Single-Cell Transcriptomics Reveals Tissue Architecture in Ovarian Carcinosarcoma

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Research

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Abstract

Background

Ovarian carcinosarcoma (OCS) is one of rarest and most challenging histologic subtype of ovarian cancer. It features remarkable cellular heterogeneity. Using single-cell RNA sequencing, we characterize the cellular composition of the OCS and identify their molecular characteristics.

Methods

we applied single-cell RNA sequencing (scRNA-seq) to resected primary OCS for the in-depth analysis of tumor cells and the TME. Immunohistochemistry (IHC) staining was used for validation.

Results

Malignant epithelial and fibroblast cells displayed a high-degree of intratumoral heterogeneity. We revealed that certain epithelial cell subclusters had high levels of drug resistance scores and many active metabolic pathways. Furthermore, $\gamma\delta$ T cells exhibited enriched IFN γ and IFN α response characteristics. Notably, we observed that macrophages were mainly M2-like macrophages with immunosuppressive properties. In addition, we found that the CD1A+/FCER1A+ DC cells were enriched with genes related to cytolytic effector pathway. Analyzing ligand-receptor interaction pairs between cell types, we identified broadly interacting cells and observed an interaction between the ANXA1+ epithelial population and FPR1+/FPR3+ myeloid cells.

Conclusion

Our findings provide a comprehensive single-cell transcriptomic landscape of human OCS and present a well-established resource for elucidating OCS diversity.

Background

Ovarian cancer is the fifth most common cause of cancer-related death in women. American cancer society has reported that approximately 21,750 women have been newly diagnosed with ovarian cancer with 13,940 deaths in 2020 [1]. Ninety percent of ovarian cancer are of the epithelial cell type [2]. Ovarian carcinosarcoma (OCS), also known as malignant mixed müllerian tumors is one of rarest and most challenging histologic subtype, accounting for 1-4% of all ovarian cancer [3]. Yet, given the rarity of OCS, there is a paucity of prospective clinical trials to guide treatment strategies. Its management has been mainly extrapolated from the treatment experience of epithelial ovarian cancer. However, some observational studies have reported that OCS has a distinct natural history compared to the epithelial ovarian cancer [3, 4]. When compared to patients with epithelial ovarian cancer, most patients with OCS

have a worse survival rate. The reported five-year overall survival (OS) rate is 29.8%, with median OS time ranging from 8 to 26 months [5, 6]. Thus, understanding the molecular alterations remains a priority in order to fully characterize this disease and provide evidence-based diagnostic and therapeutic treatments and ultimately improve survival.

Current knowledge of OCS has shown that this tumor type exhibits high heterogeneity and contains both malignant epithelial and sarcomatous elements. It have been reported to over express p53 and harbored mutations in this gene [7]. Ki-67 seems to play a critical role in OCS. Indeed, high Ki-67 levels are found in half of the OCS and associated with shorter survival [8]. Evidence from the Surveillance, Epidemiology and End Results (SEER) data suggests that patients with OCS at tumor stage II and more advanced-stage had a poor OS, with HR for death of 1.19 to 5.88. Moreover, positive immunohistochemistry staining of tumoral CD8+ T lymphocytes and mesenchymal programmed death ligand-1 (PD-L1)-negative expression were found to be associated with better survival in OCS patients [9]. Of note, one recent case report has also found that a metastatic OCS patient showed an objective response to pembrolizumab [10]. Also, increased VEGF, VEGFR3 expression and vessel number were associated with poor survival [11]. These data indicate that the tumor microenvironment (TME) may play important roles in governing the plasticity of the phenotypic traits of tumor cells, as well as in mediating the response to therapies. Unfortunately, the conventional bulk sequencing techniques have limitations in their ability to resolve tumor TME. Recent advances in single-cell transcriptomics provide new insights into tumor composition and characterization of molecular properties of the complex tissues at a cellular resolution [12–16]. However, to date, studies of OCS lack a higher-resolution formation on cellular heterogeneity. Thus, a better understanding of the tumor cells and distribution of stromal and immune cells in the TME is critical for the success of clinical strategies for diagnosis or therapies of OCS.

