

ScRNA-seq of Diverse Pheochromocytoma Patients Reveals Distinct Microenvironment Characteristics and Supports an Informative Molecular Classification System

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Abstract

Pheochromocytomas (PCCs) are rare neuroendocrine tumors that originate from chromaffin cells in the adrenal gland. However, the cellular molecular characteristics and immune microenvironment of PCCs are incompletely understood. Here, we performed single-cell RNA sequencing (scRNA-seq) on 16 tissues from 4 sporadic unclassified PCC patients and 1 hereditary PCC patient with Von Hippel-Lindau (VHL) syndrome. We found that intra-tumoral heterogeneity was less extensive than the inter-individual heterogeneity of PCCs. Further, the unclassified PCC patients were divided into two types, metabolism-type (marked by *NDUFA4L2* and *COX4I2*) and kinase-type (marked by *RET* and *PNMT*), validated by immunohistochemical staining. Trajectory analysis of tumor evolution revealed that metabolism-type PCC cells display phenotype of consistently active metabolism and increased metastasis potential, while kinase-type PCC cells showed decreased epinephrine synthesis and neuron-like phenotypes. Cell-cell communication analysis showed activation of the annexin pathway and a strong inflammation reaction in metabolism-type PCCs and activation of FGF signaling in the kinase-type PCC. Although multispectral immunofluorescence staining showed a lack of CD8⁺ T cell infiltration in both metabolism-type and kinase-type PCCs, only the kinase-type PCC exhibited downregulation of *HLA-I* molecules that possibly regulated by *RET*, suggesting the potential of combined therapy with kinase inhibitors and immunotherapy for kinase-type PCCs; in contrast, the application of immunotherapy to metabolism-type PCCs (with antigen presentation ability) is likely unsuitable. Our study presents a single-cell transcriptomics-based molecular classification and microenvironment characterization of PCCs, providing clues for potential therapeutic strategies to treat PCCs.

eLife assessment

This **valuable** study advances our understanding of the potential therapeutic strategies for the treatment of pheochromocytomas using single-cell transcriptomics. The authors propose a new molecular classification criterion based on the characterization of tumor microenvironmental features, based on **solid** evidence. The work, which could be improved further through delineating the choice of the PASS scoring system, will be of broad interest to clinicians, medical researchers, and scientists working in the field of pheochromocytoma.

Introduction

Pheochromocytomas (PCCs) are rare neuroendocrine tumors that originate from chromaffin cells in the adrenal gland (Fishbein *et al*, 2017 [↗](#); Liu *et al*, 2018 [↗](#); Nölting *et al*, 2022 [↗](#)). PCCs have been shown to display a remarkable diversity of driver alterations, including germline and somatic mutations as well as somatic fusion genes (Favier *et al*, 2015 [↗](#); Pillai *et al*, 2017 [↗](#); Toledo *et al*, 2017 [↗](#)). This diversity is reflected in the current molecular taxonomy of PCCs The Cancer Genome Atlas (TCGA), including for example the pseudo-hypoxic type (germline mutations in *SDHx*, *FH*, and *VHL*, etc.), the kinase-signaling type (germline or somatic mutations in *RET*, *NF1*, *TMEM127*, *MAX*, and *HRAS*, etc.), and the Wnt-signaling type (somatic mutations in *CSDE1* and somatic gene fusions affecting *MAML3*) (Crona *et al*, 2017 [↗](#)). However, 50~60% of PCCs fail to be classified using this system (Lenders *et al*, 2014 [↗](#)).

As understanding of PCC clinicopathological and immunophenotypic characteristics has deepened, the former concept of ‘benign or malignant PCC’ has been abandoned; since 2017, all PCCs are regarded as malignant tumors with metastatic potential according to the WHO pathological classification (Lam, 2017 [↗](#); Mete *et al*, 2022 [↗](#)). There is no single histo-morphological feature indicating the metastasis risk of PCCs; a number of multifactorial systems have been proposed (Kulkarni *et al*, 2016 [↗](#)). A cytomorphometric study reported that extra-adrenal location, coarse nodularity, confluent necrosis, and the absence of hyaline globules are associated with metastasis risk (Lewis, 1971 [↗](#)). Several scoring systems that for example consider vascular invasion, tumor size, diffuse growth, and mitotic activity have been used for risk assessment of metastasis risk of PCCs (Sherwin, 1959 [↗](#); Sisson *et al*, 1984 [↗](#); Symington & Goodall, 1953 [↗](#)). A scoring system—the Pheochromocytoma of the Adrenal gland Scaled Score (PASS)—is weighted for histologic features and has been used to separate tumors with a potential for a biologically aggressive behavior (PASS > or = 4) from tumors with benign lesion (PASS < 4) (Thompson, 2002 [↗](#)).

Immune checkpoint inhibitors (ICIs) have achieved remarkable results in a variety of solid tumors in recent years (Liu *et al*, 2022 [↗](#); Zhang *et al*, 2020 [↗](#)). Currently, there are only 2 clinical trials investigating the efficacy of ICIs for the treatment of metastatic PCCs: one is still in the recruitment stage, and the other showed 43% non-progression rate (NPR) and 0% overall response rate (ORR) in 7 PCC patients (Dai *et al*, 2020 [↗](#); Jimenez *et al*, 2022 [↗](#); Naing *et al*, 2020 [↗](#)). The composition of immune cells in the tumor immune microenvironment is an essential indicator for predicting likely responses to immunotherapy, and serves as the basis of immunotherapy (Liu *et al*, 2023 [↗](#); Liu *et al*, 2022 [↗](#)). Despite the application of immunohistochemical staining to characterize the PCC microenvironment (Calsina *et al*, 2023 [↗](#); Tufton *et al*, 2022 [↗](#)), limited knowledge exists regarding the cell composition and inter-cellular crosstalk within the PCC microenvironment.

In the present study, we performed single-cell RNA sequencing (scRNA-seq) analysis of 11 tumor tissues and 5 adjacent normal adrenal medullary tissues from 4 sporadic PCC patients with unclassified mutations and 1 hereditary PCC patient with Von Hippel-Lindau (VHL) syndrome caused by germline mutation in *VHL*. We found less intra-tumoral heterogeneity than inter-individual heterogeneity of PCCs. For the unclassified PCCs, we distinguished metabolism-type and kinase-type PCCs based on copy number variants (CNVs) and gene expression profile data. These two PCC types also displayed distinct characteristics of tumor evolution and cell-cell communication. Although multispectral immunofluorescence staining showed a lack of CD8⁺ T cell infiltration in both metabolism-type and kinase-type PCCs, only the kinase-type PCC exhibited downregulation of *HLA-I* molecules that possibly regulated by *RET*, suggesting the potential of combined therapy with kinase inhibitors and immunotherapy for kinase-type PCCs, whereas the application of immunotherapy to metabolism-type PCCs (with antigen presentation ability) is likely unsuitable. Our study presents a single-cell transcriptomics-based molecular classification and microenvironment characterization of PCCs and provides clues for potential therapeutic strategies to treat PCCs.

Results

A Landscape View of Cell Composition in PCCs

To characterize the molecular correlates of unclassified PCCs based on known prominent driver alterations, we performed single-cell RNA sequencing (scRNA-seq) on 16 collected specimens from 5 PCC patients (P1-P5). Four of these patients (P1-P4) suffered from sporadic PCCs: whole exome sequencing (WES) of these tumor tissues detected somatic mutations as compared with their adjacent normal adrenal medullary tissues, including in *JAK2*, *ARHGEF39*, *KMT2D*, and *MST1* (Supplemental Table S1); note that none of these fit into the three previously molecularly defined groups in the TCGA molecular taxonomy (Crona *et al.*, 2017). The cohort also included 1 hereditary PCC patient (P5) with Type 2 Von Hippel-Lindau (VHL) syndrome, with a germline missense mutation in *VHL* (Supplemental Table S1).

We collected 1 resected tumor specimen from P1 (P1_T1), 3 specimens from distinct intra-tumoral sites from P2 (P2_T1, P2_T2, and P2_T3) and P5 (P5_T1, P5_T2, and P5_T3), as well as 2 specimens from distinct intra-tumoral sites from P3 (P3_T1 and P3_T2), and P4 (P4_T1 and P4_T2). To enable comparisons, one normal adrenal medullary tissue adjacent to the tumor was collected from each patient (P1_A, P2_A, P3_A, P4_A, and P5_A) (Fig. 1A). The morphology of PCC cells was assessed through hematoxylin-eosin-stained tumor tissues, showing the typical cell arrangement (small alveoli surrounded by fibro-vascular stroma) and characteristic shapes (polygonal or fusiform cells) of PCCs (Lupşan *et al.*, 2016) (Supplemental Fig. S1). Meanwhile, immunocytochemistry staining showed robust expression of Chromogranin A (CGA) in these tumor tissues (Supplemental Fig. S1). These pathological evaluations validate the collected samples as PCC tumor specimens (Mete *et al.*, 2022). Additionally, we utilized the PASS scoring system to assess the histological features and evaluate the metastasis risk of PCC patients (Supplemental Table S2).

The specimens were digested into single cell suspensions, and 3'-scRNA-seq (Chromium Single Cell 3' v3 Libraries) analysis was performed on each sample. After quality control filtering to remove cells with low gene detection and high mitochondrial gene coverage, we retained 133,894 individual cells (Supplemental Fig. S2A-S2C). SCTransform normalization and principal component analysis (PCA) functions of the Seurat suite (Butler *et al.*, 2018; Satija *et al.*, 2015; Stuart *et al.*, 2019) were then employed for unsupervised dimensionality reduction prior to clustering. Uniform Manifold Approximation and Projection (UMAP) was used for visualizing the distinct specimens, patients, and tissue types (Fig. 1B and 1C).

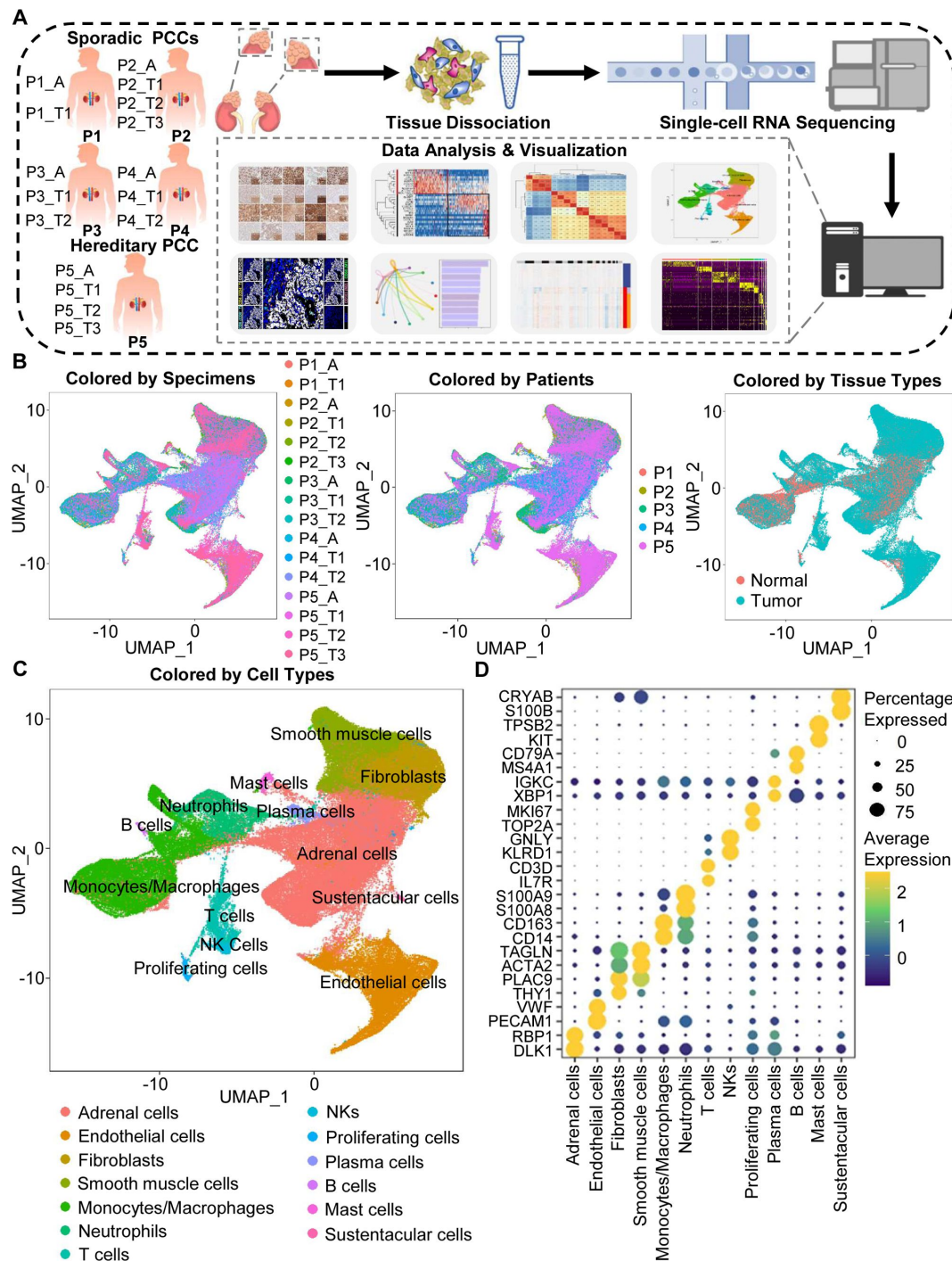


Figure 1.

