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RAPID COMMUNICATION

Integrated bulk and single-cell transcriptome data identify clinically relevant cell populations in clear cell renal cell carcinoma

Renal cell carcinoma, which consists of three main histological subtypes, namely, clear cell renal cell carcinoma (ccRCC), papillary RCC (pRCC), and chromophobe RCC (chRCC), accounts for most kidney cancer cases. ccRCC accounts for approximately 85% of all RCC cases,[1] and it is a malignant tumor with multiple molecular features and a poor prognosis. Studies have shown that ccRCC is one of the most immune and vaso-invasive cancer types.[2] Considering that ccRCC is not sensitive to either radiotherapy or chemotherapy, targeted therapies, such as vascular endothelial growth factor inhibitors/tyrosine kinase inhibitors and immunotherapy, are now the mainstay first-line therapies that are used in the clinic. However, a significant proportion of patients do not respond to the two treatments mentioned above or have high recurrence rates, which are related to the high degree of tumor heterogeneity and the tumor microenvironment (TME)[2] of ccRCC. Single-cell transcriptome sequencing provides accurate gene expression data at the single-cell level. Integrated single-cell and large transcriptome data from ccRCC samples provide comprehensive information, such as information about prognosis, metastasis, and response to sorafenib and anti-PD-1 treatment. In the present study, we aimed to integrate bulk and single-cell transcriptome data to reveal the clinically relevant cell types in renal cell carcinoma, aiming to reveal the underlying molecular mechanism.

ccRCC is an immunogenic tumor that is characterized by diverse populations of immune cells in the tumor microenvironment.[2] To reveal the intratumor heterogenicity of ccRCC, we re-analyzed the single-cell RNA sequencing data of only 7 ccRCC samples from among 14 samples that were used in a previous study[3]; from these samples, a total of 14,622 single cells were analyzed, and 7 major cell types (Fig. 1A), including cancer cells (CCs), macrophages (MPs), T cells (TCs), cycling T cells (cycling TCs), endothelial cells (ECs), vascular smooth muscle cells (vSMCs), and B cells (BCs), were identified. A subset of T cells that expressed cell cycle markers, such as *MKI67*, *CENPF*, and *CDK1*, exhibited high proliferation and were termed cycling T cells (Fig. S2A). Notably, ECs, MPs, and vSMCs accounted for the majority of the noncancer cell types in the RCC microenvironment (Fig. S2B), suggesting that these cell types played essential roles in tumor maintenance and/or progression.

To associate cell types in the tumor microenvironment with clinical characteristics, such as prognosis, metastasis, and response to sorafenib and anti-PD-1 treatment, we integrated bulk and single-cell transcriptome data to identify these cell populations. To reveal tumor cell heterogenicity, we conducted a subclustering analysis of the cancer cells and identified two cancer cell subpopulations, termed C1 and C2 (Fig. 1B). The overrepresentation enrichment analysis (ORA) of their corresponding signature genes revealed that C1 and C2 were characterized by higher expression levels of genes related to oxidative phosphorylation and interferon-gamma response (Fig. S2C), respectively. Compared with the normal tissue samples in The Cancer Genome Atlas (TCGA),[4] the ccRCC samples had higher interferon-gamma response activities and lower oxidative phosphorylation activities, further demonstrating that ccRCC was an inflammation-related malignancy and gained energy not through mitochondrial respiratory capacity. Moreover, we also divided the ccRCC patients from TCGA[4] into high and low groups based on the relative activities of these pathways. The log-rank test revealed that patients with higher oxidative phosphorylation activity or lower interferon-gamma response activity might have

