases), cystic neoplasms (120 cases), autoimmune pancreatitis (9 cases) and chronic pancreatitis (18 cases). Peripheral blood samples from the healthy volunteers were collected during routine physical examination. Their health statuses were determined by reviewing medical examination results and consulting medical history. The exclusion criteria were as follows: A history of cancer-related treatment; acute infections; blood transfusion within the past 6 months; use of drugs affecting the composition of peripheral blood, such as recombinant human erythropoietin and interleukins, within the past 2 weeks; recurrent tumours; decompensated dysfunction of organs; immune deficiency syndrome; haematological precancerous diseases; receiving immunosuppressive therapy; and confirmed coagulopathy.

Sample collection and processing

Peripheral blood sample (5 mL per person) of each participant was collected before any antitumour treatments. The collected samples were transported to the laboratory for processing within 12 hours at room temperature or within 48 hours at about 4°C. All samples had not been cryopreserved and all analysis was performed on fresh samples of blood. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by density gradient centrifugation using Ficoll. The cell precipitates were resuspended in 5 mL of pre-cooled fluorescence activated cell sorting (FACS) buffer (1×phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin), and then centrifuged at 400×g for 5 min at 4°C. The supernatant was discarded and the cell precipitates were resuspended in FACS buffer again. The number of cells was counted and the quality of samples for subsequent analysis should meet the following requirement: The number of cells should not be less than 3×10⁶ and the viability rate should be higher than 85%.

CytoF staining and data acquisition

The provider, clone number and mass tag of each antibody used in this study are showed in online supplemental table S2. Antibody labelling with mass tag was performed using the Maxpar antibody conjugation kit (Fluidigm). The concentration of mass-tagged antibody was assessed by NanoDrop. Antibody stabiliser buffer was used to adjust the concentration of labelled antibody to 200 mg/mL. Titration of conjugated antibodies should be performed to get optimal concentration for use. Obtained cells were washed with PBS, stained with 100 µL of 250 nM cisplatin (Fluidigm, South San Francisco, California, USA) for 5 min on ice to exclude dead cells and incubated in Fc receptor blocking solution before being stained with a surface antibodies cocktail for 30 min on ice. After washed with PBS, we use unique barcoding isotope combination to label individual cell samples for 30 min. Cells were washed twice with FACS buffer and fixed in 200 µL of intercalation solution (Maxpar Fix and Perm Buffer containing 250 nM 191/193Ir, Fluidigm) overnight. After fixation, the cells were washed with FACS buffer and then with Perm Buffer (eBioscience, San Diego, California, USA), and stained

with an intracellular antibodies cocktail for 30 min on ice. Cells were washed and resuspended in deionised water, added into 20% EQ beads (Fluidigm) and applied to a mass cytometer (Helios, Fluidigm). The CyTOF experiments were performed by PLTTECH (Hangzhou, China). Before each batch of samples were loaded, the instrument adjusted the signal strength of each channel according to the same beads signal (140Ce, 151Eu, 153Eu, 165Ho, 175Lu). All samples were standardised to avoid batch effect before analysis.

CytoTOF data analysis

Data for each sample were de-barcoded from the raw data using a doublet-filtering scheme with unique mass-tagged barcodes.²⁸ Each .fcs file generated from different batches were normalised using the bead normalisation method.²⁹ We used the FlowJo software (FlowJo, Ashland, Oregon, USA) to exclude debris, dead cells and doublets from the manual gating data, leaving only live single immune cells. We applied the X-shift clustering algorithm to all cells to partition the cells into distinct phenotypes based on marker expression levels,³⁰ and then annotated the cell type of each cluster according to its marker expression pattern on a heatmap of clusters and markers. The dimensionality reduction algorithm, t-distributed stochastic neighbour embedding, was used to visualise the high-dimensional data in two dimensions, to show the distribution of each cluster and marker expression and the difference among the groups or different sample types.³¹ Student's t-test statistical analysis was performed on the frequency of annotated cell populations.

Model construction

The data set was divided into three parts: training set, internal validation set and external validation set. Marker negative proportion and subgroup proportion were used as modelling features. Due to the excessive number of features, there may be some collinearity and non-significant features, so it is necessary to conduct feature screening to screen out important and useful features. The training set and internal validation set were then used to select features. The models were constructed using a random forest algorithm. In brief, features were selected according to their highest score, and 300 samples were selected randomly from negative and positive samples, respectively, to establish a random forest model. A 10-fold cross-validation method was adopted to obtain the average importance of each feature. If this process was repeated 1000 times, each feature would have 1000 feature importance values. We counted the number of features whose importance was greater than 0.01 for each feature. If the number was greater than 400, the feature was selected. Subsequently, the selected features were used as required features for modelling. Based on the screened features, the random forest model was constructed again after standardised processing of the training set, and the 10-fold cross-validation method was adopted to make each sample participate in modelling and testing, so as to eliminate the influence of sample differences on the model and find the optimal hyperparameter of the model. Then, the generalisation abilities of the constructed models were evaluated in internal validation set and external validation set, to further prove the robustness of the models. The peripheral blood immune score (PBIScore) of each participant was obtained from the models. For instance, a new sample was put into the well-trained model, and the probability of this sample belonging to malignancy was judged by each decision tree in the random forest model. The average probability obtained by all decision trees was recorded as the PBIScore of

this sample, ranging from 0 to 1. We discretised the data of AFP and CA19-9. In the hepatic group, if the AFP level was higher than 20 ng/mL (the upper limit of normal level), the value was assigned to 0; otherwise, the value was assigned to 1. We set the normal upper limit of CA19-9 at 37 U/mL and performed a similar assignment strategy. The discrete index was added to the corresponding model as a feature, and the random forest model was established again. Similarly, the score of the new sample was calculated as above method according to the new random forest model, which was denoted as integrated peripheral blood immune score (iPBIScore).

Statistical analysis

The data are presented as the mean±SD or SEM. Student's t-test, the Wilcoxon rank-sum test, or the Z test were used for statistical analysis, as appropriate. Data analysis was performed using the SPSS software (V.24; IBM, Armonk, New York, USA). A p value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of cohorts

A total of 2348 participants were included in this study, including 1131 patients with hepatic diseases, 584 patients with pancreatic diseases and 633 healthy volunteers as controls. The hepatic group was consisted of 790 patients with HCC and 341 patients with benign hepatic diseases. The pancreatic group included 376 patients with PDAC and 208 patients with benign pancreatic diseases. Healthy controls were shared by both groups. The demographic and clinicopathological characteristics of all participants are presented in online supplemental tables S3–S6.

Tumours are associated with altered peripheral immune cell subsets

The phenomenon of increased proliferation of immature neutrophils and monocytes in tumour-bearing models has been widely reviewed in previous studies,^{32,33} which indicated that changes of peripheral immune cells may reflect the existence of insidious tumours. Therefore, we speculated that similar disturbances of systemic immunity existed in patients with HCC or PDAC, which could serve as novel tumour screening markers. To reveal the profiles of peripheral immunity, we performed the CyTOF analysis to all collected PBMC samples. All CyTOF data were pre-processed and living single immune cells (CD45⁺) were retained after gating for further analysis. To exhibit the expression of different markers in various immune cells, but limited to the excessively large number of cells, we randomly selected cases from each group including 81 cases of HCC, 50 cases of benign hepatic diseases, 50 cases of PDAC, 50 cases of benign pancreatic diseases and 50 healthy volunteers. We then drew heatmap based on their CyTOF analysis results. Based on the canonical cell surface markers, we defined 32 major cell clusters, including 9 clusters of CD4⁺ T cells (CD3⁺CD4⁺), 8 clusters of CD8⁺ T cells (CD3⁺CD8⁺), 3 clusters of B cells (CD3⁻CD19⁺), 2 clusters of monocytes (CD3⁻CD19⁻CD14⁺) and 10 clusters of other subsets, such as dendritic cells (DCs, CD3⁻CD19⁻CD14⁻CD20⁻HLA-DR⁺), and NK cells (CD3⁻CD19⁻CD14⁻CD20⁻CD56⁺) (figure 1A,B).

We then analysed the differences in participants with or without malignant tumours in the hepatic group and pancreatic group, respectively, and found significant differences for some cell subsets in both scenarios (figure 1C). Compared with the participants without malignancies (named 'non-HCC', including healthy volunteers and cohort with hepatic benign

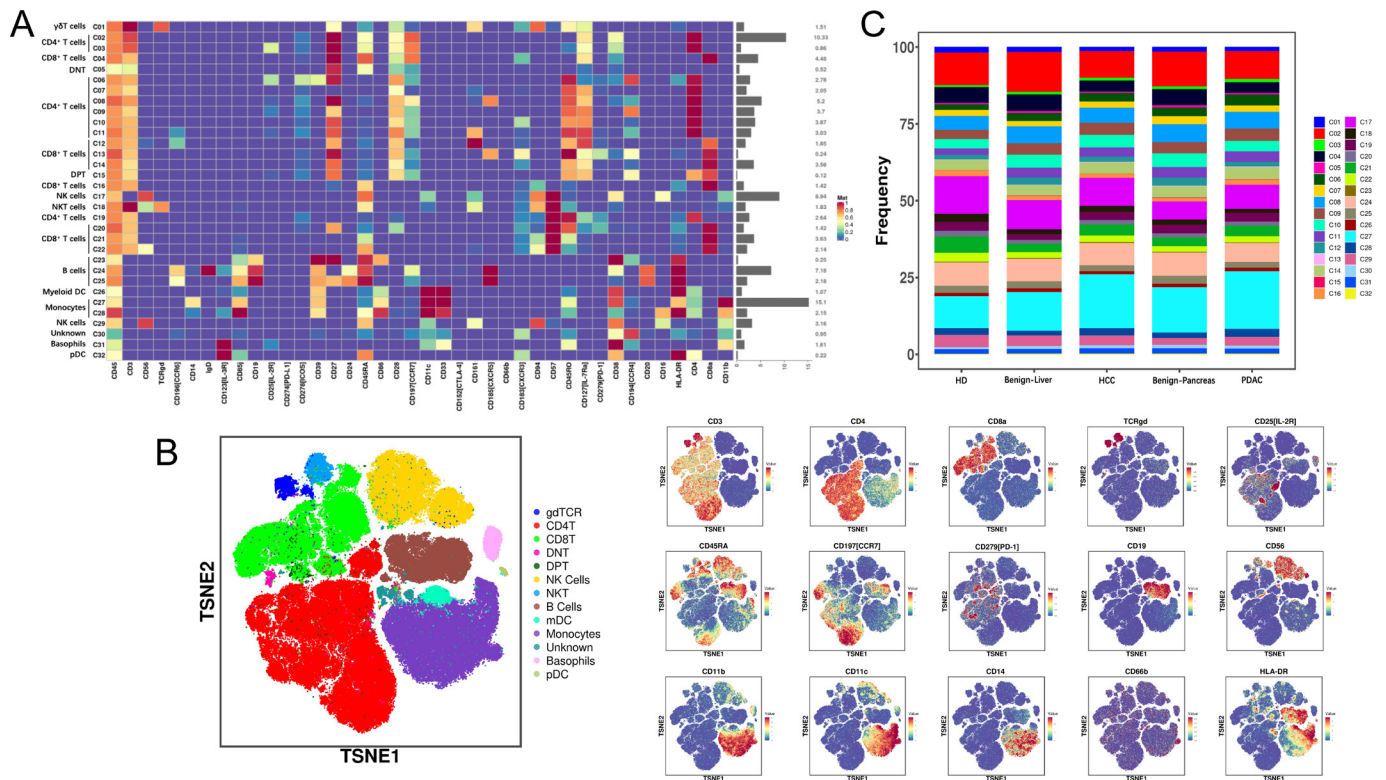


Figure 1 Profile of peripheral immune status. The CyTOF data from randomly selected cases that are representative of each cohort (including 81 HCC, 50 liver benign diseases, 50 pancreatic cancer, 50 pancreatic benign diseases and 50 healthy people) are shown. (A) Heatmap of normalised expression for markers expressed in peripheral immune cells. Types and proportions of various immune cells are presented in left and right of heatmap, respectively. (B) Visualised t-SNE map of definition of peripheral immune cells from CyTOF data of 281 selected cases and part selected classical cell surface markers used for annotation of various immune cells. (C) Frequency diagram of immune cell subsets in the different groups. Benign-liver, hepatic benign diseases; Benign-pancreas, pancreatic benign diseases; CyTOF, cytometry time-of-flight; DNT, double negative T cells; DPT, double positive T cells; double positive T cells; HD, healthy donor; HCC, hepatocellular carcinoma; mDC, myeloid dendritic cells; NK, natural killer; PDAC, pancreatic ductal adenocarcinoma; gdTCR: $\gamma\delta$ T cells; pDC, plasmacytoid dendritic cells; t-SNE, t-distributed stochastic neighbour embedding.

diseases), participants with HCC presented with decreased levels of naïve CD4⁺ T cells (C02, CD4⁺CCR7⁺CD45RA⁺), naïve CD8⁺ T cells (C04, CD8⁺CCR7⁺CD45RA⁺), effector CD8⁺ T cells (C16, CD8⁺CCR7⁺CD45RA⁺) and memory B cells (C25, CD19⁺CD27⁺), while the levels of CD39⁺ naïve CD4⁺ T cells (C06, CD4⁺CCR7⁺CD45RA⁺CD39⁺), central memory CD4⁺ T cells (C09, CD4⁺CCR7⁺CD45RA⁻), double positive T cells (C15, CD4⁺CD8⁺), plasma cells (C23, CD19⁺CD27⁺CD20⁻CD38⁺) and monocytes (C27 and C28, CD3⁻CD19⁻CD14⁺) were increased (online supplemental figure S1). Intriguingly, some subsets showed an aggravation-related change from early-stage to late-stage tumours (online supplemental figure S2). For instance, the increasement of NK cells (C17) was more significant in HCC from stage 0 to stage C, while a subset of CCR4⁺CD20⁻ B cells (C23) gradually reduced as the Barcelona Clinic Liver Cancer stage advanced.

A similar phenomenon was observed in the pancreatic group. Compared with participants without PDAC (include healthy volunteers and cohort with pancreatic benign diseases), lower levels of naïve CD4⁺ T cells (C02, CD4⁺CCR7⁺CD45RA⁺), naïve CD8⁺ T cells (C04, CD8⁺CCR7⁺CD45RA⁺), naïve B cells (C24, CD19⁺CD27⁻) and memory B cells (C25, CD19⁺CD20⁺CD27⁺) were observed in patients with PDACs, while the levels of central memory CD4⁺ T cells (C03, CD4⁺CCR7⁻CD45RA⁺), plasmacytoid DCs (C32, CD3⁻CD19⁻CD14⁻CD20⁻HLA-DR⁺CD123⁺) and monocytes (C27 and C28, CD3⁻CD19⁻CD14⁺) were higher (online supplemental figure S3). Analysis of cell subsets

between different stages of PDAC was also performed (online supplemental figure S4). The frequency of C03 subset increased as the stage of PDAC advanced, and that of C12 and C13 subsets gradually decreased with the stage of PDAC advanced. These results indicated that tumour-bearing hosts did have altered immune cell subsets in the periphery, which might be used to screen for malignancies.

Potential markers and immune cell subsets for model construction

For the HCC diagnostic model, the training cohort contained 450 participants with HCC, 150 participants with benign hepatic diseases and 300 healthy controls. The remaining 199 patients with HCC and 71 participants with hepatic diseases as well as 302 healthy controls from the leading centre were defined as the internal validation cohort to verify the efficacy of the model. Another 141 patients with HCC, 120 participants with benign hepatic diseases and 31 healthy volunteers from the 14 cooperating centres comprised the external validation cohort, which was used to confirm the generalisation ability of the models (figure 2). Participants in the pancreatic group were similarly grouped to construct and validate the diagnostic model for PDAC.