Integration Analysis across 5 PCC Patients Revealing the Cell Composition of the PCC Microenvironment.

(A) Schematic of the experimental pipeline. 11 tumor specimens and 5 adjacent normal adrenal medullary specimens were isolated from 5 PCC patients, dissociated into single-cell suspensions, and analyzed using 10x Genomics Chromium droplet scRNA-seq. (B) UMAP plots illustrating 133,894 cells from 16 specimens across 5 PCC patients. Cells color-coded by specimens, patients, and tissue types. (C) UMAP plot showing 13 main cell types from all specimens. (D) Dot plot of representative marker genes for each cell type. The color scale represents the average marker gene expression level; dot size represents the percentage of cells expressing a given marker gene.

We classified the 67 detected clusters into 13 cell types according to their expression profiles for recognized marker genes and Spearman correlation analysis between clusters: the classified cell types included adrenal cells (marked by *DLK1* and *RBP1*), endothelial cells (marked by *PECAM1* and *VWF*), fibroblasts (marked by *THY1* and *PLAC9*), smooth muscle cells (marked by *ACTA2* and *TAGLN*), monocytes/macrophages (marked by *CD14* and *CD163*), neutrophils (marked by *S100A8* and *S100A9*), T cells (marked by *IL7R* and *CD3D*), natural killer cells (NKs, marked by *KLRD1* and *GNLY*), proliferating cells (marked by *TOP2A* and *MKI67*), plasma cells (marked by *XBP1* and *IGKC*), B cells (marked by *MS4A1* and *CD79A*), mast cells (marked by *KIT* and *TPSB2*), and sustentacular cells (marked by *S100B* and *CRYAB*) (**Fig. 1C** and **1D**, Supplemental Fig. S2D and S2E). Thus, our clustering and cell type annotation analysis identified diverse adrenal cells, stromal cells, and immune cells (including lymphocytes and myeloid cells) within the PCC microenvironment (Supplemental Fig. S3).

The Intra-tumoral Heterogeneity was Less Extensive than the Inter-individual Heterogeneity of PCCs

In light of the remarkable diversity of drivers including germline and somatic mutations reported among PCCs (Crona *et al.*, 2017), we investigated PCC heterogeneity at the inter-individual and intra-tumoral levels based on the PASS system and single-cell transcriptional profiles. The PASS scores of 11 collected PCC tissues ranged from 2 to 9, showing obvious heterogeneity among the examined intra-tumoral sites and inter-individual comparisons for the 5 PCC patients (Supplemental Table S2). The Spearman correlation analysis based on average gene expression in each specimen showed that the intra-tumoral heterogeneity was less than inter-individual heterogeneity (**Fig. 2A**), inconsistent with the PASS score evaluation. We further analyzed the cell type composition in each specimen and found similar fractions of cell types between the collected intra-tumoral sites despite different PASS scores (**Fig. 2B**), indicating relatively small intra-tumoral differences in both gene expression and cell type composition. These results revealed that scRNA-seq analysis and PASS score represent different levels and dimensions of PCC heterogeneity.

The inter-individual correlation analysis indicated that P4 showed low correlation with the other three sporadic PCCs patients (P1-P3) (**Fig. 2C**), and further analysis of cell type fractions showed a significantly elevated proportion of adrenal cells (77%) in P4; the adrenal cells comprised between 16–36% of the total cells in the P1-P3 specimens (**Fig. 2D**). P4 also had an obviously distinct distribution of stromal cells vs immune cells (9% stromal cells and 14% immune cells) as compared to P1-P3 (28-62% stromal cells and 22-43% immune cells) (**Fig. 2D**). Consistent with the reported vascular endothelial hyperplasia phenotype in VHL syndrome (Vortmeyer *et al.*, 2013), it was unsurprising that we observed poor correlation between the sporadic patients (P1-P4) and the VHL patient (P5), reflecting a higher proportion of endothelial cells in P5 (35%) as compared to P1-P4 (3-10%) (**Fig. 2C** and **2D**).

Identification of Tumor Clusters through CNV analysis

To further characterize PCC cells, we collected adrenal cells, which theoretically include adrenocortical cells and chromaffin cells (Yates *et al.*, 2013). The copy number karyotyping of aneuploid tumors (CopyKAT) algorithm (Gao *et al.*, 2021) was applied, which estimates genomic copy number variants (CNVs) from scRNA-seq data by employing an integrative Bayesian segmentation approach to distinguish PCC cells from normal chromaffin cells. Our analysis identified two clusters among adrenal cells (normal cells and tumor cells), with tumor cells showing extensive chromosomal amplification and/or deletion (**Fig. 3A**). Consistently, the inferred aneuploid tumor cells highly expressed *Chromogranin B* (*CHGB*), which is a marker of PCC cells (Wiedenmann *et al.*, 1988; Winkler & Fischer-Colbrie, 1992) (**Fig. 3B** and **3C**). The inferred diploid normal cells expressing high levels of *CYP17A1* and *HSD3B2* were defined as adrenocortical cells (Kubota-Nakayama *et al.*, 2016), while the inferred diploid normal cells

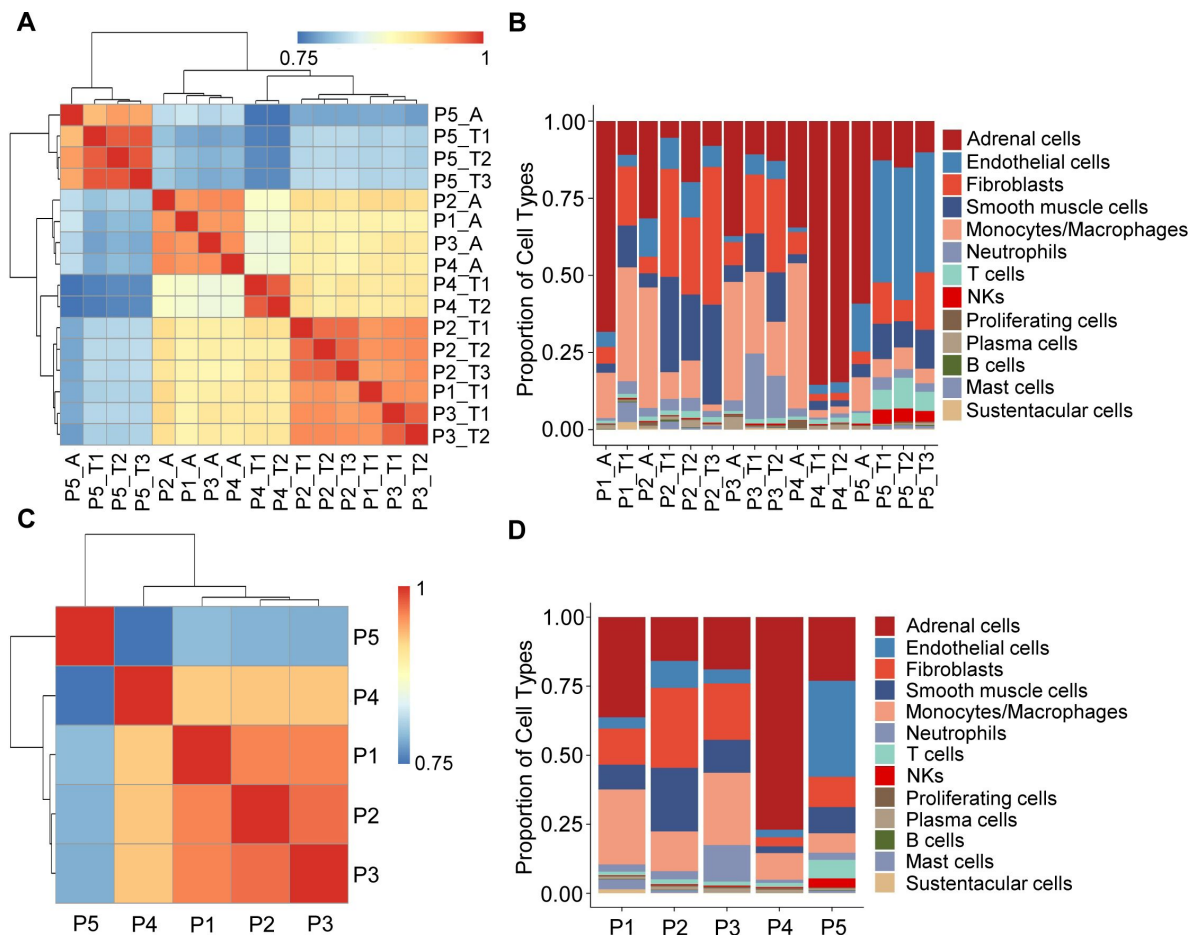


Figure 2.

Correlation Analysis Reveals Less intra-tumoral Heterogeneity than inter-individual Heterogeneity.

(A) Heatmap plotting the correlation coefficient among 16 specimens. The color keys from blue to red indicate the correlation coefficient from low to high. (B) Bar plot showing the percentage of cell types in 16 specimens. (C) Heatmap of the correlation coefficient among 5 PCC patients. The color keys from blue to red indicate the correlation coefficient from low to high. (D) Bar plot depicting the frequency distribution of cell types in 5 PCC patients.

showing low *CHGB* expression were defined as normal chromaffin cells (**Fig. 3B** and **3C**). We also found a cluster of inferred diploid normal cells with high expression of *VWF* (a marker for vascular endothelial cells) in the *VHL* patient (P5) (**Fig. 3B** and **3C**).

We noticed two CNV patterns among the inferred aneuploid tumor cells, with the distinction evident in chromosomes 3, 11, and 17 (**Fig. 3A**). These CNV profiles were reflected in distinct tumor clusters (tumor cluster A and B) through CopyKAT prediction (**Fig. 3D**). We observed that cells in inferred tumor cluster A exhibited high expression of *NDUFA4L2* and *COX4I2*, while cells in inferred tumor cluster B expressed high levels of *PNMT* and *RET* (**Fig. 3D** and **3E**), suggesting the different gene expression between tumor cluster A and B. *PNMT* is known as an enzyme that catalyzes the conversion of norepinephrine to epinephrine and that *RET* is a member of the receptor tyrosine kinase family, while *NDUFA4L2* and *COX4I2* respectively function in the oxidative phosphorylation and glycolysis pathways (Drilon *et al.*, 2018; Mahmoodi *et al.*, 2020; Sinkler *et al.*, 2017). Taken together, we distinguished PCC cells from adrenal cells through CNV analysis and found two clusters of PCC cells with different single-cell copy number profiles.

Definition of Metabolism-type and Kinase-type tumors among unclassified PCCs

To explore the gene expression profile between tumor cluster A and B, we then used the FindAllMarkers function of the Seurat suite (Butler *et al.*, 2018; Satija *et al.*, 2015; Stuart *et al.*, 2019) to identify differentially expressed genes (DEGs) of these tumor clusters (**Fig. 4A** and **4B**). A functional enrichment analysis based on gene ontology (GO) annotation was performed to further characterize the biological function of tumor clusters: the tumor cluster A DEGs showed functional enrichment for terms including generation of metabolites and energy, response to oxygen levels, response to hypoxia, and ATP metabolic process and some notably upregulated genes of tumor cluster A included known energy metabolism-related genes such as *NDUFA4L2*, *COX4I2*, *RGS4*, and *AQP1* (**Fig. 4C**). For tumor cluster B, the GO analysis showed enrichment for terms including cytoplasmic translation, neuron projection development, axon development, and cell growth, and there was obvious upregulation of *PNMT*, *RET*, and genes encoding other kinases known to function in proliferation, as well as genes that participate in the regulation of neuroendocrine functions (*e.g.*, *CALM1*, *PENK*, and *NPY*) (**Fig. 4D**).

Notably, immunocytochemistry staining of serial tumor sections showed strong accumulation of *PNMT* and *RET* in P4, while *NDUFA4L2* and *COX4I2* accumulation was characteristic for the P1-P3 and P5 sections, providing protein-level evidence to validate the differential trends detected from the scRNA-seq results (**Fig. 4E**). As a consequence, we propose a metabolism-type (P1-P3) and kinase-type (P4) classification for unclassified sporadic PCCs. Consistently, kinase-signaling PCCs by TCGA classification include germline and somatic mutations in *RET*, and are uniquely able to secrete epinephrine, owing to their high expression of *PNMT*; P5 could also be classified into the metabolism-type we defined, which is known to display reprogramming of cellular energy metabolism caused by *VHL* mutation and subsequent *HIF* dysregulation (Chappell *et al.*, 2019). When considering clinical information from 5 PCC patients, including tumor size, signs, symptoms and laboratory tests, we observed that P4 exhibited relatively higher blood pressures and the plasma levels of 3-methoxytyramine and normetanephrine compared to P1-P3, and P5 (Supplemental Table S3), consistent with the high expression of *PNMT* in the kinase-type PCC patient (**Fig. 4E**). These results support that the previously unclassified PCCs can be classified as metabolism-type or kinase-type based on transcriptional programs, with these types partially corresponding to the pseudo-hypoxic type and kinase-signaling type of PCCs from the TCGA classification.