In this study, we applied single-cell RNA sequencing (scRNA-seq) to resected primary OCS for the in-depth analysis of tumor cells and the TME. Our findings uncovered the characteristics of various cell populations of OCS and will provide important clues to develop OCS diagnosis and therapy.

Results

Single-cell transcriptional atlas and cell compositions in OCS tissues

We conducted scRNA-seq to profile one ovarian carcinosarcoma (OCS) sample with the 10×Genomics Chromium platform. This 61-year-old female patient was diagnosed as OCS at the late tumor stage IIIC. The primary ovarian tumor was in large volume (Fig. 1A). The H&E and immunohistochemistry (IHC) staining was conducted to establish the diagnosis of OCS (Fig. 1B-1F). IHC staining for cytokeratin (CK) and E-cadherin showed diffuse strong staining of the epithelial component (Fig. 1C). The mesenchymal element stained for vimentin and desmin (Fig. 1D). This tumor presented high cellular proliferation markers Ki-67 and p16 labeling indexes (Fig. 1E). In addition, overexpression of p53 was found in the OCS tumor (Fig. 1F). Studies have demonstrated that Ki-67 and p53 overexpression affect OCS overall survival [8, 17]. Recent study has been reported that pembrolizumab could provide tumor control in a

patient with metastatic OCS [10]. Here, we showed that this patient expressed focal PD1 expression (Fig. 1F), indicating that immunotherapy might be an effective treatment strategy for OCS.

To further explore the features of complex cellular components of OCS, we followed a customized workflow to isolate viable single cells from the primary surgical resections (Fig. 2A). Among the cells sequenced, 2,173 cells were retained after quality control filtering. By using a graph-based clustering method (see Materials and methods), cell clusters were annotated as epithelial cells, fibroblasts, ovarian cortex cells, endothelial cells, T cells, NK cells, NKT cells, or Myeloid cells (Fig. 2B) by established marker genes (Fig. 2C). All cell clusters identified from the OCS tumor could be visualized by a combined t-distributed stochastic neighbor embedding (t-SNE) plots (Fig. 2B). For example, endothelial cells were marked by VWF, CDH5 and ADGRL4 up-regulation, and epithelial cells were enriched with EPCAM, KRT7 and GPRC5A. Of note, the epithelial/cancer cells were the main cell type of this OCS sample and comprised 52% of the sorted cells. Fibroblasts expressed high levels of DCN, COL3A1 and LUM, and mast cells had a high level of CPA3, TPSB2 and CTSG expression. Myeloid cells were featured with HLA-DQA1, LYZ, IL1B overexpression, NK cells with GNLY, NKG7 and KLRD1 enrichment, NKT cells with FGFBP2 expression, and T cells with TRAC and CD3E overexpression. The ovarian cortex cells were enriched with TAGLN, RSG5 and THY1 expression (Fig. 2D).

Clustering-based copy-number variation resolves the malignant elements from non-cancer cells

Cancer cells are known to be associated with large-scale chromosomal alterations. Here, we applied copy-number variation (CNV) from the RNA expression data to classify epithelial cells as either cancer or non-cancer by compared with endothelial cells and macrophages (controls) [14, 18-20]. As shown in Fig. 3A and 3B, the epithelial cancer cells displayed much larger changes from the relative expression intensities across the genome. Of note, OCS contains both malignant epithelial and sarcomatous (mesenchymal) elements [3]. The sarcomatous component can be homologous (tissue physiologically native to the ovary) or heterologous (tissue foreign to the ovary). Homologous sarcomatous elements include fibrosarcoma and leiomyosarcoma, whereas the heterologous components usually contain chondrosarcoma, osteosarcoma, rhabdomyosarcoma or liposarcoma [21-23]. Thus, we also utilized CNV to verify fibroblasts and ovarian cortex cells as the malignant sarcomatous elements. As shown in Fig. 3C and 3D, we found that the fibroblasts displayed much larger chromosomal changes across the genome relative to endothelial cells and macrophages, but not the ovarian cortex cells. Furthermore, we validated the expression levels of epithelial cell markers EPCAM and KRT7 (Fig. 3E), and fibroblast markers DCN and COL3A (Fig. 3F) in human OCS tumor tissues using IHC staining. Taken together, malignant cell clustering reflected the epithelial cells and fibroblasts as the two malignant sources of OCS.