Provided that participants with malignancies had distinct immune profiles in their circulation, we first tried to identify potential parameters (eg, markers or immune cell subsets) for

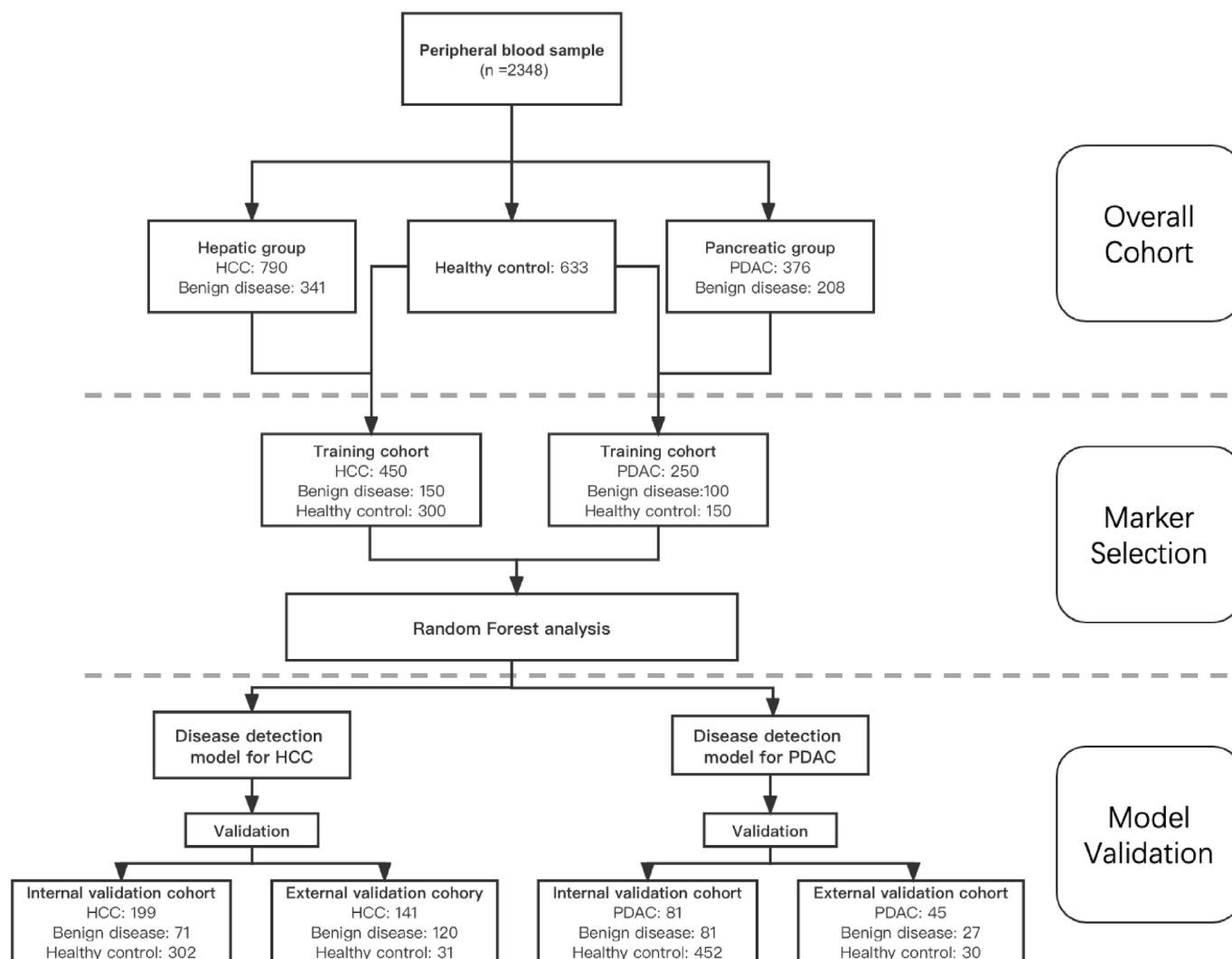


Figure 2 Workflow chart. A total of 2348 peripheral blood samples were collected prospectively from 15 centres. Healthy controls and patients with benign diseases were assigned as the non-malignant group. Marker selection was performed based on the differences between participants with or without malignancies. Healthy controls were shared with the hepatic and pancreatic groups. Random forest analysis was used to generate detection models for HCC and PDAC, respectively. The models were validated using internal and external validation cohorts. HCC, hepatocellular carcinoma; PDAC, pancreatic ductal adenocarcinoma.

tumour detection. We employed the well-established random forest algorithm to discover consequential features from the training groups of HCC and PDAC, respectively. In the hepatic group, we determined 7 markers and 16 cell subsets with the potential to discriminate HCC from non-HCC participants (online supplemental table S7). In the pancreatic group, we obtained 8 markers and 11 cell subsets for PDAC screening (online supplemental table S8). These cell subsets contained monocytes, B cells and T cells. These markers and cell subsets were then applied to construct diagnostic models.

Diagnostic and differentially diagnostic efficacies of the models based on PBIScore

In the training cohort of the hepatic group, the PBIScore of the participants with HCC was significantly higher than that of the non-HCC subjects (0.753 vs 0.247; figure 3A). Similar results were observed in the internal validation and external validation cohorts. This diagnostic model based on the PBIScore alone achieved a sensitivity of 90.2% and a specificity of 92.0%, with an area under the receiver-operating characteristic curve of 0.98

(figure 3B). In the internal validation cohort, the sensitivity was 80.9% and the specificity was 86.9% (AUC=0.91), and in the external validation cohort, the sensitivity and specificity reached 83.0% and 78.2%, respectively. We then combined the AFP level and PBIScore together (named as iPBIScore) to generate a new model, which had a higher sensitivity of 93.1% and specificity of 98.7% in the same training cohort, with an AUC of 0.99. Similarly, the sensitivity and specificity in the two validation cohorts were further improved compared with the PBIScore alone. The AUC values of the iPBIScore in the internal validation cohort and external validation cohort were 0.97 and 0.96, respectively, which were superior to that of AFP alone (AUC, 0.84–0.83).

To test our strategy in pancreatic cancer, we performed a similar study. The PBIScore was significantly higher in participants with PDAC than in non-PDAC participants for all cohorts tested (figure 4A). The model using the PBIScore alone for PDAC screening achieved a sensitivity of 92.4% and specificity of 91.2%, with an AUC of 0.98 in the training cohort (figure 4B). Our model also showed a good discrimination ability in both the internal validation cohort (AUC=0.89) and

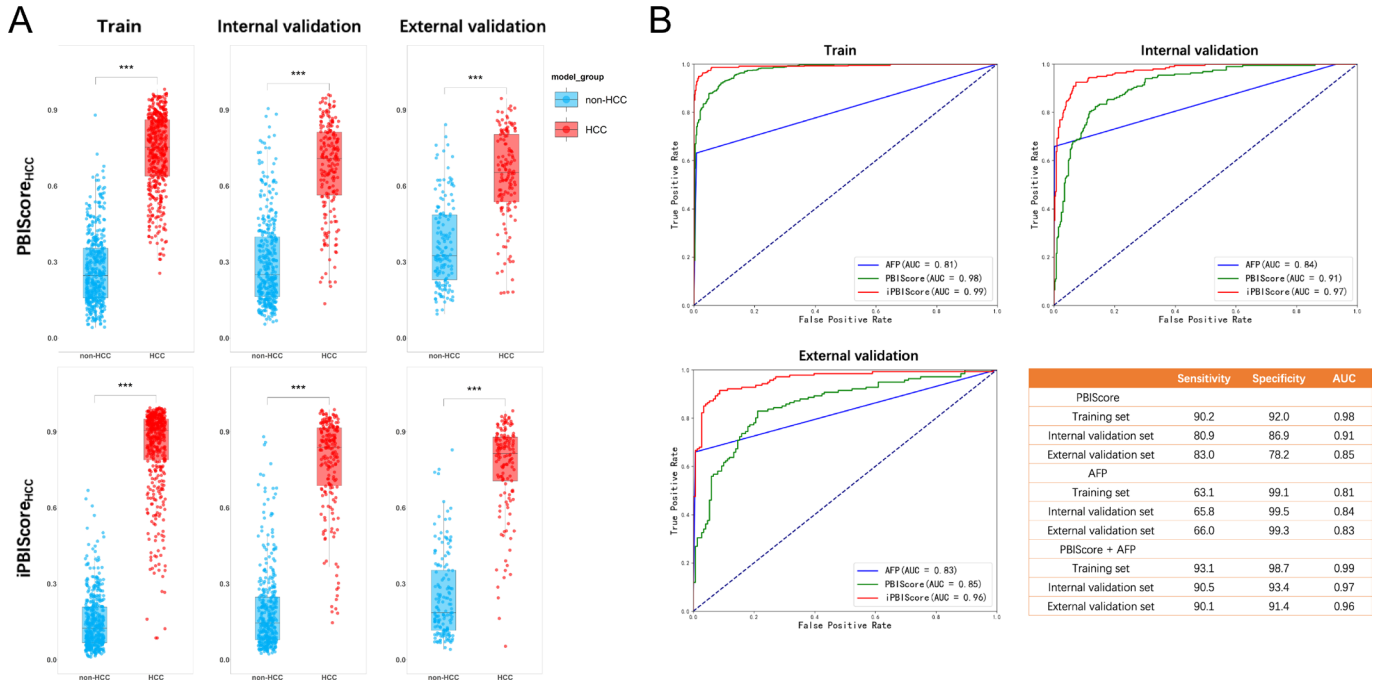


Figure 3 Development and validation of the HCC detection models. (A) The PBIScore and iPBIScore of participants with or without HCC in the training cohort, internal validation cohort and external validation cohort. (B) The ROC curves of AFP, PBIScore and iPBIScore for HCC detection in the training cohort, internal validation cohort and external validation cohort. Table showing the sensitivity, specificity and AUC value of the three strategies for HCC in different cohorts. ***, $p < 0.001$. AFP, alpha-fetoprotein; AUC, area under the curve; HCC, hepatocellular carcinoma; iPBIScore, integrated peripheral blood immune score; PBIScore, peripheral blood immune score; ROC, receiver operating characteristic.

the external validation cohort (AUC=0.89). While CA19-9, the most frequently used tumour marker in clinical practice, showed an AUC of 0.92–0.86, the combined model of PBIScore and

CA19-9 had AUC values of 0.99, 0.98 and 0.97 in the training cohort, internal validation cohort and external validation cohort, respectively.

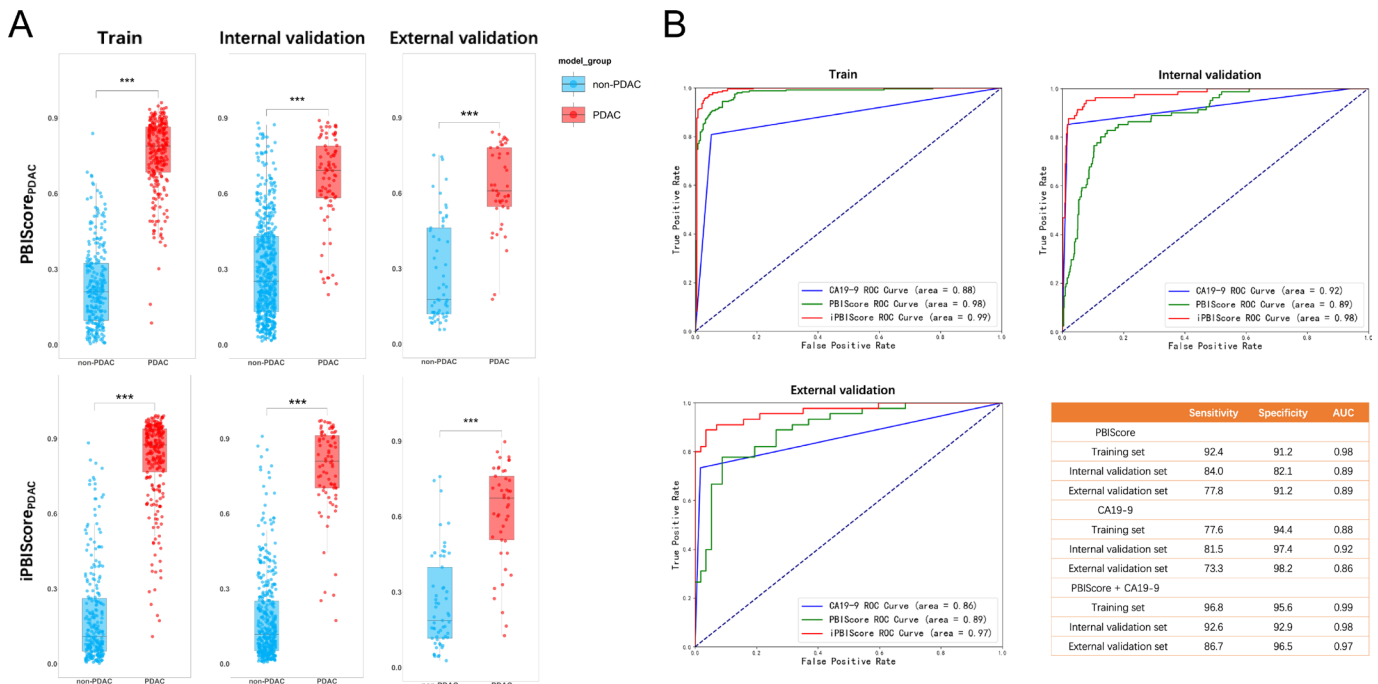


Figure 4 Development and validation of PDAC detection models. (A) The PBIScores and iPBIScore of participants with or without PDAC in the training cohort, internal validation cohort and external validation cohort. (B) The ROC curves of CA19-9, PBIScore and iPBIScore for PDAC detection in the training cohort, internal validation cohort and external validation cohort. Table showing the sensitivity, specificity and AUC value of the three strategies for PDAC in different cohorts. ***, $P < 0.0001$. AUC, area under the curve; CA19-9, carcinoma antigen 19-9; iPBIScore, integrated peripheral blood immune score; PBIScore, peripheral blood immune score; PDAC, pancreatic ductal adenocarcinoma; ROC, receiver operating characteristic.

Although the original aim of our study is to develop a tool for tumour diagnosis, we wondered the efficacy of our PBIScore-based models in differential diagnosis. We combined the internal and external validation cohorts together and excluded healthy volunteers from them to create a differential diagnosis cohort for HCC and PDAC, respectively. The iPBIScore-based model showed an AUC value of 0.97 for HCC differentiation from other hepatic benign diseases, and an AUC value of 0.92 for PDAC differentiation from other pancreatic benign diseases (online supplemental figure S5). Thus, our strategy also had satisfying performance in scenario of differential diagnosis of hepatic and pancreatic lesions.

The performance of PBIScore-based models in early-stage and tumour-marker-negative tumours

Detection of early-stage tumours and those that are negative for tumour markers is more challenging in clinical practice. To test the role of our model in these scenarios, we first analysed the performance of models in different stages of HCC and PDAC. In general, the variations between cancers and non-cancers were

more significant than the difference between stages for both HCC and PDAC, although in some cohorts, advanced tumours demonstrated a more obvious feature than early-stage tumours (online supplemental figure S6).

We then tested the PBIScore-based models in participants with HCC at stage 0 or A. Compared with an AUC of 0.81 for AFP, the PBIScore model and iPBIScore model demonstrated AUC values of 0.90 and 0.96, respectively (figure 5A). In participants with resectable PDAC, CA19-9 showed an AUC of 0.88; however, the PBIScore model and iPBIScore model achieved AUC values of 0.89 and 0.95, respectively (figure 5B). These results verified the good performance of the models to detect early-stage tumours.

AFP and CA19-9 are the most frequently used tumour markers for HCC and PDAC, respectively; therefore, patients who are negative for these tumour markers are easily overlooked. We collected all AFP-negative HCC and CA19-9-negative PDAC in the validation cohorts, and used them to test the performance of the PBIScore model. Our model showed an AUC of 0.91 in HCC and 0.86 in PDAC, respectively (figure 5C,D). Therefore, the

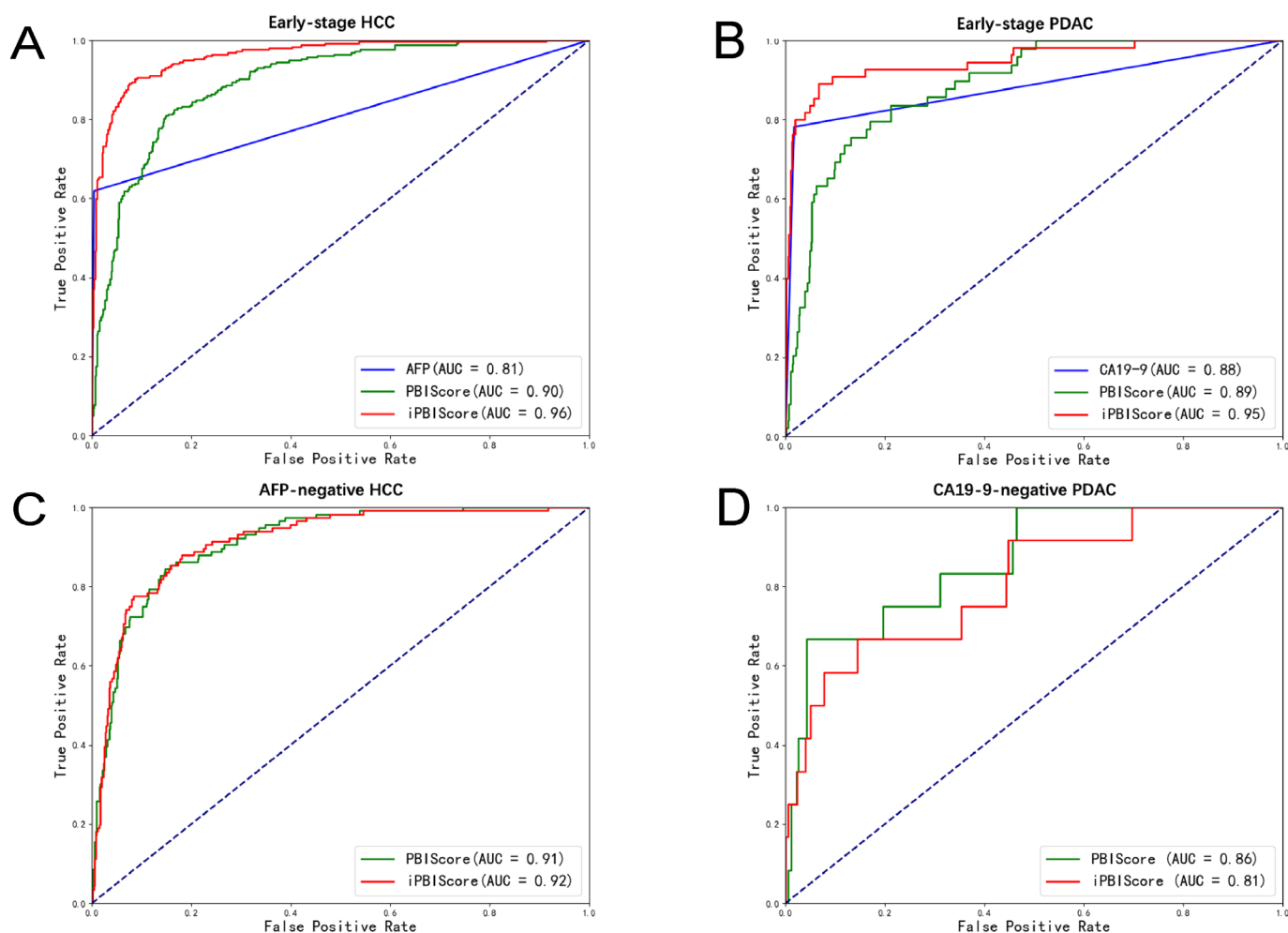


Figure 5 Performance of the tumour detection models in patients with early-stage disease or those with a normal level of tumour markers. (A) The ROC curves of AFP, PBIScore and iPBIScore for tumour detection in patients with HCC stage 0–A stage, showing an AUC of 0.81, 0.90 and 0.96, respectively. (B) The ROC curves of CA19-9, PBIScore and iPBIScore for tumour detection in patients with resectable pancreatic cancer, showing an AUC of 0.88, 0.89 and 0.95, respectively. (C) The ROC curves of PBIScore and iPBIScore for tumour detection in AFP-negative HCC, showing an AUC of 0.91 and 0.92, respectively. (D) The ROC curves of PBIScore and iPBIScore for tumour detection in CA19-9-negative PDAC, showing an AUC of 0.86 and 0.81, respectively. AFP, alpha-fetoprotein; AUC, area under the curve; CA19-9, carcinoma antigen 19-9; HCC, hepatocellular carcinoma; iPBIScore, integrated peripheral blood immune score; PBIScore, peripheral blood immune score; PDAC, pancreatic ductal adenocarcinoma; ROC, receiver operating characteristic.