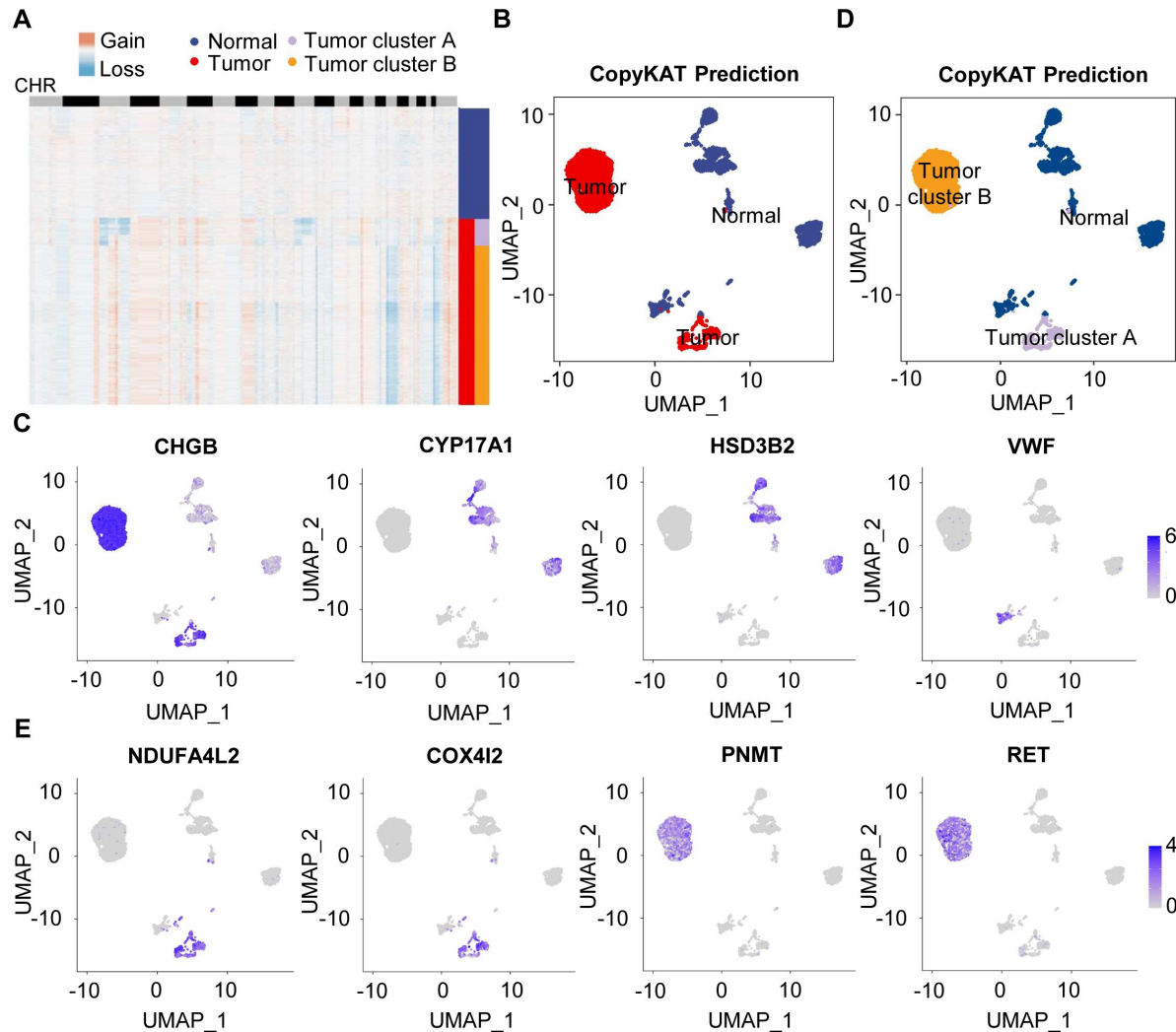


Figure 3.

Single-cell Copy Number Profiles in PCC Clusters Inferred by CopyKAT.

(A) Heatmap indicating the CNV patterns of inferred normal cells, tumor cells, tumor cluster A, and tumor cluster B. Blue, white, and red respectively indicate deletion from a chromosome, normal chromosome, and amplification on a chromosome. (B) UMAP plot of the inferred normal cells (blue) and tumor cells (red) identified by CopyKAT. (C) Feature plots showing the marker gene expression levels in inferred normal cells and tumor cells. (D) UMAP plot depicting the inferred normal cells (blue), tumor cluster A (purple), and tumor cluster B (orange) identified by CopyKAT. (E) Feature plots displaying the marker gene expression levels in inferred tumor cluster A and B.

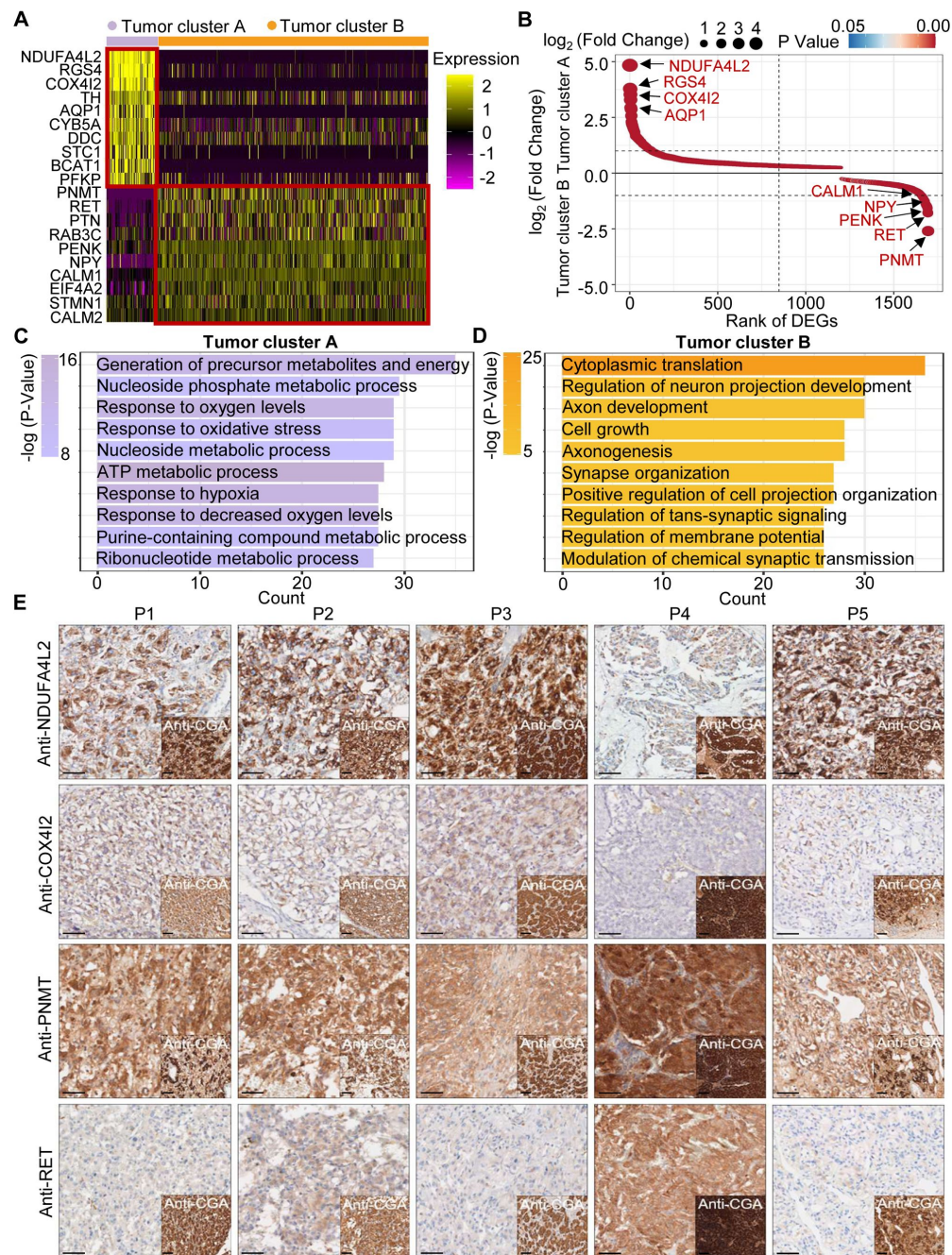


Figure 4.

PCC Patients were Classified into Metabolism-type and Kinase-type.

(A) Heatmap plotting the scaled expression patterns of major marker genes in each tumor cluster. The color keys from pink to yellow indicate relative expression levels from low to high. (B) Dot plot depicting up-regulated genes of tumor cluster A (top) and tumor cluster B (bottom). The x-axis specifies the rank of DEGs and the y-axis specifies the natural logarithm of the FC. Dotted vertical and horizontal lines reflect the filtering criteria. Dot size represents the natural logarithm of the FC of genes. The color keys from blue to red indicate the P-value from high to low. (C, D) GO enrichment analysis of the up-regulated genes in tumor cluster A (C) and tumor cluster B (D) indicating the top altered 10 terms in the biological process of gene ontology. The x-axis specifies the number of genes enriched in the pathways. The color keys from shallow to deep indicate the P-value from high to low. (E) Immunohistochemistry staining of CGA, NDUFA4L2, COX4I2, PNMT, and RET markers in formalin-fixed paraffin-embedded PCC tissue sections matched to scRNA-seq specimens. Scale bar, 100 μm .

Trajectory Analysis Reveals Subclonal Dynamics of PCC Types

We next examined tumor subclonal evolution patterns for the metabolism-type and kinase-type PCCs, by applying the Dynverse algorithm to order cells along a pseudotime trajectory (Saelens *et al.*, 2019). We first clustered metabolism-type PCC cells into 2 subclusters based on DEGs and found similar proportions of tumor subcluster 1 and 2 at both the early stage and late stage of tumor evolution (Fig. 5A and 5B). Late up-regulated genes include *S100A4* and *TAGLN* that promote epithelial-mesenchymal transition and cell invasion, which highly expressed in subcluster 2 (Fig. 5C), suggesting the higher metastasis potential of subcluster 2 as compared with subcluster 1. Metabolism-related genes such as *NDUFA4L2*, *ATP5MG*, *NDUFB4*, and *COX17* were highly expressed at early stage, while *SOD3* and *COX7A1* were up-regulated at the late stage (Fig. 5C), revealing the consistently active metabolism phenotype of metabolism-type PCCs cells. We also found the high expression of tumor suppressor gene *SPINT2* at early stage and subsequent up-regulation of the oncogene *JUNB* (Fig. 5C), revealing an increase of metastasis potential over the tumor evolution of metabolism-type PCCs. In short, these results indicate that cells from metabolism-type PCCs display phenotype of consistently active metabolism and increasingly metastasis potential.

We then clustered kinase-type PCC cells into 2 subclusters and found that the major clone has been changed from subcluster 1 to 2 along the trajectory (Fig. 5D and 5E). Further analysis of DEGs in the pseudotime trajectory showed early high expression of *CALM1* and *CALM2* in the subcluster 1, and late up-regulation of *RET* in the subcluster 2 (Fig. 5F), suggesting the persistent dysregulation of kinase signals over tumor evolution. We also noticed the early expression of *PNMT*, while neuron-specific genes such as *MAP2*, *MAP1B*, *L1CAM*, and *CNTN1* were up-regulated later in the pseudotime trajectory (Fig. 5F), suggesting the decrease of epinephrine synthesis and the appearance of neuron-like phenotypes during tumor evolution, consistent with previously reported neuron-like phenotypes in PCC cells with high expression of RET (Califano *et al.*, 1995; Powers *et al.*, 2003; Powers *et al.*, 2009). Taken together, these analyses have revealed the transcriptional clonal dynamics of metabolic-type and kinase-type PCCs.

Cell-cell Communication Analysis to Further Characterize the Tumor Microenvironment

Having characterized differences in the cell type composition, gene expression, and clonal evolution between metabolism-type and kinase-type PCCs, we subsequently expanded our investigation to cell-cell communication occurring within the distinct tumor microenvironments of these two types of PCCs. We adopted CellChat tool to quantitatively analyze inter-cellular communication networks (Jin *et al.*, 2021). We observed the activation of the annexin signaling pathway in metabolism-type PCCs (Fig. 6A). Further analysis of participated ligand-receptors showed major participation of *ANXA1-FPR1* pair in the annexin signaling pathway (Fig. 6B), and high co-expression of *ANXA1* and *FPR1* in neutrophils and monocytes/macrophages (Fig. 6C). It has been reported that the annexin signaling pathway is known to be activated under a strong inflammatory reaction (Gastardelo *et al.*, 2014; Gavins *et al.*, 2003; Gerke & Moss, 2002; Hayhoe *et al.*, 2006). Combined with our findings of a higher proportion of neutrophils and monocytes/macrophages in metabolism-type as compared with kinase-type (Supplemental Fig. S4), we speculate that metabolism-type PCCs may display elevated inflammatory responses. We then performed Gene Set Enrichment Analysis (GSEA) on metabolism-type and kinase-type PCCs by using hallmark gene sets in the MSigDB databases (Liberzon *et al.*, 2011). As expected, the inflammatory response signaling pathway showed enrichment in metabolism-type PCCs as compared with that in kinase-type PCC (Fig. 6D). Taken together, metabolism-type PCCs exhibited a strong inflammatory reaction and the activation of annexin signaling pathway.