Transcriptional landscape intra-tumoral heterogeneity of OCS epithelial cells

We performed detailed clustering analysis of the 1,127 epithelial cells using Seurat and revealed the existence of six prominent cell subpopulations, namely C0 (RPL37+ / CDH6+), C1 (SLC2A1+ / ERO1A+), C2 (RPL13A+ / CXCR4+), C3 (S100A9+ / CEACAM6+ / LY6D+), C4 (XIST+ / CCNL1+ / WEE1+) and C5 (TOP2A+ / CENPF+ / MKI67+) (Fig. 4A, Fig. S1A). To learn more about the biology underlying these cell

subgroups, we first employed the KEGG pathway analysis to identify distinct signaling pathways and interrogated for transcription factor consensus sites using single-cell regulatory network inference and clustering (SCENIC) method (Fig. 4B and 4C, Fig. S1B). These analyses identified that C3 was controlled by the transcription factors TP63, HES2 and BCL6, and enriched for antigen processing and presentation pathway. We also found this cluster cells expressed high levels of immune-related signaling pathways such as type I and II interferon-response (Fig. S1C), suggesting possible cell-cell communication between tumor cell and immune cells. C1 showed distinct signature of HIF-1 signaling pathway and expressed high level of FOSL2. C4 displayed increased levels of TAF1 and spliceosome. The C0 and C2 subpopulations indicated a link to ribosome. C5 was controlled by elevated MYBL1 as well as NFYB and CTCF, and enriched with cell cycle.

In addition, we applied metabolism-related pathway analysis for these cell clusters. As shown in Fig. 4D, increased lysine biosynthesis, lipoic acid metabolism, thiamine metabolism, oxidative phosphorylation, and drug metabolism-cytochrome P450 were observed in C0 cells. C1 was D-Glutamine and D-glutamate metabolism, lysine degradation, Vitamin B6 metabolism, nitrogen metabolism. C2 and C4 cells were in suppressive states with the lowest levels of most metabolic pathways. C3 was activated with caffeine metabolism, linoleic acid metabolism, steroid hormone biosynthesis, glycosphingolipid biosynthesis and glycosaminoglycan degradation, while C5 showed the characteristics of highly activated lysine biosynthesis, biotin metabolism, pyrimidine metabolism and cyanoamino acid metabolism. We next focused on the drug resistance analysis, and further characterized the potential drug resistant states in detail. Our data revealed that C0 as well as C1, C3 and C5 had the high levels of drug resistance scores (Fig. 4E). Interestingly, these clusters also harbored activated metabolism pathways, suggesting a potential association between metabolism and drug resistance of the tumor cells. Of note, C1 subpopulation displayed activated transcription factors and growth factor receptors by the expressing of NFKBIE and IGF1R (Fig. 4F and 4G, Fig. S1D). C5 subpopulation was positively correlated with DNA damage/repair and drug resistance, and marked by the expression of BRCA1 and TOP2A (Fig. 4F and 4G, Fig. S1D).

Distinct fibroblast subpopulations in human OCS tumor

The fibroblasts were clustered in two subpopulations (Fig. 5A). These two subclusters expressed high levels of canonical fibroblast markers such as DCN and COL3A1; however, each subcluster displayed distinct transcriptomic signatures (Fig. 5B). C0 fibroblasts accounted for the majority of the fibroblast populations (61.7%) expressed high levels of extracellular matrix (ECM) signatures, including COL6A2, COL6A1, COL3A1, COL6A3 and COL5A2. Interestingly, gene ontology (GO) analysis of this subcluster indicated significant enrichment for ECM organization and disassembly, as well as angiogenesis (Fig. 5C). C1 fibroblasts were characterized of signature genes such as SPINK6, RERGL, KERA, STEAP2, RAMP1 and TMEM176B. The GO terms enriched for this subcluster were associated with translational termination, translational elongation and SRP-dependent cotranslational protein targeting to membrane (Fig. 5C). Notably, we found that the C0 fibroblast signature was enriched with a set of metabolism-related pathways, such as linoleic acid metabolism, drug metabolism-cytochrome P450, oxidative