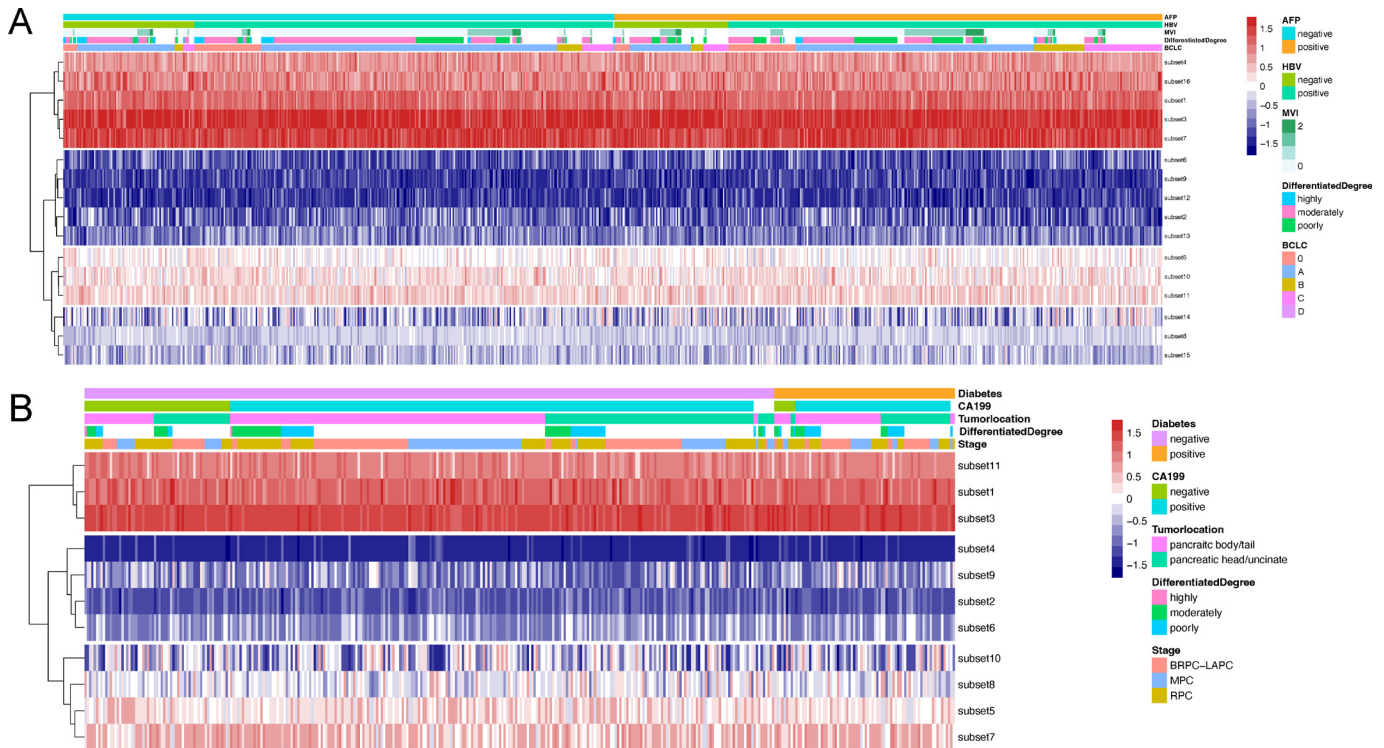


Figure 6 The relationship between the cell subsets used for model construction and the tumour features. (A) Heatmap depicting the relationship between the abundance of characteristic subgroups used to construct models and the various clinicopathological features of HCC. (B) Heatmap depicting the relationship between the abundance of characteristic subgroups used to construct the models and the various clinicopathological features of PDAC. AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CA19-9, carcinoma antigen 19-9; HBV, Hepatitis B Virus; HCC, hepatocellular carcinoma; MVI, microvascular invasion; PDAC, pancreatic ductal adenocarcinoma.

PBIScore could be a good complement for traditional tumour markers.

Relationship between clinicopathological features and peripheral immune status

Given the satisfactory performance of the PBIScore-based models, we investigated the potential subtypes among the patients with tumours. Important clinicopathological parameters, such as tumour markers, tumour differentiation degree and disease stages were analysed. We re-clustered the cell subsets included in the PBIScore-based models, and compared their proportions across these clinicopathological parameters (figure 6). Among the 790 participants with HCC, the proportion of peripheral non-classical monocyte ($CD14^+CD16^+$) and central memory $CD8^+$ T cell ($CCR7^+CD45RA^-$) was significantly less in AFP-positive participants than in AFP-negative participants (online supplemental table S9). Notably, $CD3^-CD19^-CD14^+$ monocytes and $HLA-DR^+CD38^+CD8^+$ T cells were enriched whereas $CD33^+CD14^-$ lymphocytes decreased in patients with Hepatitis B Virus (HBV) infection. The proportion of central memory $CD8^+$ T cell was higher in patients with moderate or well-differentiated HCC than that in patients with poorly-differentiated HCC. Intriguingly, advanced HCC was associated with markedly higher proportions of seven cell subsets, including monocytes, $HLA-DR^+CD38^+CD8^+$ T cells, $CD85j^-CD8^+$ T cells, $CXCR5^+CD8^+$ T cells, central memory $CD8^+$ T cells, myeloid-derived suppressor cells (MDSCs, $CD33^+CD3^-CD19^-HLA-DR^-CD11b^+$) and $CD33^-CD14^-$ lymphocytes. No featured cell subsets were identified in patients with HCC with different microvascular invasion status.

Among the 376 participants with PDAC, we noticed that tumour location and disease stage was significantly associated with many key cell subsets included in our models (online supplemental table S10). Patients with PDAC located in the body or tail of the pancreas showed more $HLA-DR^+CD38^+CD8^+$ T cells and $HLA-DR^+CD38^+CD4^+$ T cells in their peripheral blood. In contrast to HCC, the differentiation degree of PDAC did not influence these immune subsets in circulation; however, advanced PDAC demonstrated a higher level of lymphocytes, $HLA-DR^+CD38^+CD4^+$ T cells, MDSCs and $CD3^-CD19^-CD14^+$ monocytes compared with those in early-stage PDAC. Our results determined the relevance of immune cell subsets between the primary tumour and peripheral immune status, and defined some cell subsets that might have important influence in peripheral immune imbalance.

DISCUSSION

The development, progression and metastasis of a tumour are all closely related to antitumour immunity.³⁴ While much is known about the local immunity of the tumour microenvironment,³⁵ there has been little exploration of the relationship between the tumour and systemic immunity. Theoretically, the battle between the tumour and body immunity is supposed to be reflected in the peripheral blood, which constantly communicates with the tumour. For example, when the haematopoietical function of the tumour-bearing host was damaged extensively, the immature neutrophils and monocytes in the peripheral blood expanded abnormally, with some of them being metastasised to the tumour microenvironment, leading to local immunosuppression.^{32 33}

To the best of our knowledge, this is the largest study using CyTOF analysis on human peripheral blood to date and is the first multicentre report using CyTOF as a tool for tumour diagnosis. In the current study, we took advantage of the high-dimensional profiling ability of CyTOF to discover the differences in immune cells populations of peripheral blood and subsequently used selected parameters in this process to generate tumour detection models with relatively high sensitivity and specificity. The success of our strategy in early-stage HCC and PDAC suggested that hints of tumours in peripheral blood might exist soon after tumour initiation, which supports the concept of analysing systemic immunity for early tumour detection. In addition, we used the same panel and strategy for two types of tumour, and obtained relatively similar results, not only for the performance of detection models, but also in the cell subsets identified for model construction and for their underlying clinical relevance. Therefore, these findings implied that our PBIScore-based strategy is probably applicable to other tumours. Nevertheless, compared with other liquid biopsy strategies, like ctDNA and CTC, our approach has a low requirement for sample collection, with only a 5 mL sample and no need to isolate rare cells or enrich low-abundance nucleotides. Therefore, it is more customer-friendly and easier to generalise. However, some important issues need to be overcome before CyTOF technique can be translated to clinic. For instance, although CyTOF is highly sensitive, the possibility of contamination especially by heavy metals is high.³⁶ Although CyTOF was validated to be well equivalent to flow cytometry that has been clinically used, an optimal panel of CyTOF is critical.³⁷ The metals for antibody labelling should be carefully chosen according to the expression intensity of biomarkers to avoid signal 'spillover'.³⁷ The data analysis of CyTOF is challenging and are currently different in many groups. We have designed an automatic gating algorithm for our study. A uniform process including both sample and data managements is needed for clinical criteria. Moreover, the acquisition time is relatively long for CyTOF, limiting the number of samples that can be detected every day.

Our detection models have a high value of AUC, which suggested a good performance. Trade-off between the sensitivity and specificity is dependent on different clinical scenarios. Although the sensitivity was relatively low at a specificity of 99% as most diagnostic tools were, as a liquid biopsy method focusing on tumour screening, we believe both sensitivity and specificity of around 90% is acceptable. Even though the PBIScore itself is superior to AFP or CA19-9, their combination further improved the AUC value. Notably, the PBIScore and these traditional tumour markers are mutually complementary with each other, which led to the outstanding performance of the iPBIScore detection model. Tumour markers like AFP and CA19-9 are directly derived from tumour cells. However, the PBIScore reflects the interaction between the human body and the tumour, which provides another angle for tumour tracing. Concern about the value of such an interaction was raised, because it can be affected by many physiological and pathophysiological factors, such as circadian rhythm, and thus is unstable. Strikingly, our results demonstrated that this concern might be misplaced. At least the alteration of some key cell markers or subsets is stable enough to detect the tumour. Further in-depth researches are needed to understand why patients with negative tumour markers have more significant changes in their peripheral immune cells.

The present study also identified several key cell subsets that may be responsible for the efficacy of the models. Numbers of naïve CD4⁺ T cells and naïve CD8⁺ T cells decreased in the peripheral blood of tumour-bearing participants, which

limited the source of antitumour T cells in the tumour micro-environment. The frequency of CD39⁺ monocytes significantly increased in participants with HCC or PDAC (C27 and C28 in online supplemental figures S1 and S3). CD39, believed to be a metabolic checkpoint, is a rate-limiting enzyme involved in adenosine production, which plays an important immunosuppressive role in the tumour microenvironment.³⁸ Intriguingly, the frequency of HLA-DR⁺CD38⁺CD8⁺ T cells significantly increased in participants with advanced HCC (online supplemental table S9), suggesting that this cell subset was a biomarker of tumour progression in the circulation. In fact, the expression of both HLA-DR and CD38 on CD8⁺ T cells in peripheral blood was reported to be correlated with advanced tumours.³⁹ HLA-DR⁺CD38⁺CD8⁺ T cells are activated phenotype and may play key roles in antitumour immunity in both tumour microenvironment and peripheral blood.⁴⁰⁻⁴¹ Besides, in patients with HCC and those with PDAC, the proportion of monocytes increased gradually as the disease stage advanced, which was in line with previous studies showing that a high level of peripheral monocytes was found in such patients and was associated with their poor prognosis.⁴²⁻⁴⁴ Overall, these findings broadened our understanding of the underlying reasons that peripheral blood could reflect tumour existence. However, the associations between model-related key cell subsets and clinicopathological parameters of patients were only statistical based and far away from scientific causality.

There are some limitations to our research. First, all participants came from China and were not internationally representative; however, we believe that the current method can be implemented in different ethnic groups and regions worldwide, given the nature of our strategy, though the parameters of the models will be modified to some extent. Second, people with haematological system diseases and those with recent bleeding or blood transfusion were excluded from the study, which limits the targeted population of our model. Third, we only developed tumour-type-specific models for HCC and PDAC, and whether the strategy can be used for pan-cancer screening is unknown. Fourth, our validation cohorts contained a high proportion of participants with malignant diseases, which was not the case for tumour detection in the real world. A natural population-based or high-risk population-based cohort study is needed for further evaluation of our models in clinical practice. The deficiency of patients with hepatitis or cirrhosis in our non-HCC cohort did not allow us to verify the efficacy of our models in these populations, which was another drawback, as more attention would have paid in the high-risk group. Additionally, our analyses of AFP and CA19-9 were based on a discrete way rather than using continuous variables due to unavailability of accurate values within the normal range in some healthy participants. Using them as continuous variables is likely to identify the best threshold to maximise sensitivity and specificity of the models. However, dealing with them as discrete variables also have advantages, for example, it will not change the current clinical practice too much and will be easier to be accepted by physicians. Last but not least, the age and gender were not always balanced in different groups, which could introduce biases and affect the performance of our model in certain cohorts. However, malignant tumours and benign diseases naturally have distinct age and gender tendencies. Age-related or gender-related alterations of immune cells are also features of tumour, no matter they are caused by the tumour or just accompanied with it. The purpose of our study was to develop an efficient tool to diagnose cancer, and our multicentre external validation confirmed the performance of our models.

In conclusion, we verified the differences in systemic immunity in patients with solid tumours, and used large-volume, multicentre cohorts to demonstrate that PBIScore-based models show high performance for tumour detection. Therefore, we provide a ready-to-use tool for HCC and PDAC screening as an expansion of liquid biopsy. Our strategy also lays the foundation for future systemic immunity-based detection methods for pan-cancers.

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Contributors TL and QZ conceived the idea and designed the study. TL supervised the whole study. QZ, YM, MH, YW, YL, JZ, JL, YZ, TY, XS, WY, YH, HH, MX, XW, XY, WT, RL, YG, TW, JW, XW and JW collected the samples. CL and TW provided metal-conjugated antibodies and conducted sample processing. QZ, YM, CL, QK and TW analysed the data. TL, QZ, XB, CY, CZ and ZY interpreted the results. QZ and YM drafted the manuscript. All authors made revision and approved the final version of manuscript. TL is the guarantor.

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Supplementary tables**Supplementary Table S1. Centers for external validation.**

Center	Location
Zhejiang Provincial People's Hospital	Hangzhou, Zhejiang
The First Affiliated Hospital, Wenzhou University School of Medicine	Wenzhou, Zhejiang
Zhejiang Cancer Hospital	Hangzhou, Zhejiang
Sanmen People's Hospital	Taizhou, Zhejiang
Huzhou Central Hospital	Huzhou, Zhejiang
Changxing People's Hospital	Huzhou, Zhejiang
Ningbo Medical Center Lihuili Eastern Hospital	Ningbo, Zhejiang
Shengzhou People's Hospital	Shaoxing, Zhejiang
Shangyu Hospital of Chinese Medicine	Shaoxing, Zhejiang
Haining People's Hospital	Jiaxing, Zhejiang
Jiaxing Second People's Hospital	Jiaxing, Zhejiang
The Second Affiliated Hospital of Nanchang University	Nanchang, Jiangxi
The Second Hospital of Hebei Medical University	Shijiazhuang, Hebei
Jixi County People's Hospital	Xuancheng, Anhui

Supplementary Table S2. The antibody panel for CyTOF.