Considering the heterogeneity in cell type composition between sporadic and VHL patients in metabolism-type, we further analyzed inter-cellular interactions in the VHL patient. We noticed the activation of VEGF signaling pathway in P5 (Fig. 6E), and major contribution of *PLGF*-

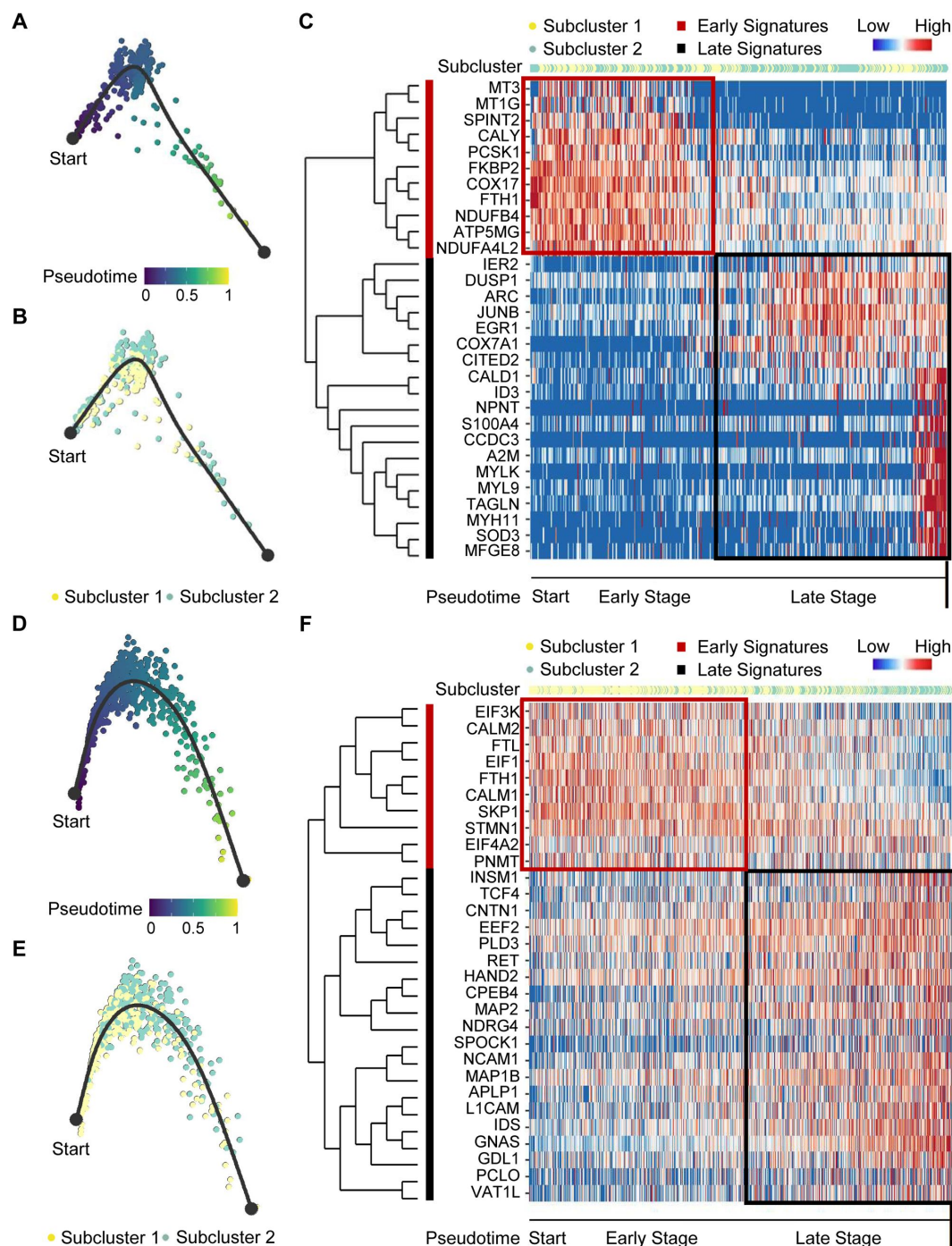


Figure 5.

Pseudotime Analysis of PCC Tumor Evolution by Dynverse.

(A, B) Pseudotime trajectory of metabolism-type PCC cells colored by pseudotime (A) and tumor subclusters (B). (C) Pseudotime heatmap plotting the expression levels of genes across the transition from beginning (left) to end (right). The color keys from blue to red indicate the gene expression levels from low to high. (D, E) Pseudotime trajectory of kinase-type PCC cells colored by pseudotime (D) and tumor subclusters (E). (F) Pseudotime heatmap showing the expression levels of genes across the transition from beginning (left) to end (right). The color keys from blue to red indicate the gene expression levels from low to high.

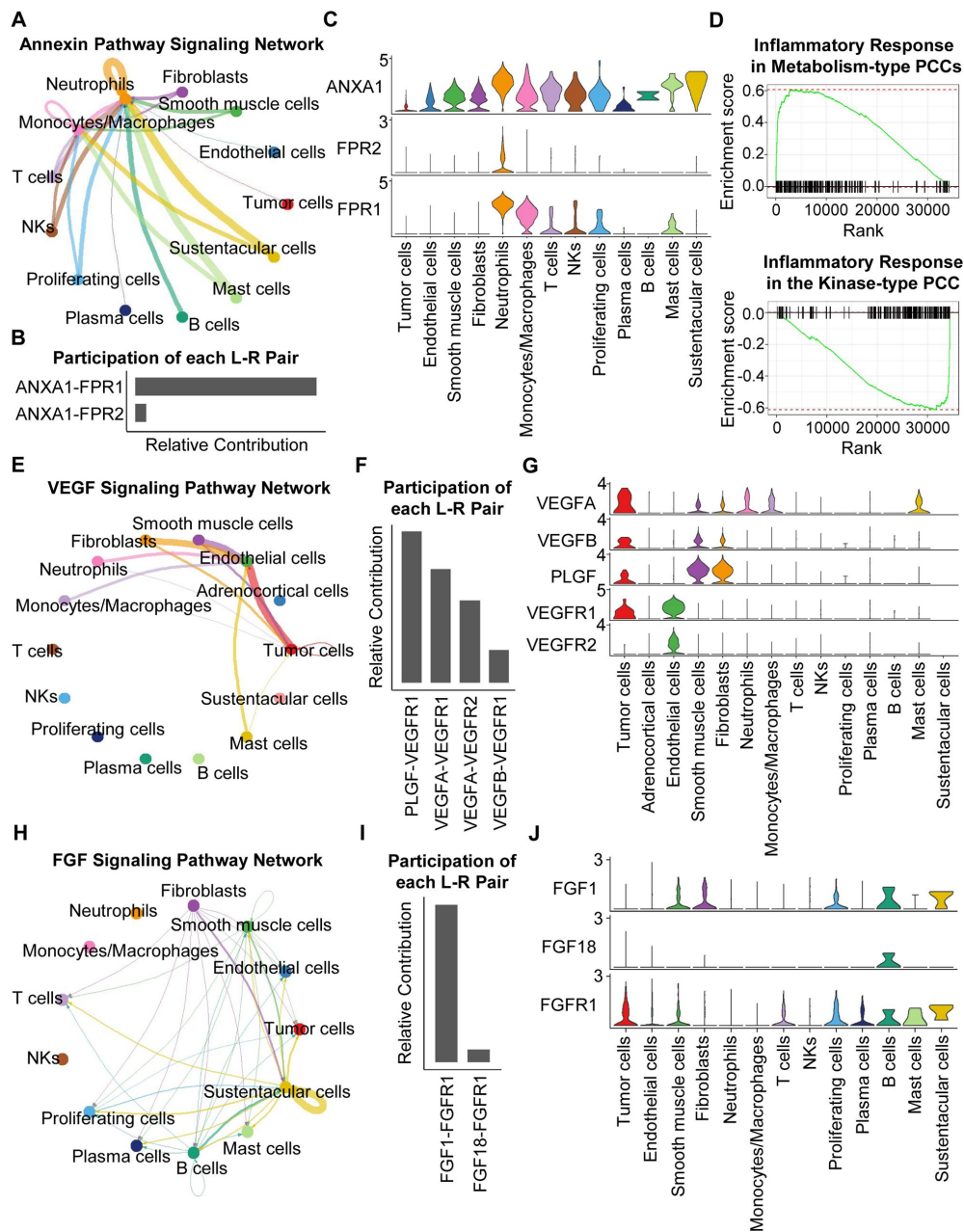


Figure 6.

CellChat Analysis Reveals Cell-cell Communication Patterns in PCC Types.

(A) Circle plot showing an inferred annexin signaling pathway network in the metabolism-type PCC microenvironment. The edges connecting the circles represent the communication probability between any two kinds of cell types. The color of the edge denotes directionality (i.e., senders vs receivers). (B) Bar graph plotting the quantification of the relative contributions of individual ligand-receptor pairs to the overall annexin signaling pathway. (C) Violin plot of the expression distribution of *ANXA1*, *FPR1*, and *FPR2* in main cell types of the metabolism-type PCC microenvironment. (D) GSEA enrichment plots of the inflammatory response signaling of metabolism-type patients (left) and the kinase-type patient (right). (E) Circle plot depicting an inferred VEGF signaling pathway. (F) Bar graph displaying the quantification of the relative contributions of individual ligand-receptor pairs to the overall VEGF communication network. (G) Violin plot plotting the expression distribution of *VEGFA*, *VEGFB*, *PLGF*, *VEGFR1*, and *VEGFR2* in main cell types of P5 PCC microenvironment. (H) Circle plot of an inferred FGF signaling pathway in the kinase-type PCC microenvironment. (I) Bar graph showing the quantification of the relative contributions of individual ligand-receptor pairs to the overall FGF signaling pathway. (J) Violin plot plotting the expression distribution of *FGF1*, *FGF18*, and *FGFR1* in main cell types of the kinase-type PCC microenvironment.

VEGFR1, *VEGFA-VEGFR1* and *VEGFA-VEGFR2* pairs to VEGF signaling pathway (Fig. 6F). Specifically, *VEGFA* and *VEGFR1* were highly expressed in tumor cells, *VEGFR1* and *VEGFR2* in endothelial cells, and *PLGF* in fibroblasts and smooth muscle cells, respectively (Fig. 6G), suggesting the crosstalk between tumor cells and fibroblasts or endothelial cells in tumor microenvironment through these ligand-receptor pairs, which mediated in the tumor angiogenesis and metastasis. The observed activation of VEGF pathway is consistent with the vascular endothelial hyperplasia caused by *VHL* mutation in VHL syndrome (Vortmeyer *et al.*, 2013) and the elevated proportion of endothelial cells in this patient (Fig. 2D).

We subsequently analyzed the kinase-type PCC patient, and identified the activation of FGF signaling network (Fig. 6H) and major involvement of *FGF1-FGFR1* in the FGF pathway (Fig. 6I). The smooth muscle cells, fibroblasts, and B cells exhibited high expression of *FGF1*, while tumor cells, smooth muscle cells, T cells, and B cells highly expressed *FGFR1* (Fig. 6J). The analyses suggested the cell-cell communication among tumor cells, stroma cells, and immune cells through the *FGF1-FGFR1* pair, which regulated the tumor cell proliferation (Kato, 2016). These results indicate that kinase-type PCCs may benefit from FGFR inhibitors, which have been shown to exert inhibitory effects on tumor proliferation (Liang *et al.*, 2012). Together, we described the inter-cellular communication networks in microenvironment of metabolism-type and kinase-type PCCs.

Immune Infiltration in the PCC Microenvironment Implies Patient Responses to Immunotherapy

We further analyzed the immune microenvironment of metabolism-type and kinase-type PCCs. Visualization of inter-cellular communication showed apparent crosstalk between tumor cells and T cells in metabolism-type patients, while we were surprised to find a lack of apparent communication between tumor cells and T cells in the kinase-type patient (Fig. 7A). These results suggested that kinase-type PCC cells could evade immune surveillance of T cells and further indicated the immune escape potential of kinase-type PCCs. Considering leukocyte antigen-class I (HLA-I) surface level is known to represent the antigen-presenting abilities of tumor cells (Brea *et al.*, 2016; Jhunjhunwala *et al.*, 2021; Oh *et al.*, 2019), we assessed *HLA-I*-expression in PCC cells: the expression levels of *HLA-A*, *HLA-B*, and *HLA-C* were significantly lower in kinase-type PCC cells than in metabolism-type PCC cells (Fig. 7B). Consistently, immunohistochemistry staining of serial sections of tumor tissues showed lower expression of HLA-A in P4 as compared to that in other patients (Fig. 7C), providing a plausible explanation for the lack of interactions detected between tumor cells and T cells in the kinase-type PCC. In short, we discovered that the kinase-type PCC displayed an impaired *HLA-I*-expression profile consistent with immune escape, while metabolism-type PCCs showed antigen-presenting abilities.

We additionally assessed the immune microenvironment of PCCs by subclustering immunotherapy-related immune cells and identified 8 clusters including central memory CD4⁺ T cells (CD4⁺ TCM, marked by *CD3D*, *CD3E*, *CD3G*, *CD4*, *CCR7*, and *IL7R*), effector memory CD4⁺ T cells (CD4⁺ TEM, marked by *CD3D*, *CD3E*, *CD3G*, *CD4*, and *IL7R*), CD8⁺ T cells (marked by *CD3D*, *CD3E*, *CD3G*, *CD8A* and *CD8B*), natural killer T cells (NKTs, marked by *CD3E*, *KLRD1* and *GNLY*), natural killer cells (NKs, marked by *KLRD1* and *GNLY*), B cells (marked by *MS4A1* and *CD79A*), M1 macrophages (marked by *CD68*), and M2 macrophages (marked by *CD68* and *CD163*) (Fig. 7D and 7E). Further analysis of immune cell type composition in metabolism-type and kinase-type PCCs showed an increase in the proportion of CD4⁺ T cells, CD8⁺ T cells, and M1 macrophages in tumor tissues as compared with that in adjacent normal adrenal medullary tissues, while the proportion of M2 macrophages in adjacent normal adrenal medullary tissues decreased as compared with that in tumor tissues (Fig. 7F).