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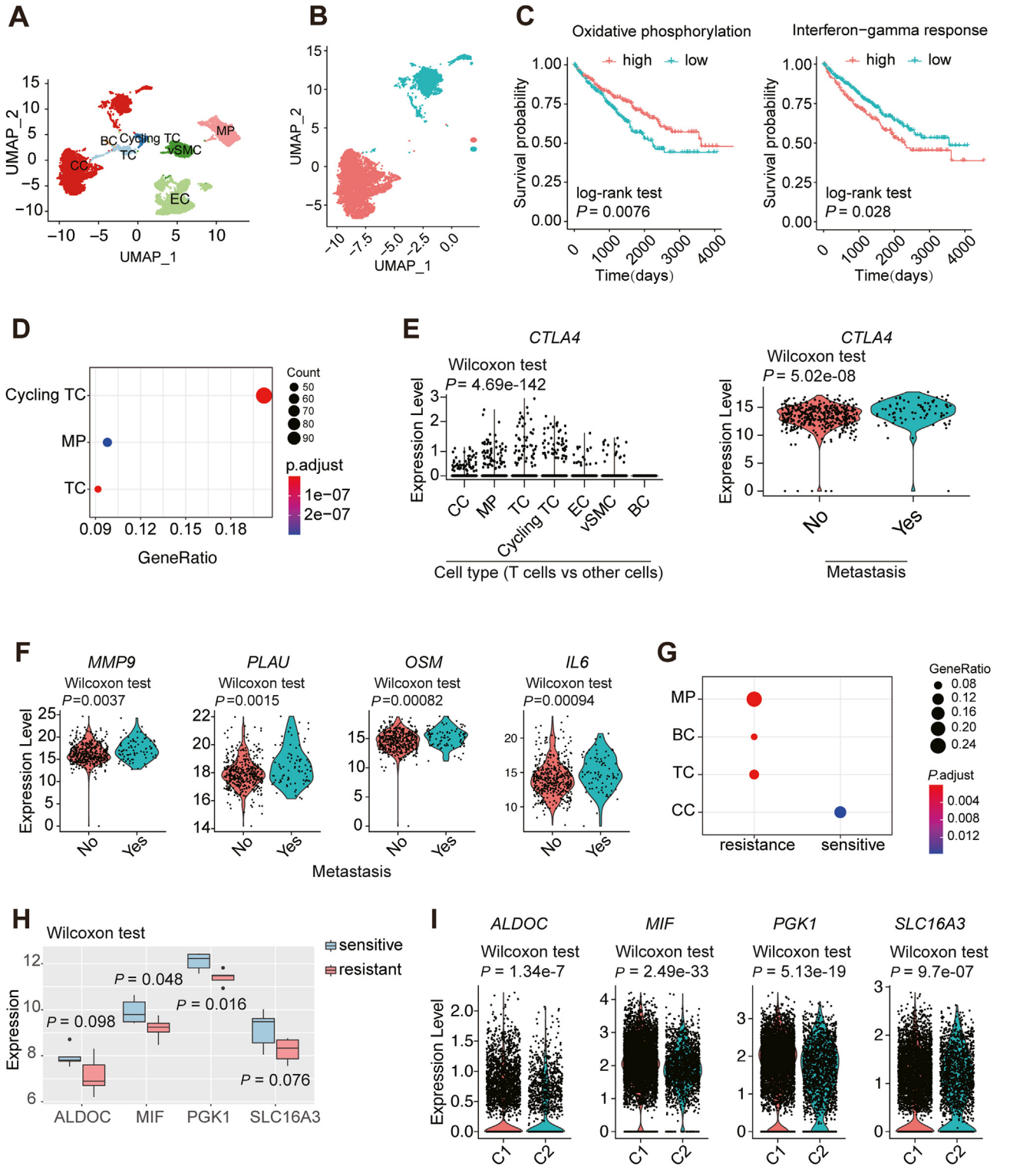

Figure 1 Integrated bulk and single-cell gene expression data were used to identify cell types associated with prognosis, metastasis, and treatment response. **(A)** UMAP analysis of the primary tumor samples. Each point represents one cell, and the point color represents the cell type. **(B)** UMAP analysis of cancer cells. **(C)** The prognostic value of the pathway scores of interferon−gamma response and oxidative phosphorylation. **(D)** The cell types enriched by metastasis-related genes. **(E)** The expression levels of the immune checkpoint gene CTLA4 in cell types of ccRCC (top) and TCGA primary tissues (bottom). **(F)** The differential expression levels of metastasis-related genes between primary samples with and without metastasis from TCGA. **(G)** The cell types enriched by genes associated with sensitivity or resistance to sorafenib treatment. **(H)** The differential expression levels of glycolysis-related genes between the sensitive and resistant tumor samples. **(I)** The differential expression levels of glycolysis-related genes between the two cancer cell subpopulations.

prolonged overall survival (Fig. 1C), suggesting that the signatures of the two subpopulations could predict prognosis.