Label	Marker	Clone	Vendor
89Y	CD45	HI30	BioLegend
115In	CD3	UCHT1	Bioxcell
141Pr	CD56	NCAM16.2	BD
142Nd	TCR γ/δ	5A6.E9	Invitrogen
143Nd	CD196 (CCR6)	G034E3	BioLegend
144Nd	CD14	M5E2	BioLegend
145Nd	IgD	IA6-2	BioLegend
146Nd	CD123 (IL-3R α)	6H6	BioLegend
147Sm	CD85j (ILT2)	GHI/75	BioLegend
148Nd	CD19	HIB19	BioLegend
149Sm	CD25 (IL-2R α)	24212	RD
150Nd	CD274 (PD-L1)	29E.2A3	BioLegend
151Eu	CD278 (ICOS)	C398.4A	BioLegend
152Sm	CD39	A1	BioLegend
153Eu	CD27	O323	BioLegend
154Sm	CD24	ML5	BioLegend
155Gd	CD45RA	HI100	BioLegend
156Gd	CD86	Fun-1	BD
157Gd	CD28	CD28.2	BioLegend
158Gd	CD197 (CCR7)	G043H7	BioLegend
159Tb	CD11c	BU15	BioLegend
160Gd	CD33	WM53	BioLegend
161Dy	CD152 (CTLA-4)	14D3	eBioscience
163Dy	CD161	HP-3G10	BioLegend
164Dy	CD185 (CXCR5)	RF8B2	BD

165Ho	CD66b	G10F5	BioLegend
166Er	CD183 (CXCR3)	G025H7	BioLegend
167Er	CD94	HP-3D9	BD
168Er	CD57	HNK-1	BioLegend
169Tm	CD45RO	UCHL1	BioLegend
170Er	CD127 (IL-7R α)	A019D5	BioLegend
171Yb	CD279 (PD-1)	EH12.2H7	BioLegend
172Yb	CD38	HIT2	BioLegend
173Yb	CD194 (CCR4)	L291H4	BioLegend
174Yb	CD20	2H7	BioLegend
175Lu	CD16	3G8	BioLegend
176Yb	HLA-DR	L243	BioLegend
197Au	CD4	RPA-T4	BioLegend
198Pt	CD8a	RPA-T8	BioLegend
209Bi	CD11b	M1/70	BioLegend

Supplementary Table S3. The demographic and clinicopathological parameters of the hepatic group

	HCC	Hepatic benign diseases	Healthy controls
Total (n)	790	341	633
Gender			
Male – no. (%)	650 (82.2)	140 (41.1) ***	350 (55.3) ***
Female – no. (%)	140 (17.8)	201 (58.9)	283 (44.7)
Age (years)			
Mean	59.0	49.4 ***	46.4 ***
Range	12-89	12-90	16-89
Hypertension	231 (29.2)	55 (16.1) ***	92 (14.5) ***
Diabetes	117 (14.8)	12 (3.5) ***	47 (7.4) ***
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.5 (2.5 – 7.0)	4.5 (2.7 – 6.1)	4.9 (3.7 – 6.1) ***
Hemoglobin, g/L (range)	141.3 (73 – 192)	138.3 (80 – 180) *	146.4 (100 – 190) ***
White blood cell, 10 ⁹ /L (range)	5.6 (1.2 – 19.5)	6.0 (2.2 – 13.8) **	6.0 (3.3 – 14.8) **
Neutrophil, 10 ⁹ /L (range)	3.6 (0.8 – 17.1)	3.7 (1.0 – 12.2)	3.4 (1.5 – 11.4)
Lymphocyte, 10 ⁹ /L (range)	1.5 (0.28 – 5.5)	1.8 (0.2 – 10.3) ***	2.0 (1.0 – 3.7) ***
Platelet, 10 ⁹ /L (range)	159.1 (24 – 616)	216.0 (44 – 419) ***	236.9 (117 – 600) ***
ALT, U/L (range)	39.1 (3 – 747)	31.6 (4 – 406) *	23.2 (3 – 105) ***
AST, U/L (range)	46.8 (8 – 1232)	29.5 (10 – 384) ***	23.0 (12 – 78) ***
Total bilirubin, µmol/L	18.7 (2.1 – 490.4)	13.5 (3.7 – 49.5) ***	12.3 (3.5 – 35.1)

	(range)		***
Albumin, g/L (range)	42.1 (21.6 – 80.0)	44.3 (25.6 – 54.6) ***	48.0 (41.3 – 54) ***
AFP value			
> 20 ng/ml	508 (64.3)	6 (1.8) ***	1 (0.2) ***
≤ 20 ng/ml	282 (35.7)	335 (98.2)	632 (99.8)
HBV infection			
Positive	611 (77.3)	91 (26.7) ***	32 (5.1) ***
Negative	178 (22.5)	241 (70.7)	601 (94.9)
NA	1 (0.2)	9 (2.6)	0 (0.0)
BCLC stage			
0	117 (14.8)	NA	NA
A	500 (63.3)	NA	NA
B	69 (8.7)	NA	NA
C-D	104 (13.2)	NA	NA
MVI grade			
0	408 (51.6)	NA	NA
1	123 (15.6)	NA	NA
2	30 (3.8)	NA	NA
NA	229 (29.0)	NA	NA
Differentiation			
Low	167 (21.1)	NA	NA
Moderately	339 (42.9)	NA	NA
High	45 (5.7)	NA	NA
Unknown	239 (30.3)	NA	NA

Note: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, Aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; MVI, microvascular invasion; NA, not

available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to HCC group).

Supplementary Table S4. The demographic and clinicopathological parameters of the training set in hepatic group

	HCC	Hepatic benign diseases	Healthy controls
Total (n)	450	150	300
Gender			
Male – no. (%)	305 (67.8)	51 (34.0) ***	168 (56.0) **
Female– no. (%)	145 (32.2)	99 (66.0)	132 (44.0)
Age (years)			
Mean	56.9	48.8 ***	54.2
Range	19-76	18-90	24-89
Hypertension	115 (25.6)	21 (14.0) **	47 (15.7) **
Diabetes	56 (12.4)	3 (2.0) ***	22 (7.3) *
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.6 (2.5 – 7.0)	4.5 (2.9 – 6.1)	4.9 (3.9 – 6.0) ***
Hemoglobin, g/L (range)	143.9 (73 – 192)	136.3 (85 – 180) ***	149.9 (103 – 184) **
White blood cell, 10 ⁹ /L (range)	5.7 (2.3 – 12.7)	6.0 (2.3 – 11.4)	6.1 (3.3 – 14.8)
Neutrophil, 10 ⁹ /L (range)	3.6 (0.9 – 10.6)	3.8 (1.0 – 10.7)	3.5 (1.5 – 10.1)
Lymphocyte, 10 ⁹ /L	1.6 (0.4 – 4.1)	1.8 (0.2 – 10.3) **	2.0 (1.0 – 3.7) ***

(range)			
Platelet, 10 ⁹ /L (range)	162.4 (25 – 616)	221.2 (82 – 418) ***	229.0 (117 – 600) ***
ALT, U/L (range)	37.6 (3 – 308)	30.0 (5 – 331) **	24.0 (6 – 78) **
AST, U/L (range)	43.0 (11 – 590)	28.2 (10 – 374) **	22.4 (14 – 40) **
Total bilirubin, µmol/L (range)	18.9 (2.1 – 490.4)	11.6 (3.7 – 34.8) ***	13.4 (3.5 – 35.1)
Albumin, g/L (range)	43.0 (23.6 – 80.0)	44.2 (25.6 – 51.7) **	47.4 (41.3 – 53.5) ***
AFP value			
> 20 ng/ml	284 (63.1)	4 (2.7) ***	0 (0.0) ***
≤ 20 ng/ml	166 (36.9)	146 (97.3)	300 (100.0)
HBV infection			
Positive	362 (80.4)	19 (12.7) ***	22 (7.3) ***
Negative	87 (19.3)	131 (87.3)	278 (92.7)
Unknown	1 (0.3)	0 (0.0)	0 (0.0)
BCLC stage			
0	65 (14.4)	NA	NA
A	292 (64.9)	NA	NA
B	39 (8.7)	NA	NA

C-D	54 (12.0)	NA	NA
MVI grade			
0	244 (54.2)	NA	NA
1	69 (15.3)	NA	NA
2	16 (3.6)	NA	NA
NA	121 (26.9)	NA	NA
Differentiation			
Low	100 (22.2)	NA	NA
Moderately	206 (45.8)	NA	NA
High	16 (3.6)	NA	NA
Unknown	128 (28.4)	NA	NA

Note: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, Aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; MVI, microvascular invasion; NA, not available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to HCC group).

Supplementary Table S5. The demographic and clinicopathological parameters of the pancreatic group

	PDAC	Pancreatic benign diseases	Healthy controls
Total (n)	376	208	632
Gender			
Male – no. (%)	221 (58.8)	81 (38.9) ***	349 (55.2)
Female – no. (%)	155 (41.2)	127 (61.1)	283 (44.8)
Age (years)			
Mean	64.3	53.2 ***	46.5 ***
Range	22-88	13-82	16-89
Hypertension	133 (35.4)	61 (29.3)	102 (16.1) *
Diabetes	78 (20.7)	22 (10.6) **	47 (7.4) ***
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.3 (2.0 – 5.9)	4.4 (2.8 – 6.3) **	4.9 (3.7 – 6.13) ***
Hemoglobin, g/L (range)	130.6 (57 – 173)	134.3 (84 – 179) **	146.4 (100 – 190) ***
White blood cell, 10 ⁹ /L (range)	6.4 (2.5 – 19.5)	6.4 (2.6 - 17.8)	6.0 (3.3 – 14.8) *
Neutrophil, 10 ⁹ /L (range)	4.5 (1.4 – 17.7)	4.3 (1.2 – 17.0)	3.4 (1.5 – 11.4) ***
Lymphocyte, 10 ⁹ /L (range)	1.3 (0.3 – 3.3)	1.6 (0.3 – 3.2) ***	2.0 (1.0 – 3.7) ***
Platelet, 10 ⁹ /L (range)	212.6 (55 – 541)	226.8 (70-801) **	236.9 (117 – 600) ***
ALT, U/L (range)	97.7 (2 – 1403)	37.1 (5 – 1226) ***	23.2 (3 – 105) ***
AST, U/L (range)	66.5 (8 – 619)	31.3 (9 – 1112) ***	22.9 (12 – 78) ***
Total bilirubin, µmol/L	59.0 (3.0 – 547.6)	16.9 (3.0 – 390.1) ***	12.3 (3.5 – 35.1) ***

(range)			
Albumin, g/L (range)	41.6 (25.0 – 52.2)	43.5 (27.7 - 54.1) ***	48.0 (41.3 – 54) ***
CA19-9 value			
> 37 U/ml	293 (77.9)	24 (11.5) ***	5 (0.8) ***
≤ 37 U/ml	83 (22.1)	184 (88.5)	627 (99.2)
Resectability			
RPC	134 (35.6)	NA	NA
BRPC-LAPC	136 (36.2)	NA	NA
MPC	106 (28.2)	NA	NA
Differentiation			
Low	53 (14.1)	NA	NA
Moderately	54 (14.4)	NA	NA
High	2 (0.5)	NA	NA
Unknown	267 (71.0)	NA	NA

Note: ALT, alanine aminotransferase; AST, Aspartate transaminase; BRPC, borderline resectable pancreatic cancer; CA19-9, carcinoma antigen 19-9; LAPC, locally advanced pancreatic cancer; MPC, metastatic pancreatic cancer; NA, not available; RPC, resectable pancreatic cancer. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to PDAC group).

Supplementary Table S6. The demographic and clinicopathological parameters of the training set in pancreatic group

	PDAC	Pancreatic benign diseases	Healthy controls
Total (n)	250	100	150
Gender			
Male– no. (%)	146 (58.4)	35 (35.0) ***	83 (55.3)
Female – no. (%)	104 (41.6)	65 (65.0)	67 (44.7)
Age (years)			
Mean	63.5	54.7 ***	60.6
Range	22-88	13-82	30-89
Hypertension	86 (34.4)	33 (33.0)	23 (15.3) ***
Diabetes	49 (19.6)	11 (11.0)	19 (12.7)
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.3 (2.3 – 5.9)	4.4 (2.8 – 5.4)	4.9 (4.2 – 5.9) ***
Hemoglobin, g/L (range)	131.6 (57 – 173)	134.5 (90 – 173)	148.7 (115 – 170) ***
White blood cell, 10 ⁹ /L (range)	6.3 (2.5 – 19.5)	6.7 (3.4 - 17.8)	6.1 (3.8 – 8.6)
Neutrophil, 10 ⁹ /L (range)	4.4 (1.4 – 17.7)	4.6 (1.2 – 17.0)	3.5 (2.1 – 5.5) ***

Lymphocyte, 10 ⁹ /L (range)	1.3 (0.3 – 3.3)	1.6 (0.3 – 3.2) ***	2.0 (1.0 – 3.3) ***
Platelet, 10 ⁹ /L (range)	211.2 (55 – 506)	227.8 (70 - 480) **	227.0 (119 – 318)
ALT, U/L (range)	96.0 (5 – 1403)	37.2 (5 – 921) ***	21.3 (6 – 105) ***
AST, U/L (range)	65.8 (8 – 619)	35.5 (9 – 1112) *	25.5 (14 – 78) ***
Total bilirubin, µmol/L (range)	59.7 (3.0 – 547.6)	18.6 (3.0 – 390.1) ***	12.3 (4.6 – 30.4) ***
Albumin, g/L (range)	42.1 (25.8 – 52.2)	43.5 (28.8 - 52.4) *	46.9 (41.3 – 53) ***
CA19-9 value			
> 37 U/ml	194 (77.6)	14 (14.0) ***	0 (0.0) ***
≤ 37 U/ml	56 (22.4)	86 (86.0)	150 (100.0)
Resectability			
RPC	79 (31.6)	NA	NA
BRPC-LAPC	104 (41.6)	NA	NA
MPC	67 (26.8)	NA	NA
Differentiation			
Low	31 (12.4)	NA	NA
Moderately	31 (12.4)	NA	NA
High	0 (0.0)	NA	NA
Unknown	188 (75.2)	NA	NA

Note: NA, not available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to PDAC group).

Supplementary Table S7 Markers and cell subsets used for HCC detection model construction

Markers or cell subsets	Feature importance	SE	Z-score	P-value
AFP	0.353122325856825	0.454	-36.552	<0.001
CD14	0.0266157507901591	6.515	-13.932	<0.001
CD152	0.0165493598488722	1.751	8.584	<0.001
CD25	0.0268740558987101	0.864	-5.537	<0.001
CD33	0.0307218964267744	8.573	-11.071	<0.001
CD45RA	0.0243438245610579	12.255	9.352	<0.001
CD85J	0.0320794521882959	6.593	-9.068	<0.001
CD86	0.0462854483540234	5.865	-15.543	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ HLA-DR ⁻ CD38 ⁻	0.0437688704892163	12.243	14.821	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁻ CD19 ⁺ IgD ⁺ CD27 ⁺	0.0444513473213629	5.56	-7.758	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ CD197 ⁺ CD45RA ⁺	0.026710817192894	11.142	12.027	<0.001
CD3 ⁻ CD19 ⁻ HLA-DR ⁻	0.0196247275687274	12.876	-9.79	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺	0.0221718539244221	8.508	11.146	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA- DR ⁺ CD8a ⁺	0.0141180611540897	3.008	7.652	<0.001
CD33 ⁻ CD14 ⁻	0.0225940563969961	8.508	-11.146	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ CD85j ⁻	0.0193691230085965	5.108	6.13	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ CXCR5 ⁺	0.0274583522068538	2.222	6.06	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA-DR ⁻ CD4 ⁺	0.044259520077781	12.346	-15.714	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺	0.0216413407150223	9.844	-8.875	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ CD45RA ⁺ CD197 ⁻	0.0307140730725735	2.371	-5.588	<0.001

CD33 ⁻ CD14 ⁻ CD3 ⁺ CD45RA ⁻ CD197 ⁺ CD8a ⁺	0.0254454238922522	3.114	9.14	<0.001
CD33 ⁺ CD3 ⁻ CD19 ⁻ HLA-DR ⁻ CD11b ⁺	0.0401007387170425	10.059	11.259	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺ CD16 ⁺	0.0255826514290803	6.768	3.488	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺	0.0153969289083717	11.627	8.775	<0.001

Supplementary Table S8 Markers and cell subsets used for PDAC detection model construction

Markers or cell subsets	Feature importance	SE	Z-score	P-value
CA19-9	0.447075072880982	0.496	-33.442	<0.001
CD86	0.0251255615948677	6.784	-12.562	<0.001
CD14	0.0314453971496788	7.04	-12.226	<0.001
CD33	0.04899000925121	9.15	-10.64	<0.001
CD4	0.0200205290640094	10.237	-3.816	<0.001
CD11b	0.0126628230877706	14.403	-4.4	<0.001
CD183	0.0100134741809992	8.382	8.895	<0.001
CD25	0.0447355631765113	0.86	-3.794	<0.001
CD152	0.017225333770737	1.331	5.815	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺	0.0199715291594582	11.571	9.064	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA-DR ⁺ CD8a ⁺	0.0459849642348083	5.01	9.34	<0.001
CD33 ⁻ CD14 ⁻	0.0451007438245495	9.129	-10.805	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD85j ⁺ CD8a ⁺	0.0309372394802058	4.554	-6.084	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺	0.0422505171663183	9.129	10.805	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ HLA-DR ⁺	0.018914250326465	6.461	8.945	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺	0.0172440965501883	9.791	-7.722	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ HLA-DR ⁺ CD38 ⁺	0.0561160245457125	1.142	9.476	<0.001
CD33 ⁻ CD14 ⁻				
CD3 ⁺ CD4 ⁺ CD25 ⁺ CD45RA ⁺ CD161 ⁻ CD127 ⁻	0.0130382951243638	7.939	-5.697	<0.001
CD33 ⁺ CD3 ⁻ CD19 ⁻ HLA-DR ⁻ CD11b ⁺	0.0326018440406268	11.173	7.482	<0.001
CD3 ⁻ CD19 ⁻ HLA-DR ⁻	0.0205467313905376	13.054	-9.008	<0.001

Supplementary Table S9. Differences in key cell subsets of HCC with certain clinicopathological parameters