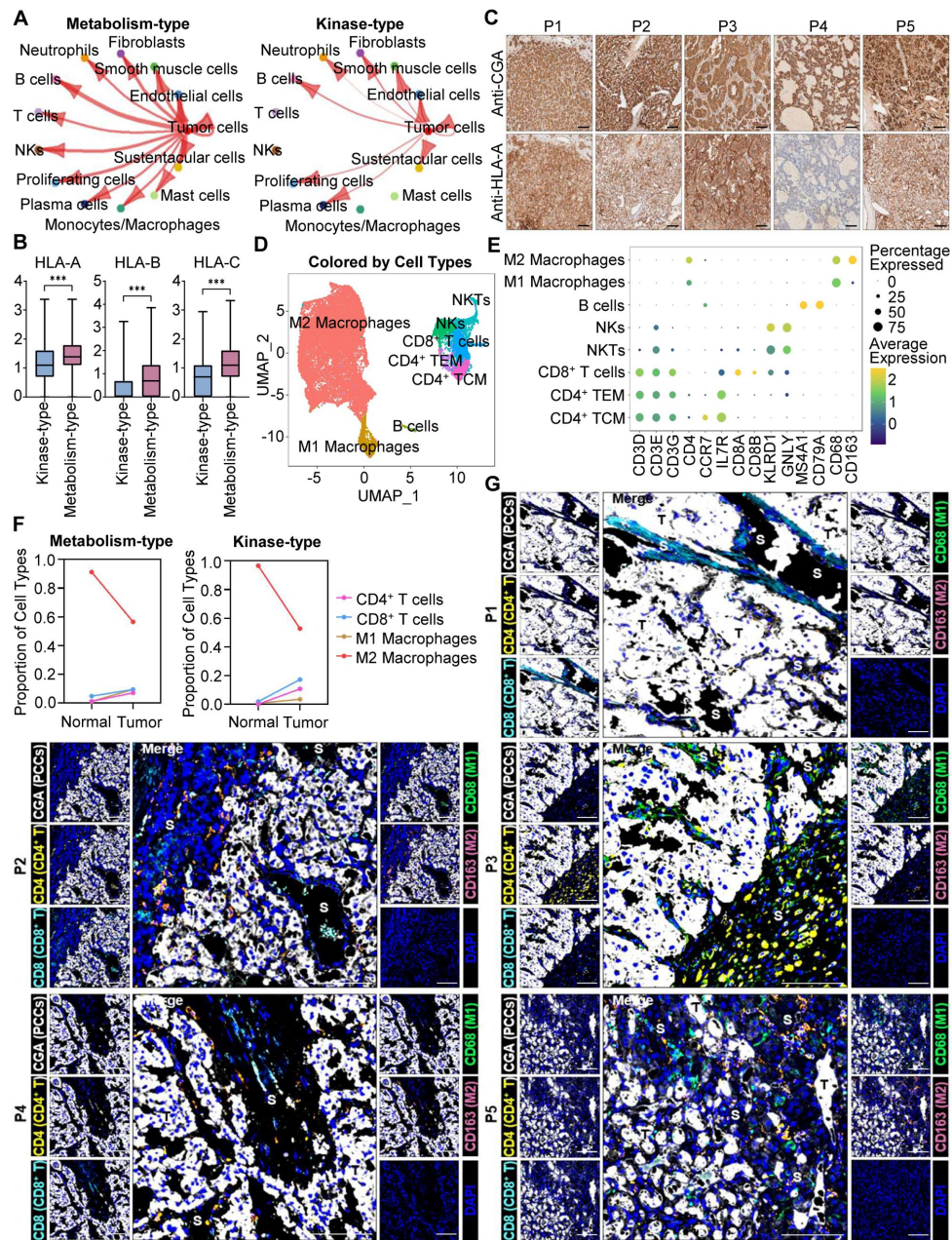


Figure 7.

Prediction of the Immune Escape Potential and Immunotherapy Response of PCC Patients.

(A) Circle plots depicting inferred inter-cellular interactions in metabolism-type (left) and kinase-type (right) PCC microenvironment. (B) Box plots showing the expression levels of *HLA-A*, *HLA-B*, and *HLA-C* in metabolism-type and kinase-type PCC patients. (C) Immunohistochemistry staining of CGA and HLA-A in formalin-fixed paraffin-embedded PCC tissue sections matched to scRNA-seq specimens. Scale bar, 100 μ m. (D) UMAP plot showing 8 immune cell types detected from all PCC specimens. (E) Dot plot of representative marker genes for each immune cell type. The color scale represents the average marker gene expression level; dot size represents the percentage of cells expressing a given marker gene. (F) Comparison of the proportion of immune cell types in tumor vs adjacent normal adrenal medullary tissues. (G) Multispectral immunofluorescent staining for the juxtaposition of PCC cells (marked by CGA), CD4⁺ T cells (marked by CD4), CD8⁺ T cells (marked by CD8), M1 macrophages (marked by CD68), and M2 macrophages (marked by CD163) in formalin-fixed paraffin-embedded PCC tissue sections matched to scRNA-seq specimens. The white, yellow, cyan, green, pink, and blue spots indicated cells with high expression of CGA, CD4, CD8, CD68, CD163, and DAPI proteins in PCC tissue sections, respectively. S, stroma; T, tumor. Scale bar, 100 μ m.

The proportion and location of infiltrating immune cells in tumor tissues have been shown to be informative regarding responsiveness to immunotherapy (Sade-Feldman *et al.*, 2018 [↗](#); Stanton & Disis, 2016 [↗](#)). In particular, the CD8⁺ T cells play an essential role in anti-tumor immunity; these cells can recognize tumor antigens displayed on the surface of tumor cells by HLA-I molecules (Jhunjhunwala *et al.*, 2021 [↗](#)). As the scRNA-seq data do not support discernment of whether immune cells have infiltrated tumors or are merely present in the sampled materials, we performed multispectral immunofluorescence (mIF) staining on CD4⁺ T cells, CD8⁺ T cells, M1 macrophages, and M2 macrophages in formalin-fixed paraffin-embedded (FFPE) tumor tissues of P1-P5. We observed that CD8⁺ T cells, as well as CD4⁺ T cells, M1 macrophages, and M2 macrophages were rare (and were only present in tumor stroma of P1-P5), indicating apparent immune escape in both metabolism-type and kinase-type PCCs (Fig. 7G [↗](#)). Combined with previously reported negative regulatory effects of kinases (such as RET, ALK, and MEK) on HLA-I expression on tumor cells (Brea *et al.*, 2016 [↗](#); Oh *et al.*, 2019 [↗](#)), we speculate that the possible reason for inability in recruiting CD8⁺ T cells of kinase-type PCCs is the down-regulation of *HLA-I* in tumor cells regulated by *RET*, while the mechanism of immune escape in metabolism-type PCCs (with antigen presentation ability) needs to be further explored. Our results also indicate that the application of immunotherapy to metabolism-type PCCs is likely unsuitable, while kinase-type PCCs may have the potential of combined therapy with kinase inhibitors and immunotherapy.

Discussion

The genomic research of PCCs has made continuous progress in recent years (Bausch *et al.*, 2017 [↗](#); Mete *et al.*, 2022 [↗](#); Neumann *et al.*, 2019 [↗](#); Nölting *et al.*, 2022 [↗](#)), but there have been obvious breakthroughs for more efficacious therapies. Isotope therapies can only relieve the clinical symptoms in 50% of patients (without reducing tumor size) (Fitzgerald *et al.*, 2006 [↗](#); Roman-Gonzalez & Jimenez, 2017 [↗](#)), while most chemotherapies and targeted therapies exhibit low remission rates and severe side effects in clinical studies (Druce *et al.*, 2009 [↗](#); Hamidi, 2019 [↗](#); O’Kane *et al.*, 2019 [↗](#); Oh *et al.*, 2012 [↗](#)), limiting the development of PCC treatments. Although immunotherapy has achieved success in the therapy of solid tumors, it is difficult to evaluate the curative effect in PCCs. To address this problem, two key features associated with the tumor immunotherapy need to be elucidated—tumor heterogeneity and the tumor microenvironment (Hegde & Chen, 2020 [↗](#); Li *et al.*, 2018 [↗](#); Liu *et al.*, 2023 [↗](#); Liu *et al.*, 2022 [↗](#); Zhang & Zhang, 2020 [↗](#)). Regarding PCC heterogeneity, genomic studies have confirmed the inter-individual heterogeneity based on driver mutations (Dahia, 2014 [↗](#); Jhavar *et al.*, 2022 [↗](#); Sarkadi *et al.*, 2022 [↗](#); Toledo *et al.*, 2017 [↗](#)). However, there is lack of relevant research on intra-tumoral heterogeneity, which has an impact on disease progression and sensitivity to immunotherapy (Vitale *et al.*, 2021 [↗](#)). For the PCC microenvironment, the cell composition and inter-cellular crosstalk remain largely unexplored. By investigating the heterogeneity and microenvironment characteristics of PCCs, we proposed a feasible method to judge the heterogeneity of PCCs, and also provided a clue for the application of immunotherapy in PCC patients.

The clinical and histopathological diagnosis of metastasis risk and heterogeneity of PCCs is particularly difficult, and is still limited by the lack of reliable prognostic markers (de Wailly *et al.*, 2012 [↗](#)). Although the PASS scoring system was proposed as a tool for discriminating potentially metastasis PCCs from benign ones, its utility is somewhat restricted due to observer variation and nonrepeatability (Agarwal *et al.*, 2010 [↗](#); Kimura *et al.*, 2014 [↗](#); Thompson, 2002 [↗](#); Wu *et al.*, 2009 [↗](#)). By integrating the PASS scoring system with scRNA-seq analysis, we observed that both methods offered complementary insights into the intra-tumoral and inter-individual heterogeneity of PCCs. Considering the intricate cellular components within the PCC microenvironment and recognizing the advantage of scRNA-seq in dissecting the tumor microenvironment (Ren *et al.*, 2021 [↗](#)), the potential integration of molecular diagnostic methods, such as single-cell sequencing, with pathological tools appears promising for implementation in the clinical practice of PCC diagnosis and care (Crona *et al.*, 2017 [↗](#); Papathomas *et al.*, 2021 [↗](#)).

According to the results of genomics research in recent years, 40~50% of PCCs have been classified into a certain molecular pathway (pseudohypoxia, kinase, or Wnt), but the remaining 50-60% are still unclassified (Crona *et al.*, 2017 [↗](#); Lenders *et al.*, 2014 [↗](#)). PCCs that classified into these molecular pathways may have the opportunity to carry out drug clinical trials against corresponding targets, such as HIF2 α , VEGF, and RET, etc. (Jhawar *et al.*, 2022 [↗](#); Nölting *et al.*, 2019 [↗](#); Toledo & Jimenez, 2018 [↗](#)), while unclassified PCCs still have no clues or basis for drug treatment. According to the WES results of the 5 patients in our study, except for one patient with *VHL* germline mutation, the others could not be classified based on detected germline or somatic mutations. Therefore, we developed a new classification of PCCs based on scRNA-seq, which are able to accurately capture the transcriptional features of PCC cells. We found that PCC cells from 4 patients highly expressed metabolism-related genes, while that from another patient exhibited high expression of kinase-related genes. Although previous studies applying bulk RNA-seq analysis of PCCs have also reported highly expressed genes (Batchu *et al.*, 2022 [↗](#); Flynn *et al.*, 2015 [↗](#)), the interpretation of bulk RNA-seq data can be complicated by interference from the large number of non-tumor cells (Huang *et al.*, 2023 [↗](#); Li *et al.*, 2022 [↗](#)), so it is difficult to determine whether the highly expressed genes are reliable molecular features of PCCs. Our study revealed that the kinase-type PCC patient (P4) exhibited higher blood pressures and plasma levels of catecholamine metabolites (3-methoxytyramine and normetanephrine). Further research is warranted to explore the correlation of our molecular classification with plasma levels of catecholamine metabolites.

The tumor immune microenvironment, defined as the interplay between tumor cell antigen presentation, immune cell infiltration, and their interactions, can be multifaceted (de Visser & Joyce, 2023 [↗](#); Hanahan, 2022 [↗](#)). Given the spatial limitations of scRNA-seq and the protein detection constraints of multispectral immunofluorescent staining, we combined these two methods to investigate immune escape mechanisms in both types of PCCs. Although our immunofluorescence staining data showed a lack of CD8⁺ T cell infiltration in both metabolism-type and kinase-type PCCs, only kinase-type PCCs exhibited downregulation of *HLA*-I molecules. The expression of *HLA*-I is a marker for tumor antigen presentation and CD8⁺ T cell infiltration (Jhunjhunwala *et al.*, 2021 [↗](#); Perea *et al.*, 2018 [↗](#); Sadagopan *et al.*, 2022 [↗](#)). Previous studies have demonstrated that kinases such as RET, MAP2K1, ALK, and FGF can promote tumor growth via helping tumor cells to evade the immune system by downregulating *HLA*-I expression (Brea *et al.*, 2016 [↗](#); Oh *et al.*, 2019 [↗](#)). The kinase-type PCCs that we defined exhibited activation of RET and FGF signals, which led us to consider the possible inhibitory effect of kinases on *HLA*-I expression in PCCs. Recently, RET inhibitors have been approved for the treatment of non-small cell lung cancer with *RET* mutation (Drilon *et al.*, 2020 [↗](#); Griesinger *et al.*, 2022 [↗](#)). For kinase-type PCCs, whether RET inhibitors can enhance the antigen presentation ability of tumor cells, and further provide chance for combination of kinase-targeted therapy and immunotherapy, needs to be verified by follow-up research. The metabolism-type PCCs, however, with antigen presentation ability and also the lack of CD8⁺ T cell infiltration, the application of immunotherapy is likely unsuitable, and the mechanism(s) of immune escape in these tumors needs to be further explored.