As distal metastasis is a hallmark of cancer progression, we found that TCs and MPs were potentially associated with ccRCC metastasis. Specifically, we compared the gene expression profiles of primary tumor tissues with distal metastasis (TNM stage IV) with those of primary tumor tissues without distal metastasis (stage I—III) from the TCGA cohort[4] to identify the differentially expressed genes associated with ccRCC metastasis. The gene set over-representation enrichment analysis of these metastasis-related genes against the cell type marker genes revealed that the metastasis-related genes were significantly enriched in the marker genes of cycling T cells (cycling TCs), T cells (TCs), and macrophages (MPs) (Table S1, q-value <0.05; Fig. 1D). Notably, some genes encoding immune checkpoint proteins, including CTLA4 (Fig. 1E), LAG3, PDCD1 (PD-1), and CD274 (PD-L1), were highly expressed in cycling TCs and TCs (Fig. S1A). We used the same method to investigate whether some MP subpopulations primarily contributed to tumor metastasis. Specifically, MPs could be further clustered into three subpopulations characterized by pro-inflammatory (IL1B, CCL4, and CCL3), M2 (MRC1, CD163, MSR1, and STAB1), and monocyte-like (FCN1, S100A6, and CD52) markers (Fig. S2D, 1E). The proinflammatory macrophage subpopulation showed high consistency with the macrophage-A subpopulation described in a previous study.[5] Interestingly, we found that proinflammatory macrophages might promote tumor metastasis, as they expressed some metastasis-related genes, such as *MMP9*, *PLAU*, *OSM*, and *IL6* (Fig. 1F, Wilcoxon test, $P < 0.05$). These results indicated that proinflammatory macrophages and exhausted T cells were associated with tumor metastasis.

Furthermore, we also investigated the cell types associated with sorafenib and anti-PD-1 treatment responses. To identify the cell types associated with sorafenib treatment outcome, we collected gene expression data from 10 tumor samples, including 5 tumors that were sensitive to sorafenib and 5 tumors that were resistant to sorafenib (Gene Expression Omnibus accession: GSE87121). The resistance genes were significantly enriched in the marker genes of immune cells such as MPs, BCs, and TCs, while the sensitive genes were enriched in marker genes of CCs (Fig. 1G). Notably, the genes associated with sensitivity to sorafenib that were expressed by CCs, including *ALDOC*, *MIF*, *PGK1*, and *SLC16A3*, were involved in glycolysis (Fig. 1H, Wilcoxon test, $P < 0.05$). Glycolysis-related genes were expressed at higher levels in cancer cell subpopulation C1 than in C2 (Fig. 1I, $P < 0.001$). These results indicated that metabolic reprogramming of cancer cells might be a signature of sensitivity sorafenib treatment.

As ccRCC is highly infiltrated by T cells (TCs), we then investigated the cell types associated with the response to anti-PD-1 immunotherapy. The sensitive or resistance genes that were differentially expressed in tumors that responded to nivolumab treatment or tumors that did not respond to nivolumab treatment were identified by differential gene expression analysis of a previously published dataset[5] (GEO accession: GSE67501). The ORA revealed that the resistance genes were enriched in the marker genes of cancer cells (CCs) (Table S2, $P < 0.05$). Furthermore, we also investigated the cell—cell contact between CCs and TCs. CCs were predicted to interact with TCs via SPP1-CD44, MIF (macrophage migration inhibitory factor)-(CD74/CD44/CXCR4), and CD70-CD27 (Fig. S2F). Notably, recombinant human secreted phosphoprotein 1 (SPP1), also called osteopontin (OPN), was expressed by CCs, and the network centrality scores for the SPP1-induced signaling pathway revealed that CCs and TCs had high importance in sending and receiving signals (Fig. S2G). Therefore, we found that CC marker genes were associated with anti-PD-1 immunotherapy resistance, and we speculated that CCs were predicted to interact with TCs via SPP1-CD44. SPP1/CD44 is considered an immune checkpoint that controls $CD8^+$ T-cell activation and tumor immune evasion. Consistently, we compared $CD44^+$ TCs with $CD44^-$ TCs and observed that $CD44^-$ TCs had a higher expression of granzyme than $CD44^+$ TCs (Fig. S2H), suggesting that SPP1-CD44 signaling might inhibit T-cell activation in ccRCC.

Taken together, we re-analyzed ccRCC single-cell and bulk RNAseq data in a public database, and we identified 7 major cell types. Specifically, we observed two subpopulations in cancer cells, termed C1 and C2, characterized by higher expression levels of genes related to oxidative phosphorylation and interferon-gamma response, respectively. We found that exhausted T cells and proinflammatory cells might potentially promote ccRCC metastasis, and metabolic reprogramming of cancer cells is associated with sensitivity to sorafenib treatment. Moreover, cancer cells may attenuate the response to anti-PD-1 immunotherapy by inhibiting T-cell activation. In summary, we integrated bulk and single-cell gene expression data to identify the cell types associated with prognosis, metastasis, and treatment response, improving our understanding of the functional roles of cell populations in the ccRCC microenvironment.

Conflict of interests

The authors declare that there is no competing interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.007.

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