	Cell subsets	AFP		Differentiation Degree		HBV		MVI		BCLC stage	
		t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value
subset1	CD33-CD14-CD3+CD4+HLA_DR-CD38-	1.376	0.169	5.311	0.07	0.329	0.742	1.553	0.46	2.579	0.461
subset2	CD33-CD14-CD3-CD19+IGD+CD27+	-0.411	0.681	0.963	0.618	1.343	0.179	1.331	0.514	6.882	0.076
subset3	CD33-CD14-CD3+CD4+CD197+CD45RA+	0.435	0.663	3.252	0.197	0.133	0.894	0.108	0.947	3.615	0.306
subset4	CD3-CD19-HLA_DR-	0.333	0.739	0.346	0.841	-0.277	0.782	1.235	0.539	7.752	0.051
subset5	CD3-CD19-CD14+	-1.634	0.102	1.916	0.384	2.384	0.017	0.075	0.963	32.23	<0.001
subset6	CD33-CD14-CD3+CD38+HLA_DR+CD8a+	-0.555	0.579	4.416	0.11	2.438	0.015	0.1	0.951	17.525	<0.001
subset7	CD33-CD14-	1.634	0.102	1.916	0.384	-2.384	0.017	0.075	0.963	32.23	<0.001
subset8	CD33-CD14-CD3+CD8a+CD85j-	1.798	0.072	0.848	0.655	1.398	0.162	0.605	0.739	33.67	<0.001
subset9	CD33-CD14-CD3+CD8a+CXCR5+PD_L1+	-0.264	0.792	0.135	0.935	0.758	0.449	3.379	0.185	9.501	0.023
subset10	CD33-CD14-CD3+CD38+HLA_DR-CD4+	-1.119	0.263	4.039	0.133	-0.688	0.491	1.01	0.603	1.897	0.594
subset11	CD33-CD14-CD3+CD8a+	-0.538	0.591	0.13	0.937	0.755	0.45	3.337	0.189	1.645	0.649
subset12	CD33-CD14-CD3+CD4+CD45RA+CD197-	0.175	0.861	1.348	0.51	-0.166	0.868	0.642	0.725	3.081	0.379
subset13	CD33-CD14-CD3+CD45RA-CD197+CD8a+	-2.383	0.017	6.226	0.044	-0.579	0.563	0.786	0.675	8.024	0.046
subset14	CD33+CD3-CD19-HLA_DR-CD11b+	-0.534	0.594	0.613	0.736	-0.255	0.799	0.005	0.997	21.181	<0.001
subset15	CD3-CD19-CD14+CD16+	-2.788	0.005	0.116	0.944	-0.639	0.523	0.048	0.976	1.368	0.713
subset16	CD33-CD14-CD3+CD4+	0.39	0.697	0.248	0.883	1.473	0.141	1.38	0.502	0.754	0.861

Negative as control

Poorly differentiation control

Negative as control

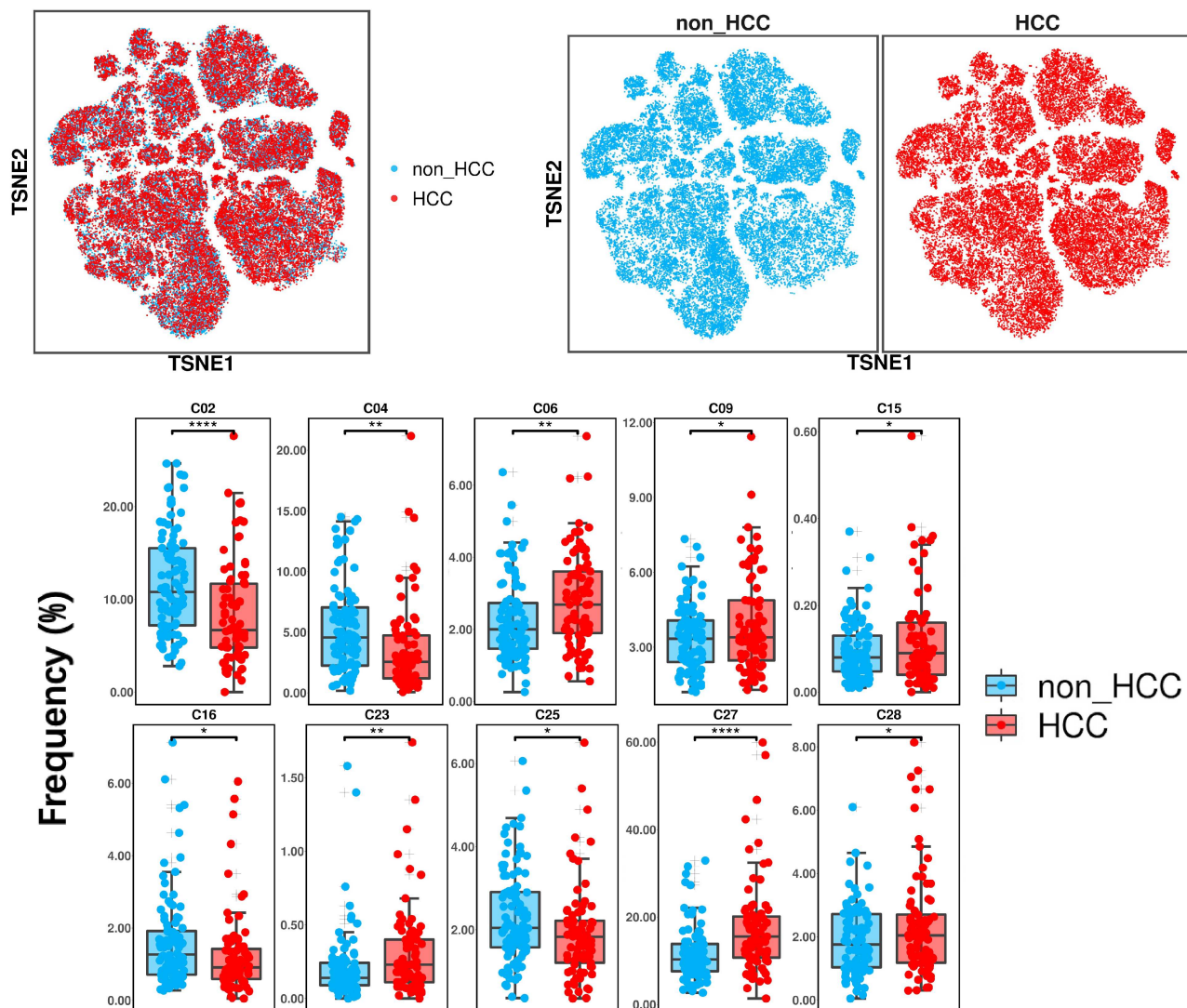
M0 as control

Stage 0 as control

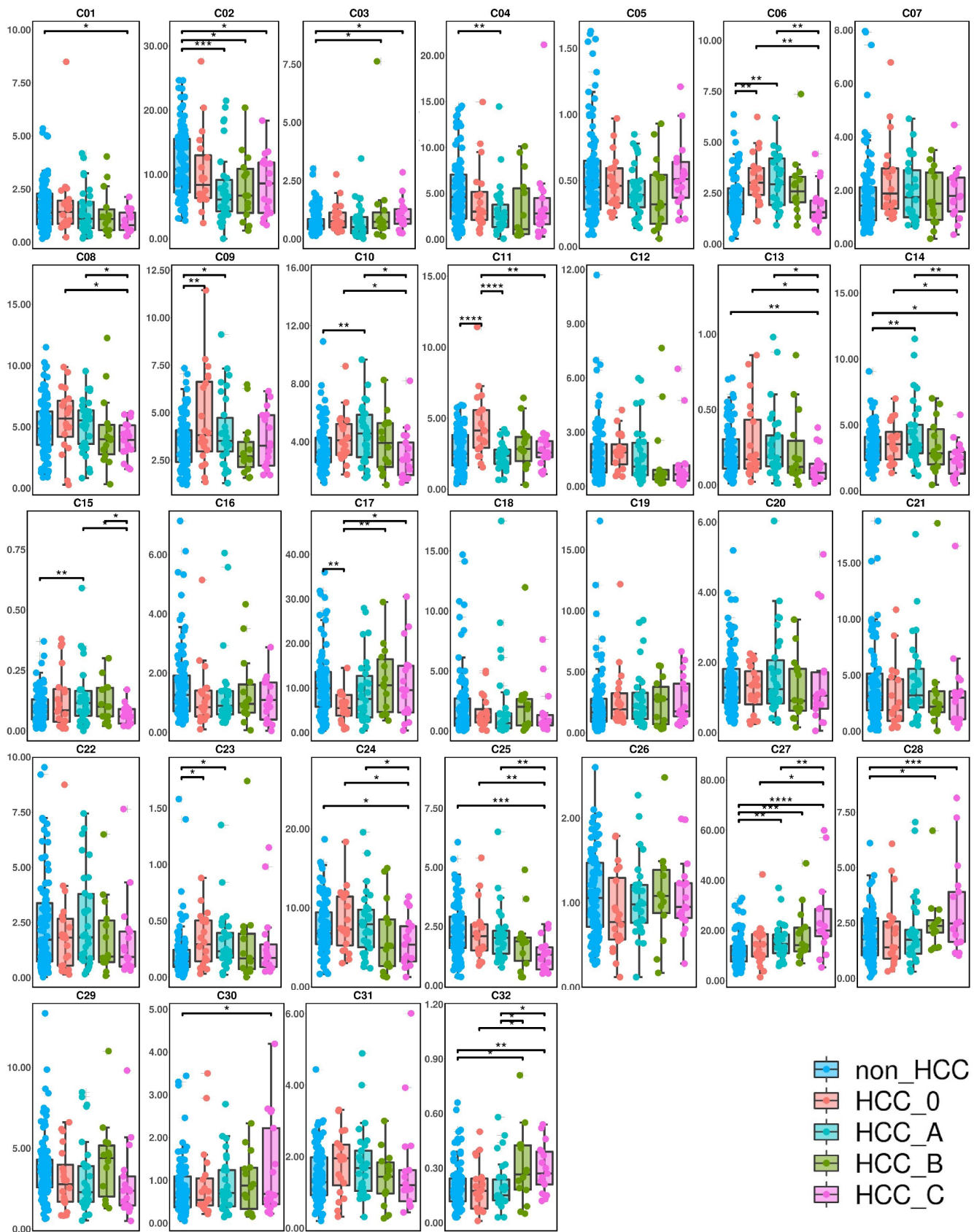
Supplementary Table S10. Differences in key cell subsets of PDAC with certain clinicopathological parameters

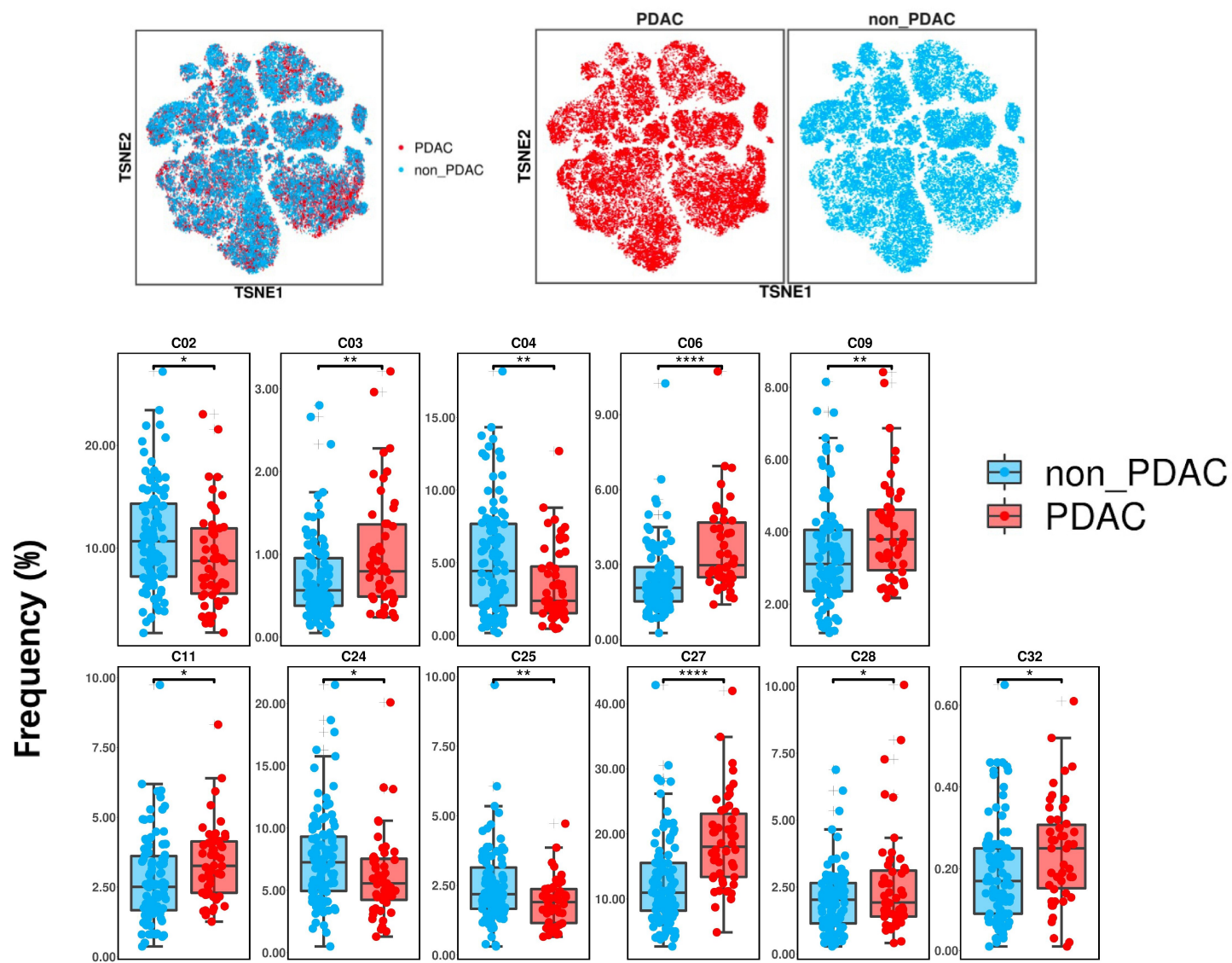
Cell subsets	CA199		Diabetes		Differentiation degree		Tumor location		Disease stage		
	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	
subset1	CD33-CD14-CD3+CD4+	-0.935	0.35	-0.821	0.535	-0.412	0.681	-1.461	0.144	1.026	0.599
subset2	CD33-CD14-CD3+CD38+HLA_DR+CD8+	0.17	0.965	-1.089	0.276	0.938	0.97	2.919	0.005	1.05	0.592
subset3	CD33-CD14-	-0.105	0.916	-1.268	0.205	-1.551	0.121	-1.128	0.259	18.941	<0.001
subset4	CD33-CD14-CD3+CD85+CD8+	-0.436	0.663	-1.112	0.266	-0.57	0.569	-1.33	0.183	0.183	0.912
subset5	CD3-CD19-CD14+	0.105	0.916	1.268	0.205	1.551	0.121	1.128	0.259	18.941	<0.001
subset6	CD33-CD14-CD3+CD8+HLA_DR+	0.139	0.889	-1.555	0.12	0.663	0.507	2.627	0.009	1.126	0.57
subset7	CD33-CD14-CD3+CD8+	0.898	0.369	-0.306	0.759	0.357	0.721	1.874	0.061	0.761	0.684
subset8	CD33-CD14-CD3+CD4+HLA_DR+CD38+	-0.72	0.472	-0.063	0.946	0.657	0.511	2.101	0.035	6.973	0.018
subset9	CD33-CD14-CD3+CD4+CD25+CD45RA+CD161-CD127-	1.299	0.194	-1.29	0.2	-1.37	0.171	0.02	0.984	7.072	0.029
subset10	CD33+CD3-CD19-HLA_DR-CD11b+	0.632	0.527	1.555	0.125	0.595	0.552	1.626	0.104	7.121	0.028
subset11	CD3-CD19-HLA_DR-	-0.738	0.461	0.391	0.696	-0.909	0.393	-1.739	0.072	3.828	0.147

Negative as control Negative as control Poorly differentiation as control Pancreatic head/uncinate as control Resectable pancreatic cancer as control