There are potential limitations of our study. Despite the patient cohort in our study including a hereditary PCC patient, only one patient with a *VHL* germline mutation was included; other types of hereditary PCCs with germline mutations (for example in *SDHx* and *FH*) were not included. The number of sporadic cases was also limited, mainly caused by the low incidence of PCCs. In addition, the specimens were all from primary tumors, while no metastatic tumors were included. Together, these considerations underscore the necessary for additional studies of PCCs in the future.

In summary, our study presents the molecular classification and tumor microenvironment characterization of PCCs through scRNA-seq. The intra-tumoral heterogeneity is lower than the inter-individual heterogeneity of PCCs. Among unclassified PCCs, we defined metabolism-type and kinase-type at the single-cell transcriptome level. We observed a lack of CD8⁺ T cell infiltration in both metabolism-type and kinase-type PCCs. The kinase-type PCC showed downregulation of *HLA*-

that possibly regulated by *RET*, suggesting the potential of combined therapy with kinase inhibitors and immunotherapy in kinase-type PCCs. For the metabolism-type PCCs, which have antigen presentation ability and also exhibit immune escape, the application of immunotherapy is likely unsuitable. The proposal of this molecular classification and our characterization of these PCC types can contribute to strategy development and clinical trials of PCC treatments in the future.

Methods

Ethical Regulations

The research presented here complies with all relevant local, national, and international regulations. For all PCC patient specimens, informed written consent was obtained prior to donation. The Peking University First Hospital Review Board (Protocol 300-001) approved the study.

Patient Cohort

Five PCC patients were included, and all patients had signed the consent forms at the Department of Urology in Peking University First Hospital. Fresh tumor specimens were collected during surgical resection. The sporadic patients (P1-P4) were performed surgical resections of the tumors at right adrenals, and the VHL patient (P5) underwent left adrenalectomy. We collected 1 resected tumor specimen from P1 (P1_T1), 3 specimens from distinct intra-tumoral sites from P2 (P2_T1, P2_T2, and P2_T3) or P5 (P5_T1, P5_T2, and P5_T3), and 2 specimens from distinct intra-tumoral sites from P3 (P3_T1 and P3_T2), or P4 (P4_T1 and P4_T2). To enable comparisons, one normal adrenal medullary tissue adjacent to the tumor was collected from each patient (P1_A, P2_A, P3_A, P4_A, and P5_A). A total of sixteen specimens were carefully dissected under the microscope and confirmed by a qualified pathologist.

Tissue Processing

The fresh tumor specimens were stored in the tissue preservation solution (JSENb) and washed with Hanks Balanced Salt Solution (HBSS, HyClone) for 3 times and minced into 1-2 mm pieces. Then the tissue pieces were digested with 2 ml tissue dissociation solution at 37°C for 15 min in 15 ml centrifuge tube with sustained agitation. After digestion, using 40 µm sterile strainers to filter the samples and centrifuging the samples at 1,000 rpm for 5 min. Then the supernatant was discarded, and the sediment was resuspended in 1 ml phosphate buffered saline (PBS, Solarbio). To remove the red blood cells, 2 ml red blood cell lysis buffer (BD) was added at 25°C for 10 min. The solution was then centrifuged at 500 g for 5 min and suspended in PBS. The cells were stained with trypan blue (Sigma) and microscopically evaluated.

Single-cell Library Construction and Sequencing

Utilizing the 10x Genomics Chromium Single Cell 3' v3 Library Kit and Chromium instrument, approximately 17,500 cells were partitioned into nanoliter droplets to achieve single-cell resolution for a maximum of 10,000 individual cells per sample. The resulting cDNA was tagged with a common 16 nt cell barcode and 10 nt Unique Molecular Identifier during the RT reaction. Full-length cDNA from poly-A mRNA transcripts was enzymatically fragmented and size selected to optimize the cDNA amplicon size (approximately 400 bp) for library construction (10x Genomics). The concentration of the 10x single-cell library was accurately determined through qPCR (Kapa Biosystems) to produce cluster counts appropriate for the HiSeq4000 or NovaSeq6000 platform (Illumina). In all, 26 × 98 bp (3' v3 libraries) sequence data were generated targeting between 25 and 50K read pairs/cell, which provided digital gene expression profiles for each individual cell.

General scRNA-seq Data Analysis

For single-cell RNA-seq analysis, we used Cell Ranger (10x Genomics, v.6.1.2) to pre-process the single-cell RNA-seq data after obtaining the paired-end raw reads. Cell barcodes and unique molecular identifiers (UMIs) of the library were extracted from read 1. Then, the reads were split according to their cell (barcode) IDs, and the UMI sequences from read 2 were simultaneously recorded for each cell. Quality control on these raw readings was subsequently performed to eliminate adapter contamination, duplicates, and low-quality bases. After filtering barcodes and low-quality readings that were not related to cells, we mapped the cleaned readings to the human genome (GRCh38) and retained the uniquely mapped readings for UMIs counts. Next, we estimated the accurate molecular counts and generated a UMI count matrix for each cell by counting UMIs for each sample. Finally, we generated a gene-barcode matrix that showed the barcoded cells and gene expression counts.

The R package Seurat (v.4.0.2) was used for all subsequent analysis (Butler *et al.*, 2018 [\[link\]](#); Satija *et al.*, 2015 [\[link\]](#); Stuart *et al.*, 2019 [\[link\]](#)). For quality control of single-cell RNA-seq, a series of quality filters was applied to the data to remove those barcodes which fell into any one of these categories: too few genes expressed (possible debris), too many UMIs associated (possible more than one cell), and too high mitochondrial gene expression (possible dead cell). The cutoffs for these filters were as follows: the minimum number (no less than 200) and maximum number (no more than 5000) were used in controlling the number of genes; the maximum number (no more than 30%) was used for the quality control of the percentage of mitochondrial genes. Low-quality cells and outliers were discarded, and the single cells that passed the QC criteria were used for downstream analyses.

Cell Clustering and Cell Type Annotation

The Seurat software package (v.4.0.2) was used to perform cell clustering analysis to identify major cell types (Butler *et al.*, 2018 [\[link\]](#); Satija *et al.*, 2015 [\[link\]](#); Stuart *et al.*, 2019 [\[link\]](#)). All Seurat objects constructed from the filtered UMI-based gene expression matrixes of given samples were merged. We first applied the SCTransform (v.0.3.2) function to implement normalization, variance stabilization, and feature selection through a regularized negative binomial model. Then, the principal component analysis (PCA) was applied for linear dimensionality reduction with the top 3000 variable genes. According to standard steps implemented in Seurat, highly variable numbers of principal components (PCs) 1-50 were selected and used for clustering using the Uniform Manifold Approximation and Projection (UMAP) method. We identified cell types of these clusters based on the expression of canonic cell type markers or inferred by CellMarker database (Zhang *et al.*, 2019 [\[link\]](#)). The cluster markers were also certified using the FindAllMarkers function of the Seurat suite, and cell types were manually annotated based on certified markers finally.

Correlation Analysis of scRNA-seq

After integration, for each cluster, each sample, and each patient, we compared the gene expression to others to identify the significant highly expressed genes (adjusted P-value < 0.05 and log fold change > 0). Then the average gene expressions in each cluster, each sample, and each patient were calculated. The pairwise correlations were then estimated.

DEGs Identification and Enrichment Analysis

The cluster-specific genes were identified by running Seurat containing the function of FindAllMarkers on a log-transformed expression matrix (min. pct = 0.25, only. Pos = TRUE, and logfc.threshold = 0.25). We also identified the differentially over-expressed genes between two clusters with the Wilcoxon Rank-Sum Test with the FindMarkers function in Seurat (adjusted P-value < 0.05, only.pos = T, and logfc.threshold = 0.1), and the cluster-specific overrepresented GO biological process was calculated with the compareCluster function in the clusterProfiler package (v.4.2.2) of R (Yu *et al.*, 2012 [\[link\]](#)). We also used the GSEA with the curated gene sets to identify the

pathways that were induced or repressed in between the cell clusters. In brief, the mean gene expression level was calculated and the log twofold change (FC) between the specific cell cluster and the other cells was applied as the test statistic. The 50-hallmark gene sets in the MSigDB databases (<https://www.gsea-msigdb.org/gsea/msigdb>) were used for the GSEA analysis (Liberzon *et al.*, 2011).

Single-cell RNA CNV Detection and Clustering

All cells were classified as either normal or tumor based on the genome-wide copy number profiles computed from the gene expression UMI matrix using the Bayesian segmentation approach, CopyKat (v.1.0.8) (Gao *et al.*, 2021). Aneuploid single cells with genome-wide copy number aberrations were anticipated to be tumor cells, while diploid cells were presumed to be normal cells. The CopyKat-based predictions were subsequently validated by single-cell gene expression profiles.

Trajectory Analysis of scRNA-Seq Data

In order to perform a detailed comparison among different trajectory modeling tools, the Dynverse (v.0.1.2) tool was used (Saelens *et al.*, 2019). Based on the scoring system provided by Dynverse and following a careful inspection of the generated trajectories, we applied ‘SCORPIUS’ and ‘EMBEDDR’ to infer the development trajectories. Finally, we selected the genes that were differentially expressed on different stages through the trajectory and plotted the pseudotime heatmap.

Cell-cell Communication Analysis

Cell-cell interactions based on the expression of known ligand-receptor pairs in different cell types were inferred using CellChatDB (v.1.1.3) (Jin *et al.*, 2021). In brief, we followed the official workflow and loaded the normalized counts into CellChat and applied the preprocessing functions ‘identifyOverExpressedGenes’, ‘identifyOverExpressedInteractions’, and ‘projectData’ with standard parameters set. As database we selected the ‘Secreted Signaling’ pathways and used the pre-compiled human ‘Protein-Protein-Interactions’ as a priori network information. For the main analyses the core functions ‘computeCommunProb’, ‘computeCommunProbPathway’, and ‘aggregateNet’ were applied using standard parameters and fixed randomization seeds. Finally, to determine the senders and receivers in the network, the function ‘netAnalysis_signalingRole’ was applied on the ‘netP’ data slot.

Whole Exome Sequencing and Analysis

Genomic DNA extracted from tumor tissues were sent for whole exome sequencing. The exomes were captured using the Agilent SureSelect Human All Exon V6 Kit and the enriched exome libraries were constructed and sequenced on the Illumina NovaSeq 6000 platform to generate WES data (150 bp paired-end reads, > 100×) according to standard manufacturer protocols. The cleaned reads were aligned to the human reference genome sequence UCSC Build 19 (hg19) using Burrows-Wheeler Aligner (BWA) (v.0.7.12) (Li & Durbin, 2009). All aligned BAM were then performed through the same bioinformatics pipeline according to GATK Best Practices (v.3.8) (McKenna *et al.*, 2010). We obtained germline variants based on variant calling from GATK-HaplotypeCaller. We then used GATK-MuTect2 to call somatic variants in tumors and obtained a high-confidence mutation set after rigorous filtering by GATK-FilterMutectCalls. All variants were annotated using ANNOVAR (v.2018Apr16) (Wang *et al.*, 2010).

Immunocytochemistry and Multispectral Immunofluorescent Staining

Immunocytochemistry and multispectral immunofluorescent staining experiments were conducted according to standard protocols using antibodies against formalin-fixed paraffin-embedded (FFPE) tissue specimens. The antibodies used are listed as follows: CGA (ABclonal, A9576), NDUFA4L2 (Proteintech, 66050-1-1g), COX4I2 (Santa, sc-100522), PNMT (Abcam, ab154282), RET (Abcam, ab134100), CD4 (Abcam, ab133616), CD8 (Invitrogen, MA1-80231), CD68 (Invitrogen, MA5-12407), CD163 (Abcam, ab182422), and HLA-A (ABclonal, A11406).

Data availability

Fastq raw data and processed data can be requested by contacting the corresponding authors. Human transcriptome reference used for our analysis is available at 10x Genomics website (<https://cf.10xgenomics.com/supp/cell-exp/refdata-cellranger-GRCh38-3.0.0.tar.gz>) or in zenodo (<https://zenodo.org/record/4114854/files/refdata-cellranger-GRCh38-3.0.0.tar.gz?download=1>).

Code availability

The single-cell RNA sequencing data was processed using cellranger v.6.1.2 (<https://www.10xgenomics.com/>) and analyzed with the R package Seurat v.4.0.2 (<https://satijalab.org/seurat/>). Custom code that was created for scRNA-seq analysis can be requested by contacting the corresponding authors.

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Author contributions

Z.Z., L.S., and K.G. conceived the idea and designed the study. Y.X. prepared samples for scRNA-seq. S.Q. performed scRNA-seq data analysis. Q.S., S.F., W.A., Y.X., and W.H. performed immunocytochemistry and multispectral immunofluorescent staining. S.Y., K.Z., and X.Z. provided technical assistance. Z.Z., L.S., and Y.W. provided guidance in data analysis and interpretation of the results. S.Q. drafted the manuscript, with significant input from Z.Z., L.S., and K.G. All authors reviewed the manuscript prior to submission.

Conflict of interest statements

The authors declare no conflicts of interest that pertain to this work.