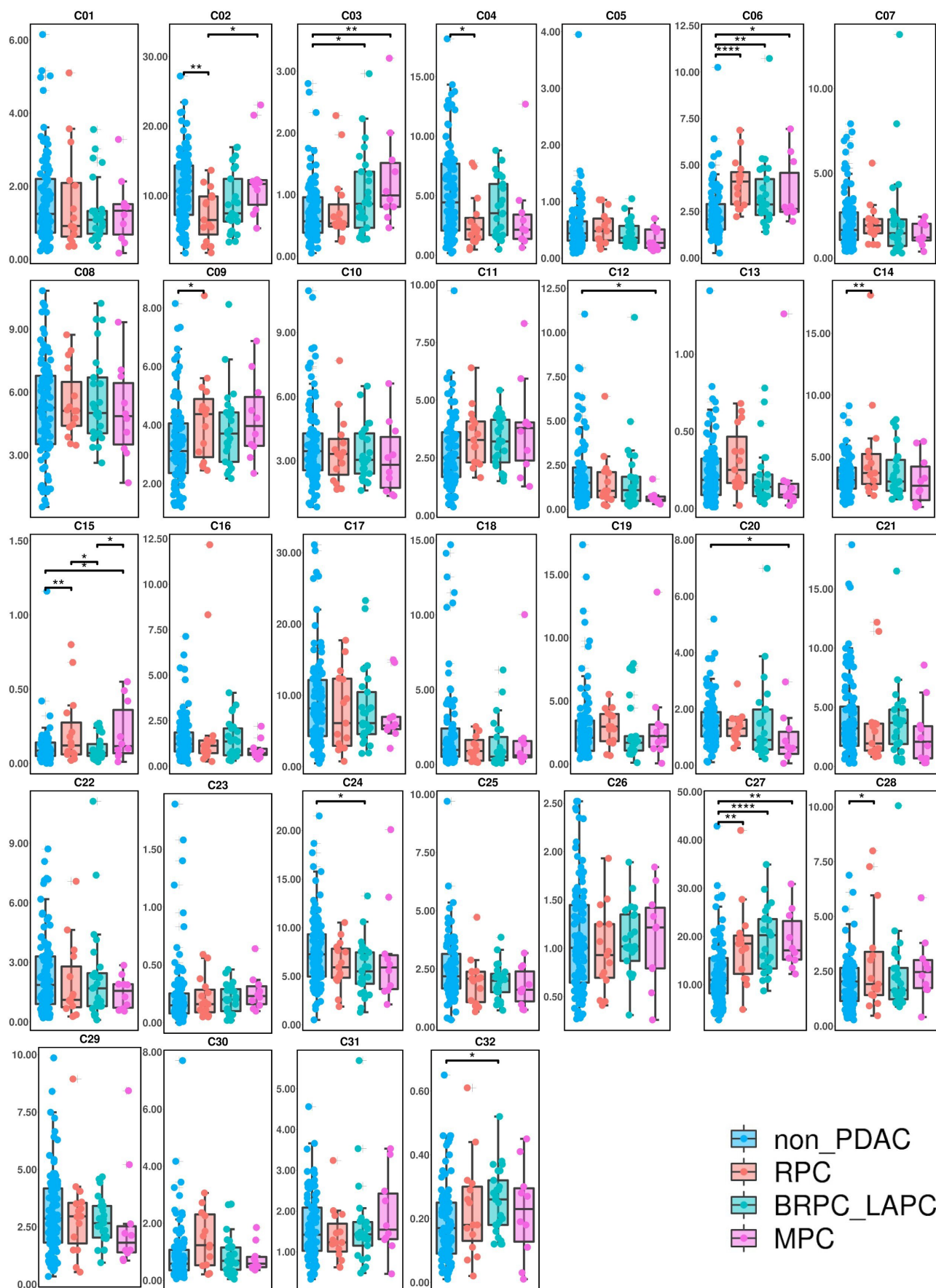


Frequency (%)

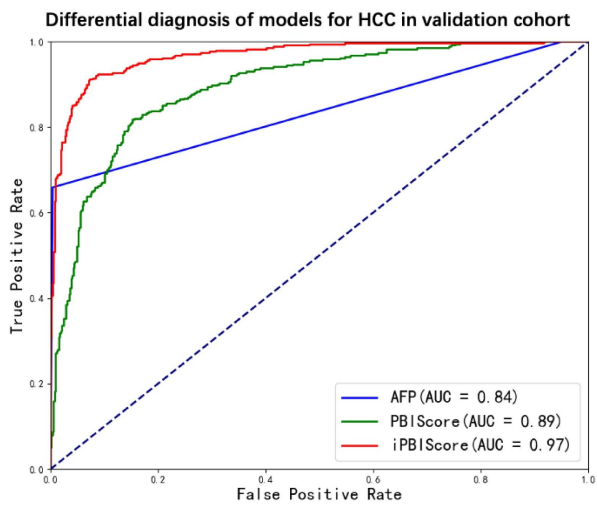




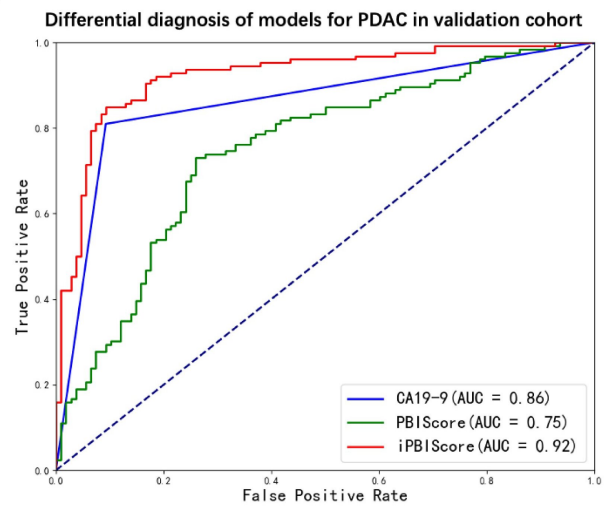
Frequency (%)

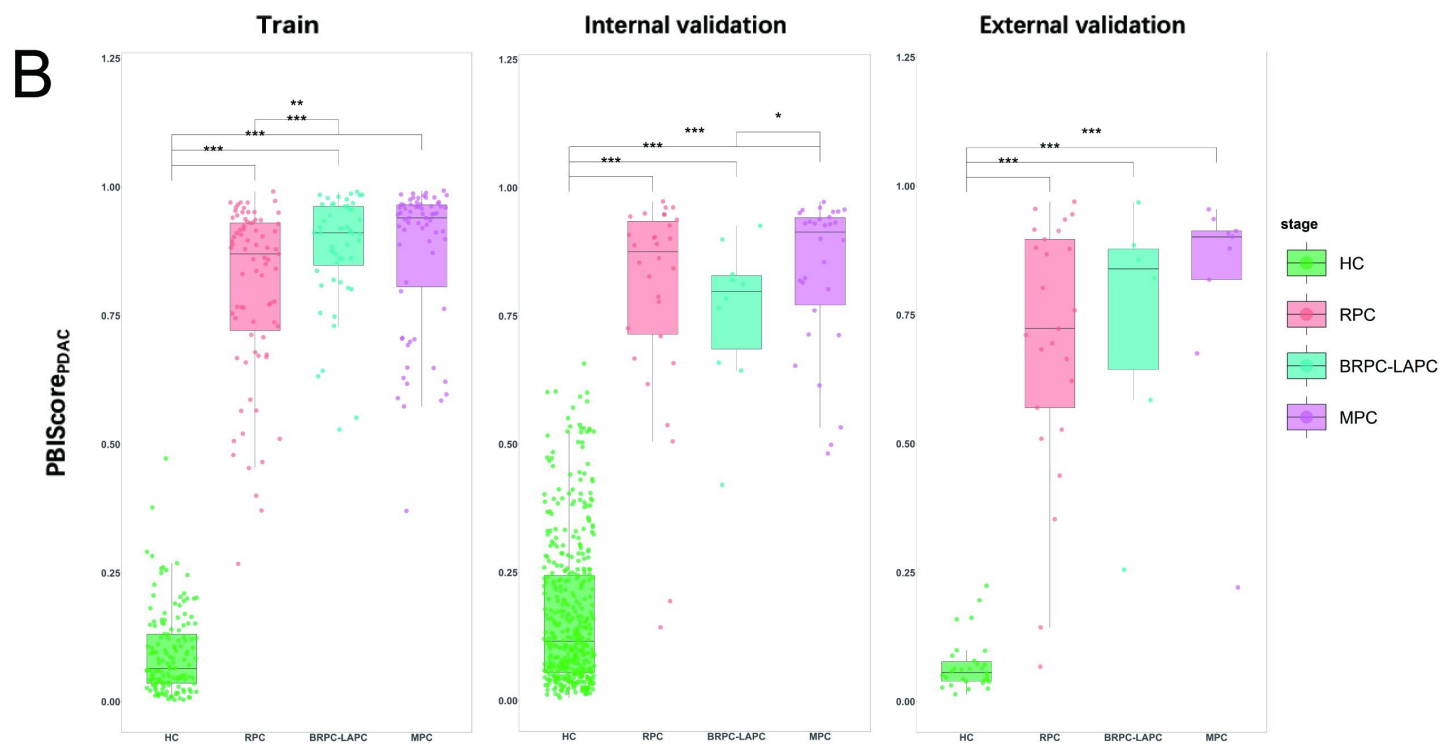
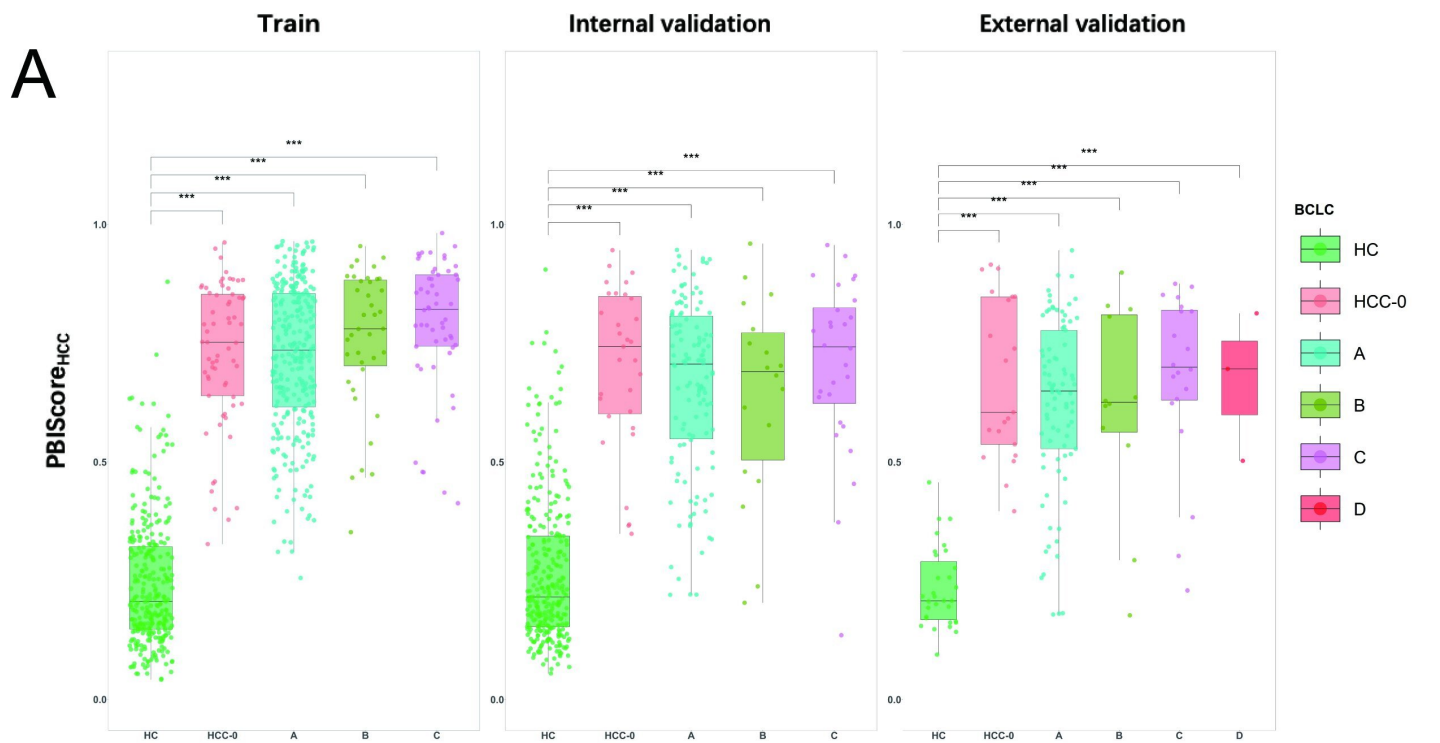


A



B





Supplementary tables**Supplementary Table S1. Centers for external validation.**

Center	Location
Zhejiang Provincial People's Hospital	Hangzhou, Zhejiang
The First Affiliated Hospital, Wenzhou University School of Medicine	Wenzhou, Zhejiang
Zhejiang Cancer Hospital	Hangzhou, Zhejiang
Sanmen People's Hospital	Taizhou, Zhejiang
Huzhou Central Hospital	Huzhou, Zhejiang
Changxing People's Hospital	Huzhou, Zhejiang
Ningbo Medical Center Lihuili Eastern Hospital	Ningbo, Zhejiang
Shengzhou People's Hospital	Shaoxing, Zhejiang
Shangyu Hospital of Chinese Medicine	Shaoxing, Zhejiang
Haining People's Hospital	Jiaxing, Zhejiang
Jiaxing Second People's Hospital	Jiaxing, Zhejiang
The Second Affiliated Hospital of Nanchang University	Nanchang, Jiangxi
The Second Hospital of Hebei Medical University	Shijiazhuang, Hebei
Jixi County People's Hospital	Xuancheng, Anhui

Supplementary Table S2. The antibody panel for CyTOF.

Label	Marker	Clone	Vendor
89Y	CD45	HI30	BioLegend
115In	CD3	UCHT1	Bioxcell
141Pr	CD56	NCAM16.2	BD
142Nd	TCR γ/δ	5A6.E9	Invitrogen
143Nd	CD196 (CCR6)	G034E3	BioLegend
144Nd	CD14	M5E2	BioLegend
145Nd	IgD	IA6-2	BioLegend
146Nd	CD123 (IL-3R α)	6H6	BioLegend
147Sm	CD85j (ILT2)	GHI/75	BioLegend
148Nd	CD19	HIB19	BioLegend
149Sm	CD25 (IL-2R α)	24212	RD
150Nd	CD274 (PD-L1)	29E.2A3	BioLegend
151Eu	CD278 (ICOS)	C398.4A	BioLegend
152Sm	CD39	A1	BioLegend
153Eu	CD27	O323	BioLegend
154Sm	CD24	ML5	BioLegend
155Gd	CD45RA	HI100	BioLegend
156Gd	CD86	Fun-1	BD
157Gd	CD28	CD28.2	BioLegend
158Gd	CD197 (CCR7)	G043H7	BioLegend
159Tb	CD11c	BU15	BioLegend
160Gd	CD33	WM53	BioLegend
161Dy	CD152 (CTLA-4)	14D3	eBioscience
163Dy	CD161	HP-3G10	BioLegend
164Dy	CD185 (CXCR5)	RF8B2	BD

165Ho	CD66b	G10F5	BioLegend
166Er	CD183 (CXCR3)	G025H7	BioLegend
167Er	CD94	HP-3D9	BD
168Er	CD57	HNK-1	BioLegend
169Tm	CD45RO	UCHL1	BioLegend
170Er	CD127 (IL-7R α)	A019D5	BioLegend
171Yb	CD279 (PD-1)	EH12.2H7	BioLegend
172Yb	CD38	HIT2	BioLegend
173Yb	CD194 (CCR4)	L291H4	BioLegend
174Yb	CD20	2H7	BioLegend
175Lu	CD16	3G8	BioLegend
176Yb	HLA-DR	L243	BioLegend
197Au	CD4	RPA-T4	BioLegend
198Pt	CD8a	RPA-T8	BioLegend
209Bi	CD11b	M1/70	BioLegend

Supplementary Table S3. The demographic and clinicopathological parameters of the hepatic group

	HCC	Hepatic benign diseases	Healthy controls
Total (n)	790	341	633
Gender			
Male – no. (%)	650 (82.2)	140 (41.1) ***	350 (55.3) ***
Female – no. (%)	140 (17.8)	201 (58.9)	283 (44.7)
Age (years)			
Mean	59.0	49.4 ***	46.4 ***
Range	12-89	12-90	16-89
Hypertension	231 (29.2)	55 (16.1) ***	92 (14.5) ***
Diabetes	117 (14.8)	12 (3.5) ***	47 (7.4) ***
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.5 (2.5 – 7.0)	4.5 (2.7 – 6.1)	4.9 (3.7 – 6.1) ***
Hemoglobin, g/L (range)	141.3 (73 – 192)	138.3 (80 – 180) *	146.4 (100 – 190) ***
White blood cell, 10 ⁹ /L (range)	5.6 (1.2 – 19.5)	6.0 (2.2 – 13.8) **	6.0 (3.3 – 14.8) **
Neutrophil, 10 ⁹ /L (range)	3.6 (0.8 – 17.1)	3.7 (1.0 – 12.2)	3.4 (1.5 – 11.4)
Lymphocyte, 10 ⁹ /L (range)	1.5 (0.28 – 5.5)	1.8 (0.2 – 10.3) ***	2.0 (1.0 – 3.7) ***
Platelet, 10 ⁹ /L (range)	159.1 (24 – 616)	216.0 (44 – 419) ***	236.9 (117 – 600) ***
ALT, U/L (range)	39.1 (3 – 747)	31.6 (4 – 406) *	23.2 (3 – 105) ***
AST, U/L (range)	46.8 (8 – 1232)	29.5 (10 – 384) ***	23.0 (12 – 78) ***
Total bilirubin, µmol/L	18.7 (2.1 – 490.4)	13.5 (3.7 – 49.5) ***	12.3 (3.5 – 35.1)

	(range)		***
Albumin, g/L (range)	42.1 (21.6 – 80.0)	44.3 (25.6 – 54.6) ***	48.0 (41.3 – 54) ***
AFP value			
> 20 ng/ml	508 (64.3)	6 (1.8) ***	1 (0.2) ***
≤ 20 ng/ml	282 (35.7)	335 (98.2)	632 (99.8)
HBV infection			
Positive	611 (77.3)	91 (26.7) ***	32 (5.1) ***
Negative	178 (22.5)	241 (70.7)	601 (94.9)
NA	1 (0.2)	9 (2.6)	0 (0.0)
BCLC stage			
0	117 (14.8)	NA	NA
A	500 (63.3)	NA	NA
B	69 (8.7)	NA	NA
C-D	104 (13.2)	NA	NA
MVI grade			
0	408 (51.6)	NA	NA
1	123 (15.6)	NA	NA
2	30 (3.8)	NA	NA
NA	229 (29.0)	NA	NA
Differentiation			
Low	167 (21.1)	NA	NA
Moderately	339 (42.9)	NA	NA
High	45 (5.7)	NA	NA
Unknown	239 (30.3)	NA	NA

Note: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, Aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; MVI, microvascular invasion; NA, not

available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to HCC group).