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Reviewer #2 (Public Review):

Pheochromocytoma (PCC), a rare neuroendocrine tumor, is currently considered malignant, but non-surgical treatment options are very limited and there is an urgent need for more basic research to support the development of new therapeutic approaches. In the present work, the authors described the intra- and inter-tumor heterogeneity by performing scRNA-seq on tumor samples from five patients with PCC, and evaluated the corresponding PASS scores.

Strengths: The tumor microenvironment of PCC was characterized and potential molecular classification criteria based on single-cell transcriptomics were proposed, offering new theoretical possibilities for the treatment of PCC. The article is logically written and the results are clearly presented.

Weaknesses: I still have concerns about some of the article's content. My main concerns are: In this study, the authors seem to have demonstrated the inaccuracy of a subjective score

(PASS) by another objective means (scrRNA-seq). In fact, the multiparametric scoring systems such as PASS are no longer endorsed in the 2022 WHO guidelines. The PASS scoring system does not have a high positive predictive value for risk stratification of PCC metastasis, but "rule-out" of metastasis risk with a PASS score of <4 seems to be fairly reliable. Could the authors please explain why the PASS scores were chosen rather than the GAPP, m-GAPP, or COPPS scoring systems? If possible, please try to emphasize the importance and necessity of using the PASS scoring system, either by replacing it with a more acceptable scoring system or by deleting the relevant part, which does not seem to be very relevant to the subject of the article.

Moreover, I noted the following statement in the text "There are no studies reporting the composition of immune cells in PCCs. The few published studies investigating the immune microenvironment of PCCs have been limited to the expression of PDL1 at the histological level and to assessment of the tumor mutation burden (TMB) at the genomic level, and these results only seem to suggest that PCCs are immune-cold (Bratslavsky et al, 2019; Guo et al, 2019; Pinato et al, 2017)." This statement is very wrong. The reason for this error may be that the authors did not adequately search and read the relevant literature. I noticed that almost all references in this paper are dated 2021 and earlier, which is surprising. Please update the references cited in this paper in a comprehensive and detailed manner; referring to literature published too early may lead to inadequate discussion or even one-sided or incorrect conclusions and conjectures.

For example, the text statement "Combined with previously reported negative regulatory effects of kinases (such as RET, ALK, and MEK) on HLA-I expression on tumor cells (Brea et al., 2016; Oh et al., 2019), we speculate that the possible reason for inability in recruiting CD8+ T cells of kinase-type PCCs is the downregulation of HLA-I in tumor cells regulated by RET, while the mechanism of immune escape in metabolism-type PCCs (with antigen presentation ability) needs to be further explored. Our results also indicate that the application of immunotherapy to metabolism-type PCCs is likely unsuitable, while kinase-type PCCs may have the potential of combined therapy with kinase inhibitors and immunotherapy." is rather one-sided; in fact, the presence of immune escape in PCC, as the malignancy with the lowest tumor mutation compliance, has been well characterized, and the low number of infiltrating T cells in tumor tissue may be influenced by a variety of factors, such as the release of catecholamines, the expression of inhibitory receptors on the surface of T cells, and so on, although genetic mutation still plays the most crucial role. The Discussion section also has a lot of information that needs to be updated or corrected and expanded, so please rewrite the above section with sufficiently updated references.

Below I have listed some references for the authors to read:

- Tufton N, Hearnden RJ, Berney DM, et al. The immune cell infiltrate in the tumour microenvironment of pheochromocytomas and paragangliomas. *Endocr Relat Cancer*. 2022;29(11):589-598. Published 2022 Sep 19. doi:10.1530/ERC-22-0020
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- Celada L, Cubiella T, San-Juan-Guardado J, et al. Pseudohypoxia in paraganglioma and pheochromocytoma is associated with an immunosuppressive phenotype. *J Pathol*. 2023;259(1):103-114. doi:10.1002/path.6026
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<https://doi.org/10.7554/eLife.87586.2.sa1>

Reviewer #3 (Public Review):

The main findings of this study are as follows: (1) The authors defined "metabolism-type" and "kinase-type" in unclassified sporadic PCC patients through the single-cell transcriptomics-based differentially expressed genes and functional enrichment analyses. (2) They identified the limitation of Pheochromocytoma of the Adrenal gland Scaled Score (PASS) system and suggested the combination of molecular diagnostic methods like scRNA-seq with pathological tools like PASS in aiding the clinical evaluation of PCCs. (3) Analysis of the PCC microenvironment revealed a lack of immune cell infiltration in both metabolism-type and kinase-type PCCs, while only the kinase-type PCC patient exhibited the low expression of HLA-I that potentially regulated by RET, providing clues for the combined therapy with kinase inhibitors and immunotherapy in kinase-type PCC patients.

The main strength of this manuscript is that it involves scRNA-seq analysis of an extremely rare tumor type-PCCs, which presents a single-cell transcriptomics-based molecular classification and microenvironment characterization of PCCs and further provides clues for potential therapeutic strategies to treat PCCs. The authors also validated the scRNA-seq analysis results (such as the expression levels of marker genes and the distribution of immune cells in the PCC microenvironment) with immunocytochemistry and multispectral immunofluorescent staining. In summary, the findings in this manuscript are quite interesting and significant, which will potentially be valuable for the molecular classification of PCCs.

<https://doi.org/10.7554/eLife.87586.2.sa0>

Author Response

The following is the authors' response to the original reviews.

eLife assessment

This study presents a valuable finding for the treatment of PCCs by sequencing 16 tumor specimens from five patients with pheochromocytomas by single-cell transcriptomics and proposing a new molecular classification criterion based on the sequencing results and characterization of tumor microenvironmental features. The evidence supporting the claims of the authors is solid, although the inclusion of more patient samples would strengthen the study's conclusions. The work will be of interest to clinicians or medical biologists working on rare pheochromocytomas (PCCs).

Firstly, we sincerely appreciate the positive feedback from the editor and extend our gratitude to the three reviewers for their meticulous review and valuable comments. Our detailed responses to each recommendation are outlined below.

Response to reviewers' recommendations***Reviewer #1 (Recommendations for The Authors):***

1. *Transcriptomal clonal dynamics of different PCCs is well written. However for conclusion sample size needs to be more.*

Acknowledging the rarity of PCCs with an incidence of approximately 0.2 to 0.6 cases per 100,000 person-years (Farrugia & Charalampopoulos, 2019; Neumann et al, 2019), our study recognizes the limitation in sample size, as discussed in the limitations section (Page 22). In response to this concern, we are committed to undertaking further research with an

expanded sample size to bolster the robustness of our conclusions, seeking a more comprehensive understanding of tumor microenvironment characterization and molecular classification in PCCs. We appreciate the valuable guidance provided by the reviewer.

1. *Clinical, biochemistry data of 5 cases can be analysed. Any findings in different categories as per postulated classification can be noted for further studies. Example: epinephrine levels*

We have now included the clinical information of 5 PCC patients, encompassing signs and symptoms, the tumor size, and laboratory test results in the revised manuscript as Supplemental Table S3 (Page 11-12). Notably, our analysis revealed that the kinase-type PCC patient (P4) exhibited higher blood pressures and plasma levels of catecholamine metabolites (3-methoxytyramine and normetanephrine) compared to metabolism-type PCC patients (P1-P3, and P5). This observation aligns with the elevated expression of phenylethanolamine N-methyltransferase (PNMT), an enzyme involved in the biosynthesis of catecholamine and linked to hypertension, in P4, as identified in the scRNA-seq data (Figure 4B and 4D) (Kennedy et al, 1993; Konosu-Fukaya et al, 2018; Nguyen et al, 2015). As suggested, we plan to conduct further research to explore the correlation of our molecular classification with plasma levels of catecholamine metabolites, and the relevant points have been discussed in the revision (Page 20).

We would like to take this chance to again thank the reviewer for the careful review and very helpful guidance about how to improve our study.

References for Reviewer #1:

Farrugia FA, Charalampopoulos A (2019) Pheochromocytoma. *Endocrine regulations* 53: 191-212
Neumann HPH, Young WF, Jr., Eng C (2019) Pheochromocytoma and Paraganglioma. *The New England journal of medicine* 381: 552-565

Kennedy B, Elayan H, Ziegler MG (1993) Glucocorticoid hypertension and nonadrenal phenylethanolamine N-methyltransferase. *Hypertension (Dallas, Tex : 1979)* 21: 415-419

Konosu-Fukaya S, Omata K, Tezuka Y, Ono Y, Aoyama Y, Satoh F, Fujishima F, Sasano H, Nakamura Y (2018) Catecholamine-Synthesizing Enzymes in Pheochromocytoma and Extraadrenal Paraganglioma. *Endocrine pathology* 29: 302-309

Nguyen P, Khurana S, Peltsch H, Grandbois J, Eibl J, Crispo J, Ansell D, Tai TC (2015) Prenatal glucocorticoid exposure programs adrenal PNMT expression and adult hypertension. *The Journal of endocrinology* 227: 117-127

Reviewer #2 (Recommendations for The Authors):

1. *Please revise all references to "malignant potential", "malignant behavior", etc. throughout the article, including the abstract and introduction, and replace them with the word "metastasis" as appropriate. Since all PCCs are malignant non-epithelial neuroendocrine neoplasms originating from the paraganglia, which are themselves malignant tumors, it is unacceptable to describe them as "malignant potential" or "malignant potential". Please review the 2022 WHO/IARC classification and description of pheochromocytoma/paraganglioma (reference: Mete O, Asa SL, Gill AJ, Kimura N, de Krijger RR, Tischler A. Overview of the 2022 WHO Classification of Paragangliomas and Pheochromocytomas. *Endocr Pathol.* 2022;33(1):90-114. doi:10.1007/s12022-022-09704-6).*

As suggested, we have replaced all occurrences of “malignant potential” or “malignant behavior” with “metastasis” throughout the revised manuscript. We have also included a citation to the 2022 WHO/IARC classification for further clarity.

- *Similarly, it is not advisable to use the PASS score to predict "malignant" PCC; this type of scoring system evaluates the "metastasis risk" or the "metastasis potential" of PCC.*

We appreciate the reviewer for this insight and have revised our statements accordingly.

- *Also, "MALIGNANT CHAFFIN CELLS" needs to be modified; in fact, it is the "tumor cell of PCC" that the authors are trying to express.*

As suggested, we have amended the term “malignant chromaffin cells” to “PCC cells” in the revised manuscript (Page 9-10).

1. *How does the PASS score specifically relate to intra-tumor heterogeneity as reflected by scRNA-seq? In fact, the PASS score evaluates the histological or pathological invasiveness of PCC, and different sections of the same tumor tissue may have different histological manifestations, which may affect the score; however, scRNA-seq analyzes the cellular composition of the tumor, which is not the same as the information reflected by the PASS score. Both represent different levels and dimensions of intra-tumor heterogeneity and should be analyzed together. Please specifically list, one by one, the proportion of each item score of the PASS system and cell type of scRNA-seq for each sample and the results of the comparisons with each other to better present the conclusions.*

As suggested, we have included the proportion of each item score from the PASS system in the revised manuscript as Supplemental Table S2 (Page 8). Integrating this data with the cell type composition of each sample from Figure 2B, our analysis suggests that intra-tumor heterogeneity, as assessed by the PASS system, is more extensive compared to scRNA-seq. We concur with the reviewer’s judgement that scRNA-seq analysis and PASS score represent different levels and dimensions of intratumor heterogeneity, and we have adjusted our claim throughout the revised manuscript accordingly (Page 8, 9, and 19).

1. *Where is the specific mutation site of the VHL gene in patient 5? Please advise.*

The VHL gene mutation site, c.499C>T (missense mutation), in patient 5 was identified through whole exome sequencing (WES) analysis. We have now added the information to Supplemental Table S1 in the revised manuscript (Page 6).

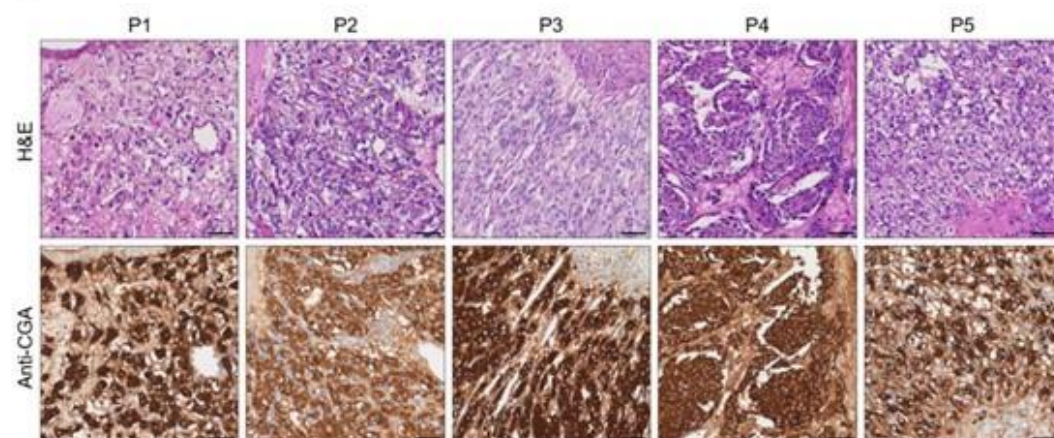
1. *Please revise Supplementary Figure 1, the scale should not appear in the picture of the staining result of P5.*

As suggested, we have adjusted the position of the scale bar.

Author response image 1.

Hematoxylin-eosin staining and immunohistochemistry staining of CGA marker in formalin-fixed paraffin-embedded PCC tissue sections matched to scRNA-seq specimens. Scale bar, 100 μ m.