Supplementary Table S4. The demographic and clinicopathological parameters of the training set in hepatic group

	HCC	Hepatic benign diseases	Healthy controls
Total (n)	450	150	300
Gender			
Male – no. (%)	305 (67.8)	51 (34.0) ***	168 (56.0) **
Female– no. (%)	145 (32.2)	99 (66.0)	132 (44.0)
Age (years)			
Mean	56.9	48.8 ***	54.2
Range	19-76	18-90	24-89
Hypertension	115 (25.6)	21 (14.0) **	47 (15.7) **
Diabetes	56 (12.4)	3 (2.0) ***	22 (7.3) *
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.6 (2.5 – 7.0)	4.5 (2.9 – 6.1)	4.9 (3.9 – 6.0) ***
Hemoglobin, g/L (range)	143.9 (73 – 192)	136.3 (85 – 180) ***	149.9 (103 – 184) **
White blood cell, 10 ⁹ /L (range)	5.7 (2.3 – 12.7)	6.0 (2.3 – 11.4)	6.1 (3.3 – 14.8)
Neutrophil, 10 ⁹ /L (range)	3.6 (0.9 – 10.6)	3.8 (1.0 – 10.7)	3.5 (1.5 – 10.1)
Lymphocyte, 10 ⁹ /L	1.6 (0.4 – 4.1)	1.8 (0.2 – 10.3) **	2.0 (1.0 – 3.7) ***

(range)			
Platelet, 10 ⁹ /L (range)	162.4 (25 – 616)	221.2 (82 – 418) ***	229.0 (117 – 600) ***
ALT, U/L (range)	37.6 (3 – 308)	30.0 (5 – 331) **	24.0 (6 – 78) **
AST, U/L (range)	43.0 (11 – 590)	28.2 (10 – 374) **	22.4 (14 – 40) **
Total bilirubin, µmol/L (range)	18.9 (2.1 – 490.4)	11.6 (3.7 – 34.8) ***	13.4 (3.5 – 35.1)
Albumin, g/L (range)	43.0 (23.6 – 80.0)	44.2 (25.6 – 51.7) **	47.4 (41.3 – 53.5) ***
AFP value			
> 20 ng/ml	284 (63.1)	4 (2.7) ***	0 (0.0) ***
≤ 20 ng/ml	166 (36.9)	146 (97.3)	300 (100.0)
HBV infection			
Positive	362 (80.4)	19 (12.7) ***	22 (7.3) ***
Negative	87 (19.3)	131 (87.3)	278 (92.7)
Unknown	1 (0.3)	0 (0.0)	0 (0.0)
BCLC stage			
0	65 (14.4)	NA	NA
A	292 (64.9)	NA	NA
B	39 (8.7)	NA	NA

C-D	54 (12.0)	NA	NA
MVI grade			
0	244 (54.2)	NA	NA
1	69 (15.3)	NA	NA
2	16 (3.6)	NA	NA
NA	121 (26.9)	NA	NA
Differentiation			
Low	100 (22.2)	NA	NA
Moderately	206 (45.8)	NA	NA
High	16 (3.6)	NA	NA
Unknown	128 (28.4)	NA	NA

Note: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, Aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; MVI, microvascular invasion; NA, not available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to HCC group).

Supplementary Table S5. The demographic and clinicopathological parameters of the pancreatic group

	PDAC	Pancreatic benign diseases	Healthy controls
Total (n)	376	208	632
Gender			
Male – no. (%)	221 (58.8)	81 (38.9) ***	349 (55.2)
Female – no. (%)	155 (41.2)	127 (61.1)	283 (44.8)
Age (years)			
Mean	64.3	53.2 ***	46.5 ***
Range	22-88	13-82	16-89
Hypertension	133 (35.4)	61 (29.3)	102 (16.1) *
Diabetes	78 (20.7)	22 (10.6) **	47 (7.4) ***
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.3 (2.0 – 5.9)	4.4 (2.8 – 6.3) **	4.9 (3.7 – 6.13) ***
Hemoglobin, g/L (range)	130.6 (57 – 173)	134.3 (84 – 179) **	146.4 (100 – 190) ***
White blood cell, 10 ⁹ /L (range)	6.4 (2.5 – 19.5)	6.4 (2.6 - 17.8)	6.0 (3.3 – 14.8) *
Neutrophil, 10 ⁹ /L (range)	4.5 (1.4 – 17.7)	4.3 (1.2 – 17.0)	3.4 (1.5 – 11.4) ***
Lymphocyte, 10 ⁹ /L (range)	1.3 (0.3 – 3.3)	1.6 (0.3 – 3.2) ***	2.0 (1.0 – 3.7) ***
Platelet, 10 ⁹ /L (range)	212.6 (55 – 541)	226.8 (70-801) **	236.9 (117 – 600) ***
ALT, U/L (range)	97.7 (2 – 1403)	37.1 (5 – 1226) ***	23.2 (3 – 105) ***
AST, U/L (range)	66.5 (8 – 619)	31.3 (9 – 1112) ***	22.9 (12 – 78) ***
Total bilirubin, µmol/L	59.0 (3.0 – 547.6)	16.9 (3.0 – 390.1) ***	12.3 (3.5 – 35.1) ***

(range)			
Albumin, g/L (range)	41.6 (25.0 – 52.2)	43.5 (27.7 - 54.1) ***	48.0 (41.3 – 54) ***
CA19-9 value			
> 37 U/ml	293 (77.9)	24 (11.5) ***	5 (0.8) ***
≤ 37 U/ml	83 (22.1)	184 (88.5)	627 (99.2)
Resectability			
RPC	134 (35.6)	NA	NA
BRPC-LAPC	136 (36.2)	NA	NA
MPC	106 (28.2)	NA	NA
Differentiation			
Low	53 (14.1)	NA	NA
Moderately	54 (14.4)	NA	NA
High	2 (0.5)	NA	NA
Unknown	267 (71.0)	NA	NA

Note: ALT, alanine aminotransferase; AST, Aspartate transaminase; BRPC, borderline resectable pancreatic cancer; CA19-9, carcinoma antigen 19-9; LAPC, locally advanced pancreatic cancer; MPC, metastatic pancreatic cancer; NA, not available; RPC, resectable pancreatic cancer. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to PDAC group).

Supplementary Table S6. The demographic and clinicopathological parameters of the training set in pancreatic group

	PDAC	Pancreatic benign diseases	Healthy controls
Total (n)	250	100	150
Gender			
Male– no. (%)	146 (58.4)	35 (35.0) ***	83 (55.3)
Female – no. (%)	104 (41.6)	65 (65.0)	67 (44.7)
Age (years)			
Mean	63.5	54.7 ***	60.6
Range	22-88	13-82	30-89
Hypertension	86 (34.4)	33 (33.0)	23 (15.3) ***
Diabetes	49 (19.6)	11 (11.0)	19 (12.7)
Laboratory tests (mean)			
Red blood cell, 10 ¹² /L (range)	4.3 (2.3 – 5.9)	4.4 (2.8 – 5.4)	4.9 (4.2 – 5.9) ***
Hemoglobin, g/L (range)	131.6 (57 – 173)	134.5 (90 – 173)	148.7 (115 – 170) ***
White blood cell, 10 ⁹ /L (range)	6.3 (2.5 – 19.5)	6.7 (3.4 - 17.8)	6.1 (3.8 – 8.6)
Neutrophil, 10 ⁹ /L (range)	4.4 (1.4 – 17.7)	4.6 (1.2 – 17.0)	3.5 (2.1 – 5.5) ***

Lymphocyte, 10 ⁹ /L (range)	1.3 (0.3 – 3.3)	1.6 (0.3 – 3.2) ***	2.0 (1.0 – 3.3) ***
Platelet, 10 ⁹ /L (range)	211.2 (55 – 506)	227.8 (70 - 480) **	227.0 (119 – 318)
ALT, U/L (range)	96.0 (5 – 1403)	37.2 (5 – 921) ***	21.3 (6 – 105) ***
AST, U/L (range)	65.8 (8 – 619)	35.5 (9 – 1112) *	25.5 (14 – 78) ***
Total bilirubin, µmol/L (range)	59.7 (3.0 – 547.6)	18.6 (3.0 – 390.1) ***	12.3 (4.6 – 30.4) ***
Albumin, g/L (range)	42.1 (25.8 – 52.2)	43.5 (28.8 - 52.4) *	46.9 (41.3 – 53) ***
CA19-9 value			
> 37 U/ml	194 (77.6)	14 (14.0) ***	0 (0.0) ***
≤ 37 U/ml	56 (22.4)	86 (86.0)	150 (100.0)
Resectability			
RPC	79 (31.6)	NA	NA
BRPC-LAPC	104 (41.6)	NA	NA
MPC	67 (26.8)	NA	NA
Differentiation			
Low	31 (12.4)	NA	NA
Moderately	31 (12.4)	NA	NA
High	0 (0.0)	NA	NA
Unknown	188 (75.2)	NA	NA

Note: NA, not available. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to PDAC group).

Supplementary Table S7 Markers and cell subsets used for HCC detection model construction

Markers or cell subsets	Feature importance	SE	Z-score	P-value
AFP	0.353122325856825	0.454	-36.552	<0.001
CD14	0.0266157507901591	6.515	-13.932	<0.001
CD152	0.0165493598488722	1.751	8.584	<0.001
CD25	0.0268740558987101	0.864	-5.537	<0.001
CD33	0.0307218964267744	8.573	-11.071	<0.001
CD45RA	0.0243438245610579	12.255	9.352	<0.001
CD85J	0.0320794521882959	6.593	-9.068	<0.001
CD86	0.0462854483540234	5.865	-15.543	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ HLA-DR ⁻ CD38 ⁻	0.0437688704892163	12.243	14.821	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁻ CD19 ⁺ IgD ⁺ CD27 ⁺	0.0444513473213629	5.56	-7.758	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ CD197 ⁺ CD45RA ⁺	0.026710817192894	11.142	12.027	<0.001
CD3 ⁻ CD19 ⁻ HLA-DR ⁻	0.0196247275687274	12.876	-9.79	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺	0.0221718539244221	8.508	11.146	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA- DR ⁺ CD8a ⁺	0.0141180611540897	3.008	7.652	<0.001
CD33 ⁻ CD14 ⁻	0.0225940563969961	8.508	-11.146	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ CD85j ⁻	0.0193691230085965	5.108	6.13	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ CXCR5 ⁺	0.0274583522068538	2.222	6.06	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA-DR ⁻ CD4 ⁺	0.044259520077781	12.346	-15.714	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺	0.0216413407150223	9.844	-8.875	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ CD45RA ⁺ CD197 ⁻	0.0307140730725735	2.371	-5.588	<0.001

CD33 ⁻ CD14 ⁻ CD3 ⁺ CD45RA ⁻ CD197 ⁺ CD8a ⁺	0.0254454238922522	3.114	9.14	<0.001
CD33 ⁺ CD3 ⁻ CD19 ⁻ HLA-DR ⁻ CD11b ⁺	0.0401007387170425	10.059	11.259	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺ CD16 ⁺	0.0255826514290803	6.768	3.488	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺	0.0153969289083717	11.627	8.775	<0.001

Supplementary Table S8 Markers and cell subsets used for PDAC detection model construction

Markers or cell subsets	Feature importance	SE	Z-score	P-value
CA19-9	0.447075072880982	0.496	-33.442	<0.001
CD86	0.0251255615948677	6.784	-12.562	<0.001
CD14	0.0314453971496788	7.04	-12.226	<0.001
CD33	0.04899000925121	9.15	-10.64	<0.001
CD4	0.0200205290640094	10.237	-3.816	<0.001
CD11b	0.0126628230877706	14.403	-4.4	<0.001
CD183	0.0100134741809992	8.382	8.895	<0.001
CD25	0.0447355631765113	0.86	-3.794	<0.001
CD152	0.017225333770737	1.331	5.815	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺	0.0199715291594582	11.571	9.064	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD38 ⁺ HLA-DR ⁺ CD8a ⁺	0.0459849642348083	5.01	9.34	<0.001
CD33 ⁻ CD14 ⁻	0.0451007438245495	9.129	-10.805	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD85j ⁺ CD8a ⁺	0.0309372394802058	4.554	-6.084	<0.001
CD3 ⁻ CD19 ⁻ CD14 ⁺	0.0422505171663183	9.129	10.805	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺ HLA-DR ⁺	0.018914250326465	6.461	8.945	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD8a ⁺	0.0172440965501883	9.791	-7.722	<0.001
CD33 ⁻ CD14 ⁻ CD3 ⁺ CD4 ⁺ HLA-DR ⁺ CD38 ⁺	0.0561160245457125	1.142	9.476	<0.001
CD33 ⁻ CD14 ⁻				
CD3 ⁺ CD4 ⁺ CD25 ⁺ CD45RA ⁺ CD161 ⁻ CD127 ⁻	0.0130382951243638	7.939	-5.697	<0.001
CD33 ⁺ CD3 ⁻ CD19 ⁻ HLA-DR ⁻ CD11b ⁺	0.0326018440406268	11.173	7.482	<0.001
CD3 ⁻ CD19 ⁻ HLA-DR ⁻	0.0205467313905376	13.054	-9.008	<0.001

Supplementary Table S9. Differences in key cell subsets of HCC with certain clinicopathological parameters

	Cell subsets	AFP		Differentiation Degree		HBV		MVI		BCLC stage	
		t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value
subset1	CD33-CD14-CD3+CD4+HLA_DR-CD38-	1.376	0.169	5.311	0.07	0.329	0.742	1.553	0.46	2.579	0.461
subset2	CD33-CD14-CD3-CD19+IGD+CD27+	-0.411	0.681	0.963	0.618	1.343	0.179	1.331	0.514	6.882	0.076
subset3	CD33-CD14-CD3+CD4+CD197+CD45RA+	0.435	0.663	3.252	0.197	0.133	0.894	0.108	0.947	3.615	0.306
subset4	CD3-CD19-HLA_DR-	0.333	0.739	0.346	0.841	-0.277	0.782	1.235	0.539	7.752	0.051
subset5	CD3-CD19-CD14+	-1.634	0.102	1.916	0.384	2.384	0.017	0.075	0.963	32.23	<0.001
subset6	CD33-CD14-CD3+CD38+HLA_DR+CD8a+	-0.555	0.579	4.416	0.11	2.438	0.015	0.1	0.951	17.525	<0.001
subset7	CD33-CD14-	1.634	0.102	1.916	0.384	-2.384	0.017	0.075	0.963	32.23	<0.001
subset8	CD33-CD14-CD3+CD8a+CD85j-	1.798	0.072	0.848	0.655	1.398	0.162	0.605	0.739	33.67	<0.001
subset9	CD33-CD14-CD3+CD8a+CXCR5+PD_L1+	-0.264	0.792	0.135	0.935	0.758	0.449	3.379	0.185	9.501	0.023
subset10	CD33-CD14-CD3+CD38+HLA_DR-CD4+	-1.119	0.263	4.039	0.133	-0.688	0.491	1.01	0.603	1.897	0.594
subset11	CD33-CD14-CD3+CD8a+	-0.538	0.591	0.13	0.937	0.755	0.45	3.337	0.189	1.645	0.649
subset12	CD33-CD14-CD3+CD4+CD45RA+CD197-	0.175	0.861	1.348	0.51	-0.166	0.868	0.642	0.725	3.081	0.379
subset13	CD33-CD14-CD3+CD45RA-CD197+CD8a+	-2.383	0.017	6.226	0.044	-0.579	0.563	0.786	0.675	8.024	0.046
subset14	CD33+CD3-CD19-HLA_DR-CD11b+	-0.534	0.594	0.613	0.736	-0.255	0.799	0.005	0.997	21.181	<0.001
subset15	CD3-CD19-CD14+CD16+	-2.788	0.005	0.116	0.944	-0.639	0.523	0.048	0.976	1.368	0.713
subset16	CD33-CD14-CD3+CD4+	0.39	0.697	0.248	0.883	1.473	0.141	1.38	0.502	0.754	0.861
		Negative as control		Poorly differentiation control		Negative as control		M0 as control		Stage 0 as control	

Supplementary Table S10. Differences in key cell subsets of PDAC with certain clinicopathological parameters

Cell subsets	CA199		Diabetes		Differentiation degree		Tumor location		Disease stage		
	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	
subset1	CD33-CD14-CD3+CD4+	-0.935	0.35	-0.821	0.535	-0.412	0.681	-1.461	0.144	1.026	0.599
subset2	CD33-CD14-CD3+CD38+HLA_DR+CD8+	0.17	0.965	-1.089	0.276	0.938	0.37	2.919	0.005	1.05	0.592
subset3	CD33-CD14-	-0.105	0.916	-1.268	0.205	-1.551	0.121	-1.128	0.259	18.941	<0.001
subset4	CD33-CD14-CD3+CD85+CD8+	-0.436	0.663	-1.112	0.266	-0.57	0.569	-1.33	0.183	0.183	0.912
subset5	CD3-CD19-CD14+	0.105	0.916	1.268	0.205	1.551	0.121	1.128	0.259	18.941	<0.001
subset6	CD33-CD14-CD3+CD8+HLA_DR+	0.139	0.889	-1.555	0.12	0.663	0.507	2.627	0.009	1.126	0.57
subset7	CD33-CD14-CD3+CD8+	0.898	0.369	-0.306	0.759	0.357	0.721	1.874	0.061	0.761	0.684
subset8	CD33-CD14-CD3+CD4+HLA_DR+CD38+	-0.72	0.472	-0.063	0.946	0.657	0.511	2.101	0.035	6.973	0.018
subset9	CD33-CD14-CD3+CD4+CD25+CD45RA+CD161-CD127-	1.299	0.194	-1.29	0.2	-1.37	0.171	0.02	0.984	7.072	0.029
subset10	CD33+CD3-CD19-HLA_DR-CD11b+	0.632	0.527	1.555	0.125	0.595	0.552	1.626	0.104	7.121	0.028
subset11	CD3-CD19-HLA_DR-	-0.738	0.461	0.391	0.696	-0.909	0.363	-1.739	0.072	3.828	0.147

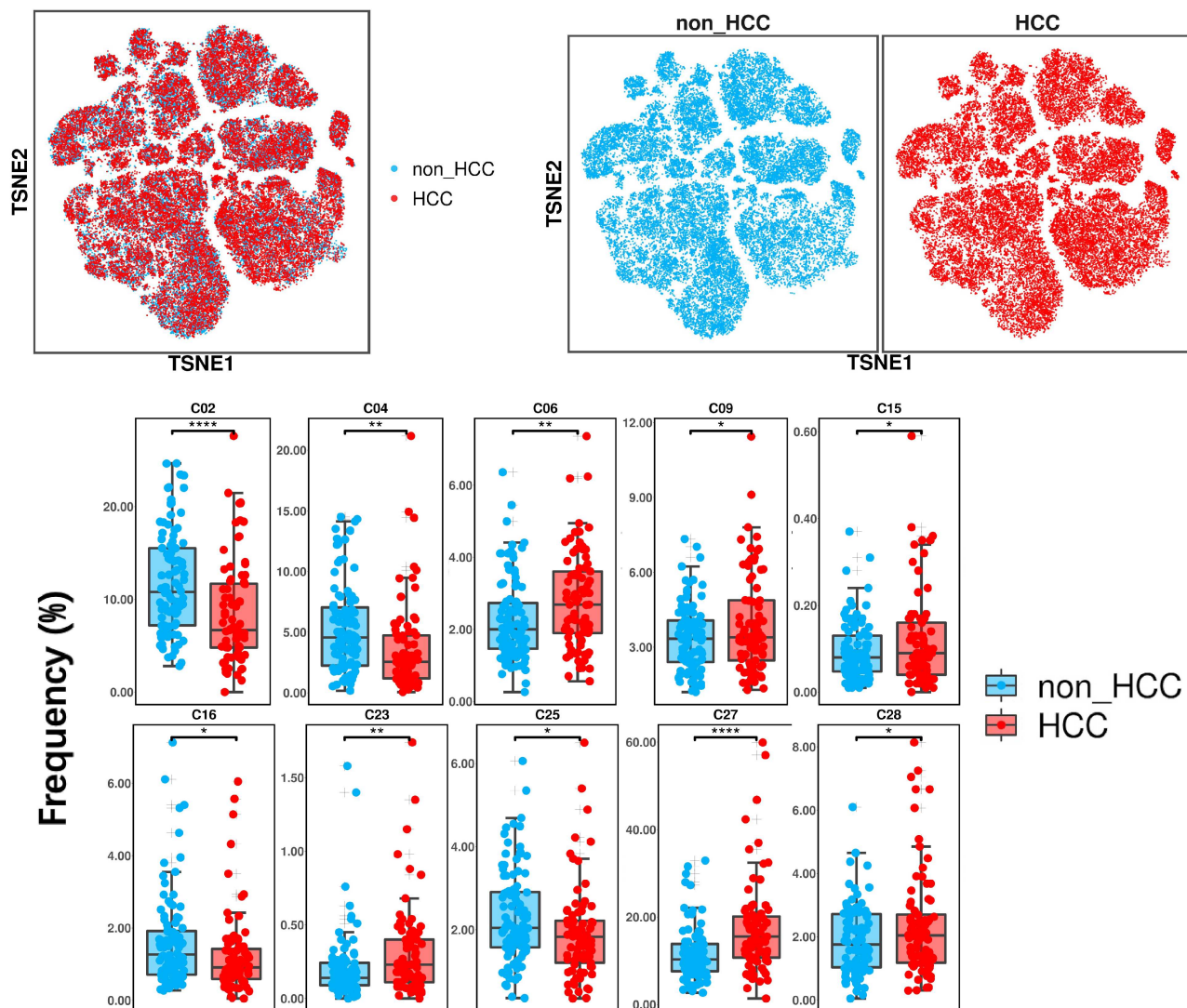
Negative as control

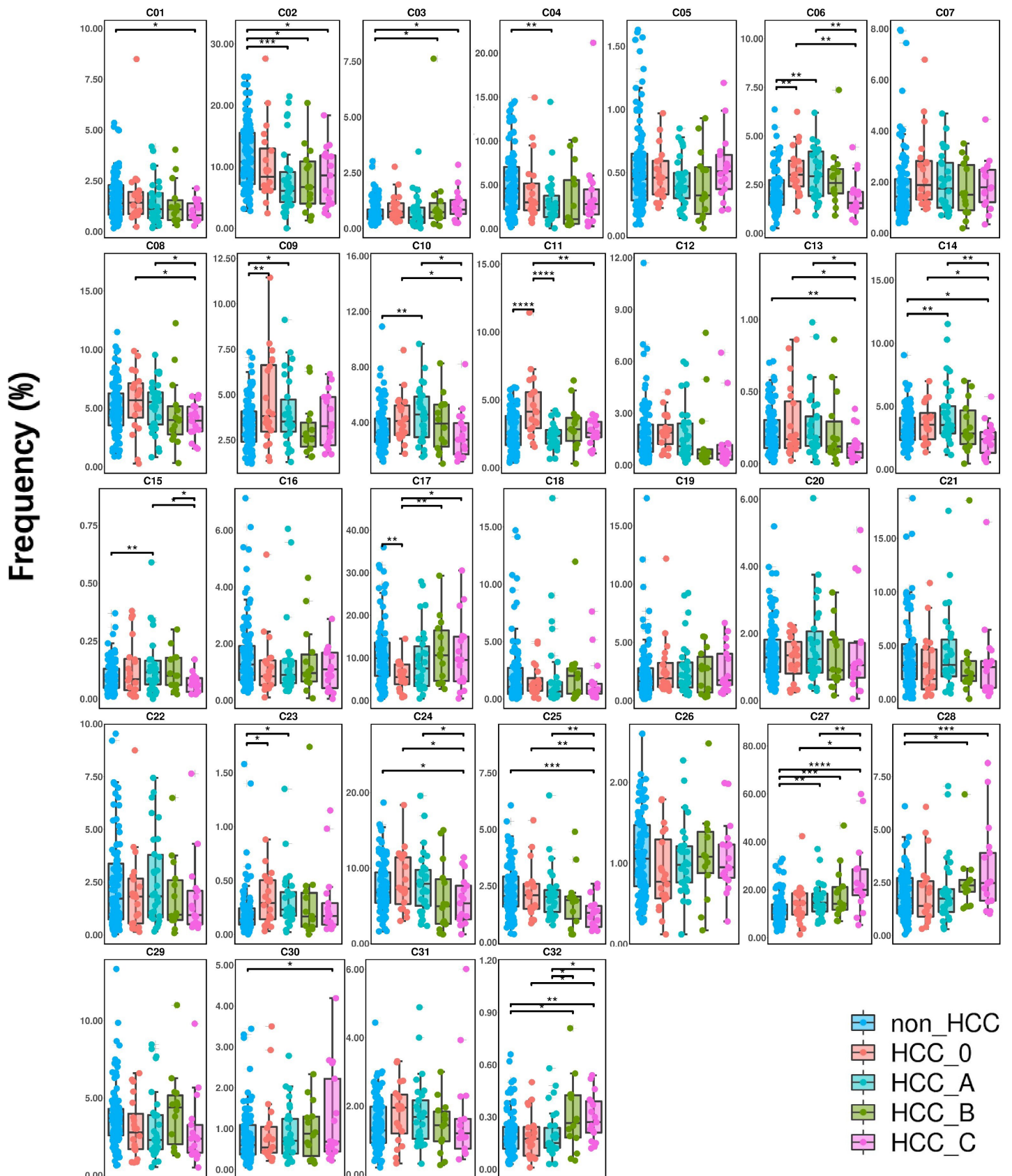
Negative as control

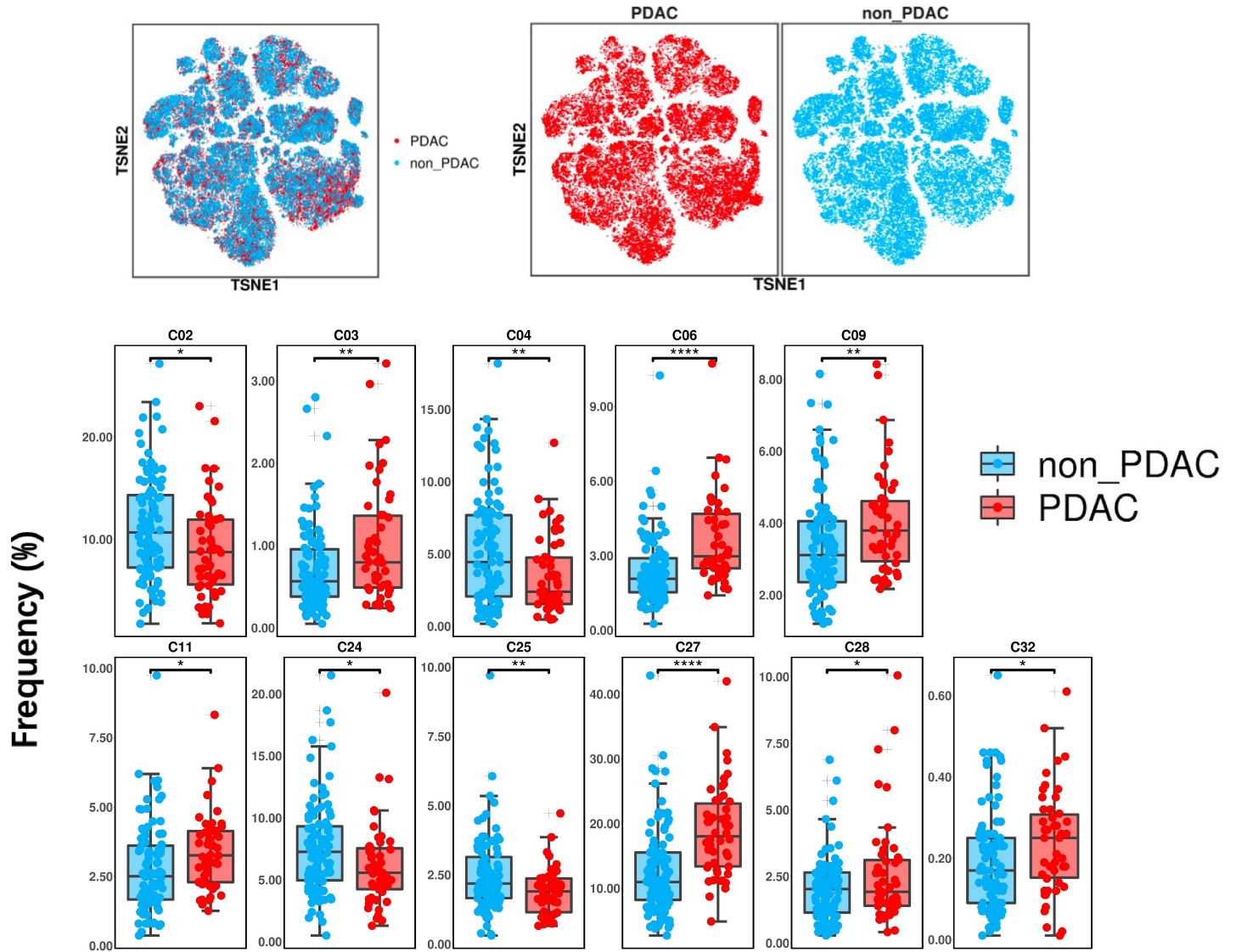
Poorly differentiation as control

Pancreatic head/uncinate as control

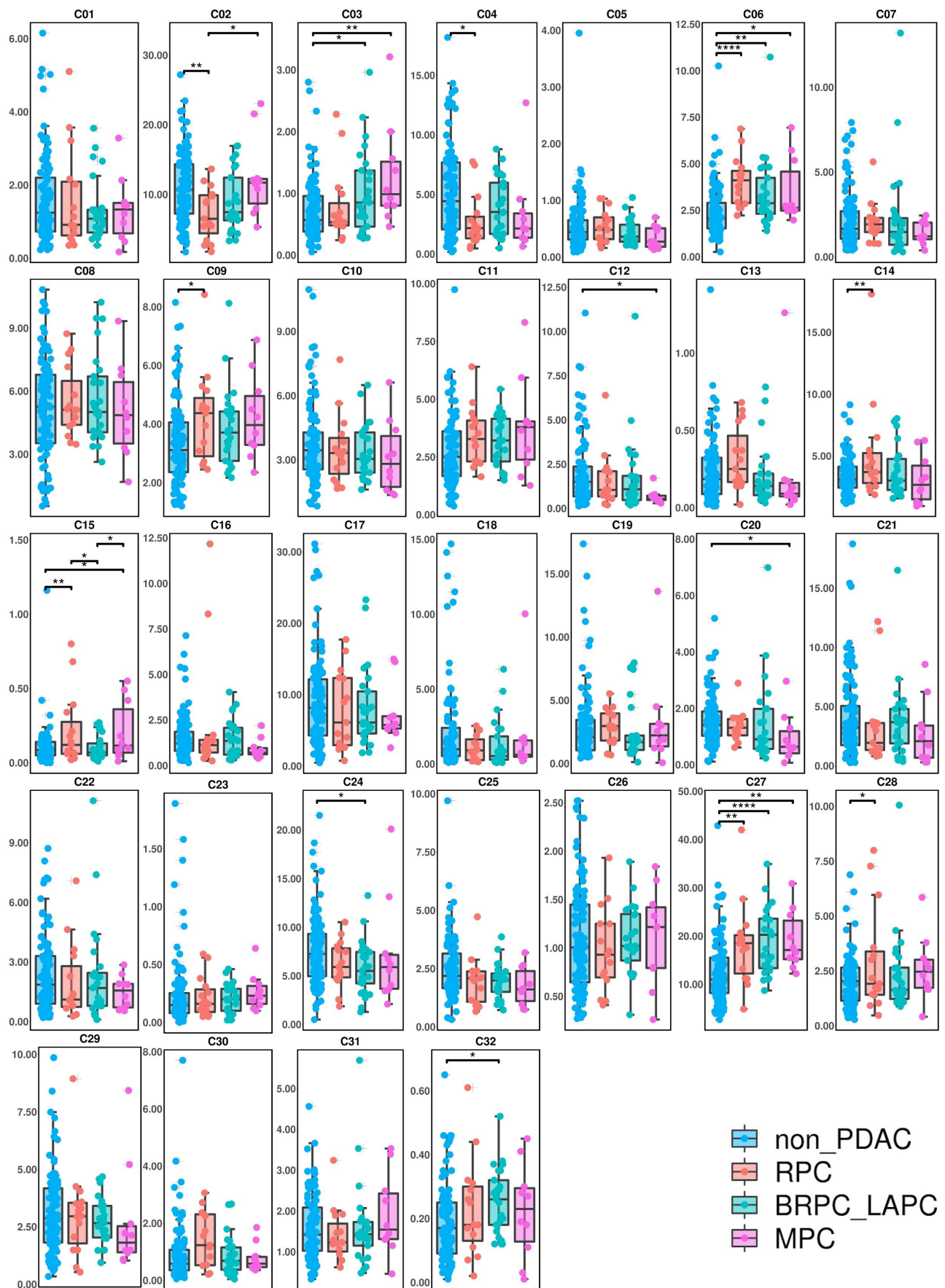
Resectable pancreatic cancer as control



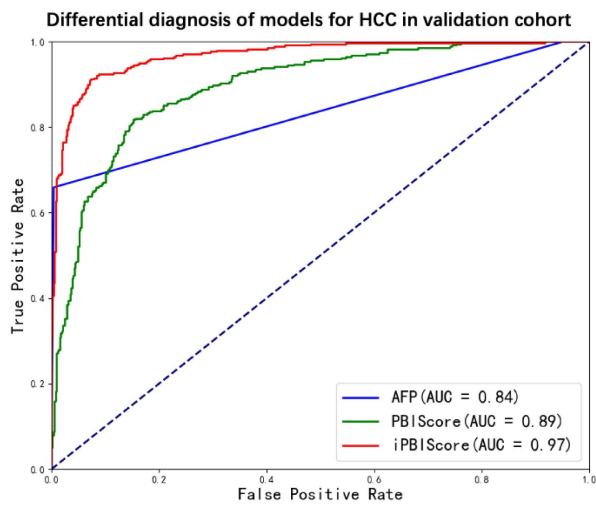




Frequency (%)



A



B