S1



1. What were the clinical presentation and biochemical findings in the five patients?

The information regarding tumor sizes, signs and symptoms, and plasma levels of catecholamine metabolites [3-methoxytyramine (3-MT), metanephrine (MN), and normetanephrine (NMN)] has been added to the revised manuscript as Supplemental Table S3 (Page 11-12).

- Were there any preoperative symptoms of hypertension?

With the exception of P2, preoperative symptoms of hypertension were observed in all PCC patients. The information has been added to the revised manuscript as Supplemental Table S3 (Page 11-12).

- What was the size and catecholamine secretion phenotype of each tumor? What was the relationship between these data and the scRNA-seq results?

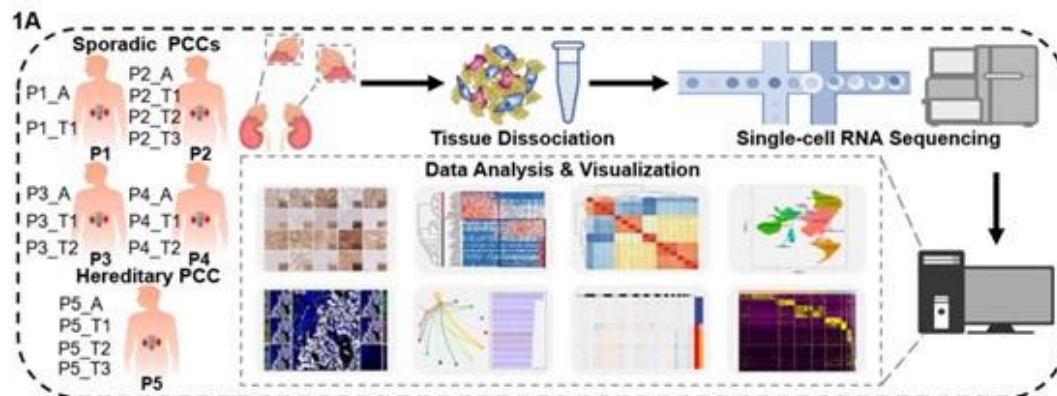
The secretion phenotype showed that the kinase-type PCC patient (P4) exhibited higher plasma levels of catecholamine metabolites (3-methoxytyramine and normetanephrine) compared to metabolism-type PCC patients (P1-P3, and P5). This observation aligns with the elevated expression of phenylethanolamine N-methyltransferase (PNMT), an enzyme involved in the biosynthesis of catecholamine and linked to hypertension, in P4, as identified in the scRNA-seq data (Figure 4B and 4D) (Kennedy et al, 1993; Konosu-Fukaya et al, 2018; Nguyen et al, 2015). Meanwhile, we have not observed the correlation between tumor sizes and molecular classification. We have now included tumor sizes and laboratory test results of 5 PCC patients in the revised manuscript as Supplemental Table S3 (Page 11-12), and the relevant points have been discussed in the revision (Page 20).

1. Please revise Figure 1A, the meaning shown in the figure appears to dissociate the tissues of the patient's normal adrenal glands, which can be misleading.

We appreciate the reviewer for raising this concern. The schematic in Figure 1A has been revised accordingly.

Author response image 2.

(1A) Schematic of the experimental pipeline. 11 tumor specimens and 5 adjacent normal adrenal medullary specimens were isolated from 5 PCC patients, dissociated into single-cell suspensions, and analyzed using 10x Genomics Chromium droplet scRNA-seq.



- Please revise the figure note for Figure 1B, where the symbol (B) appears twice.

As suggested, we have revised the figure legends for Figure 1B and 1C (Page 42).

1. Please indicate in the figure legends and text what exactly is meant by "adjacent specimens"? medulla? cortex? normal tissue? I believe the authors mean adjacent normal adrenal medullary tissue, please check the article.

As suggested, we have revised the term "adjacent specimens" to "adjacent normal adrenal medullary tissues" throughout the revised manuscript.

1. Please review the pathologic diagnostic criteria of this study in light of the 2022 WHO/IARC guidelines for pathologic diagnosis: "For the pathological diagnosis, the inclusion criteria were neuroendocrine neoplasm originating from the adrenal medulla and retroperitoneal origin, i.e. pheochromocytoma and paraganglioma, with consistent morphologic and immunohistochemical confirmation in relevant cases and positivity for chromogranin A and synaptophysin. The exclusion criteria were adrenocortical neoplasm and metastatic tumors." It is not rigorous enough to diagnose a tumor as PCC based on positive CgA immunohistochemical staining results alone.

We have revised the statements about pathologic diagnostic criteria in accordance with the suggestion and have cited the reference (Page 6).

We would like to express our gratitude to the reviewer for the thorough review and invaluable guidance provided to enhance the quality of our study.

References for Reviewer #2:

Kennedy B, Elayan H, Ziegler MG (1993) Glucocorticoid hypertension and nonadrenal phenylethanolamine N-methyltransferase. *Hypertension* (Dallas, Tex: 1979) 21: 415419

Konosu-Fukaya S, Omata K, Tezuka Y, Ono Y, Aoyama Y, Satoh F, Fujishima F, Sasano H, Nakamura Y (2018) Catecholamine-Synthesizing Enzymes in Pheochromocytoma and

Nguyen P, Khurana S, Peltsch H, Grandbois J, Eibl J, Crispo J, Ansell D, Tai TC (2015) Prenatal glucocorticoid exposure programs adrenal PNMT expression and adult hypertension. The Journal of endocrinology 227: 117-127

Reviewer #3 (Recommendations For The Authors):

I have several concerns and suggestions, which if addressed would improve the manuscript.

- 1. The statements of “plasmas” in the manuscript and figures are confusing, which should be revised as “plasma cells”.*

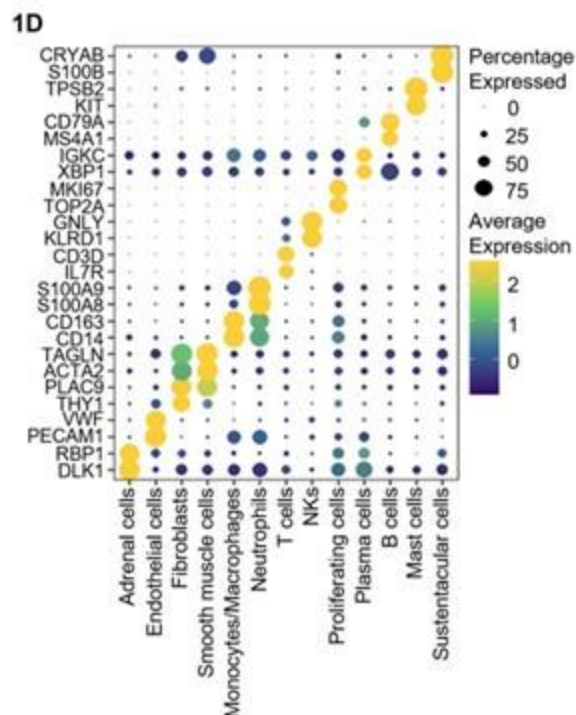
As suggested, we have revised the terminology from “plasmas” to “plasma cells” throughout the revised manuscript and figures.

- 1. The marker genes used for defining plasma cells (IGHG1 and IGLC2) showed low expressing percentage in Figure 1D. Please consider providing other genes as the marker of plasma cells.*

As suggested, we performed additional analysis to pinpoint marker genes for accurate definition of plasma cells. Applying stricter statistical criteria (cut-off pvalue < 0.05, log2 fold change ≥ 1.5 , and expressing percentage ≥ 0.6), we identified XBP1 (a transcription factor playing key roles in the final stages of plasma cell development) and IGKC (a type of light-chain immunoglobulins) (Todd et al, 2009; Poulsen et al, 2002) as top significant differentially expressed genes (DEGs) suitable for defining plasma cells. These data are now presented as Figure 1D in the revised manuscript (Page 7).

Author response image 3.

(1D) Dot plot of representative marker genes for each cell type. The color scale represents the average marker gene expression level; dot size represents the percentage of cells expressing a given marker gene.



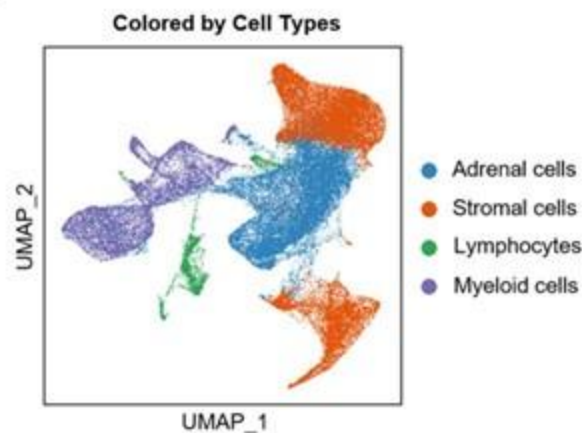
1. The statement “Our clustering and cell type annotation analysis identified diverse adrenal cells, stromal cells, and immune cells within the PCC microenvironment” seems not be exhibited in Figure 1, so the clustering result of adrenal cells, stromal cells, and immune cells need to be added.

As suggested, we performed clustering analysis for adrenal cells, stromal cells, and immune cells (including lymphocytes and myeloid cells), and visualized by the Uniform Manifold Approximation and Projection (UMAP) plot. These data have been added to the revised manuscript as Supplemental Figure S3 (Page 8).

Author response image 4.

Integration Analysis across 5 PCC Patients Revealing the Cell Type Composition of the PCC Microenvironment. UMAP plot depicting the distribution of adrenal cells, stromal cells, and immune cells (including lymphocytes and myeloid cells) within the PCC microenvironment.

S3



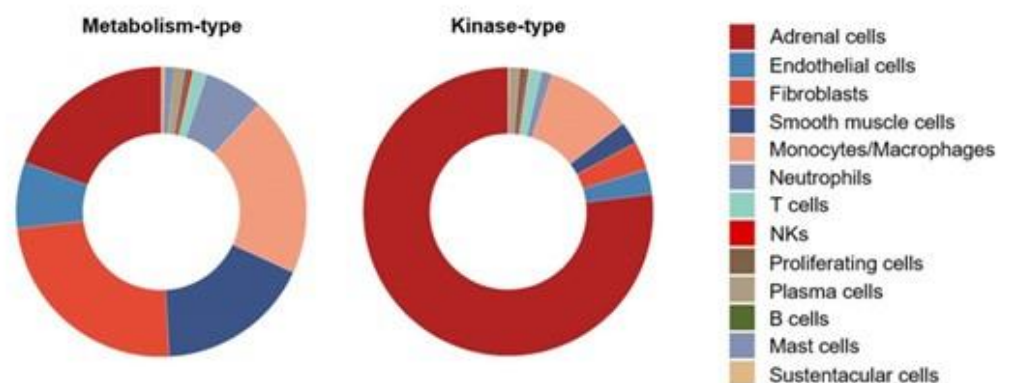
1. Given the classification of “metabolism-type PCCs” and “kinase-type PCCs” have not been presented in Figure 2D, the statement “Combined with our findings of a higher proportion of neutrophils and monocytes/macrophages in metabolism-type as compared with kinase-type” in Result 6 should be supported by using additional data.

As suggested, we performed additional analysis to evaluate the proportion of neutrophils and monocytes/macrophages in metabolism-type and kinasetype PCC patients. These data have been added to the revised manuscript as Supplemental Figure S4 (Page 14).

Author response image 5.

The frequency distribution of cell types within the microenvironment of metabolism-type and kinase-type PCC patients.

S4



1. What makes the difference of scRNA-seq analysis and multispectral immunofluorescent staining in judging the immune escape of PCCs? Please provide an explanation.

We appreciate the reviewer's concern. scRNA-seq lacks spatial details, and multispectral immunofluorescent staining is constrained in the number of detected proteins. To address this, we employed both methods for analysis. scRNA-seq revealed limited communication between tumor and T cells, with lower HLA-I expression in kinase-type PCCs compared to

metabolism-type PCCs. This was supported by multispectral staining using antibodies against CD4+ T cells, CD8+ T cells, M1 macrophages, or M2 macrophages markers, indicating sparse immune cell infiltration around tumor cells, mainly in the stroma (Figure 7A and 7B). This dual approach strengthens our understanding of immune escape in both PCC types. The explanation has been added to the revised manuscript (Page 21).

1. *Figure 7G missed the scale bar for the staining results of marker proteins. Please add the scale bar into the figure.*

As suggested, we have added to the scale bar accordingly.

1. *In the method part of the manuscript, the authors should describe the minimum and maximum number used for quality control of the number of genes and the percentage of mitochondrial genes.*

For quality control, we established a minimum threshold of no less than 200 genes and a maximum threshold of no more than 5000 genes. Additionally, the quality control process included a maximum threshold of 30% for mitochondrial genes. These specific criteria have been added to the methods section of the revised manuscript (Page 25-26).

We express our gratitude to the reviewer for their supportive recommendations and invaluable guidance on enhancing the rigor of our data.

References for Reviewer #3:

Todd DJ, McHeyzer-Williams LJ, Kowal C, Lee AH, Volpe BT, Diamond B, McHeyzer-Williams MG, Glimcher LH (2009) XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *The Journal of experimental medicine* 206: 2151-2159

Poulsen TS, Silahtaroglu AN, Gisselø CG, Tommerup N, Johnsen HE (2002) Detection of illegitimate rearrangements within the immunoglobulin light chain loci in B cell malignancies using end sequenced probes. *Leukemia* 16: 2148-2155