phosphorylation and citrate cycle (TCA cycle) (Fig. 5D). However, most of these metabolic pathways were suppressed in C1 fibroblasts. The C1 signature was related to lysine biosynthesis (Fig. 5D). Next, we investigated whether these subtypes were correlated with known CAF types. However, we failed to group them into one certain CAF type (Fig. S2A). Interestingly, the C0 subtype was related to matrix CAFs (mCAFs), myofibroblastic CAFs (myCAFs), antigen-presenting CAFs (apCAFs) and inflammatory CAFs (iCAFs). As shown in Fig. 5E and 5F, the C0 fibroblasts expressed high levels of the type I interferon-response genes, such as IFITM3, IFI6, IFITM2, NAMPT, ADAR, LY6E, ISG15, and the type II interferon-response genes, such as STAT1, CDKN1A, HLA-A, HLA-B, HLA-C and HLA-E. This subcluster also expressed high levels of growth factors including PGF, VEGFB, IGF2, PTN, BMP2, BMP7, NRP1, GDF11, GPI and VEGFA (Fig. 5G), chemokines including CCL2, CXCL1, CCL28 and CXCL14 (Fig. S2B), and interleukins including IL11 and IL7 (Fig. S2C). These features of the C0 fibroblasts suggest an enrichment in the interplay between these fibroblasts and OCS cells.

Analysis of T cell and NK cell subpopulations defining the immune states in human OCS tumor

Tumor-infiltrating immune cells have been shown to play key roles in response to immunotherapy and tumor immune evasion [24]. As shown in the tSNE plots, the T and NK cells exhibited 9 distinct subclusters (Fig. 6A, Fig. S3A). According to their main marker genes, these subclusters were further grouped into seven subtypes: CD4⁺ T cells (CD3D+CD3E+CD4+CD8A⁻, subcluster 1 and 8); CD8⁺ T cells (CD3D+ CD3E+CD8A+CD4⁻, subcluster 4); gamma-delta ($\gamma\delta$) T cells (CD3D+ CD3E+TRDC+HOPX+CD8A-CD4⁻, subcluster 0 and 7); cycling immune cells (TOP2A+MKI67⁺, subcluster 6); NK cells (NKG7+GNLY+CD3D-CD3E⁻, subcluster 2); and NKT cells (NKG7+GNLY+CD3D+CD3E⁺, subcluster 5) (Fig. 6A and 6B, Fig. S3B). The subcluster 3 contained low quality cells, and were not further analyzed in this study. In addition, we found that NK cells, NKT cells and CD8⁺ T cells (subcluster 2, 5 and 4) expressed high levels of cytotoxic markers such as CST7, GZMA, GZMB, IFNG, NKG7 and PRF1 (Fig. 6C). Furthermore, the CD8⁺ T cells (subcluster 4) expressed a certain number of exhaustion markers, such as LAG3, PDCD1 and TIGIT, the $\gamma\delta$ T cells (subcluster 7) expressed high level of exhaustion marker HAVCR2, and the CD4⁺ T cells (subcluster 8) expressed high level of TIGIT, suggesting that these cells became exhausted (Fig. 6D). Notably, the subcluster 8 CD4⁺ T cells were characterized by marker genes related to Tregs (Fig. 6E), and display immune checkpoint inhibition characteristics with high expression of CTLA4 and TIGIT (Fig. 6F, Fig. S3C). The $\gamma\delta$ T cells play important roles in both innate and adaptive immune systems and certain subtype could contribute to IFN γ production [25, 26]. Similarly, hallmark pathways analysis in our study also revealed that the subcluster 7 of $\gamma\delta$ T cells were enriched in IFN γ and IFN α response, suggesting that this subcluster might be IFN γ -producing $\gamma\delta$ T cells (Fig. 6G).

OCS tumor contains distinct myeloid cell subtypes

Tumor-infiltrating myeloid cells, comprising of monocytes, macrophages, dendritic cells (DC), and neutrophils, have emerged as key regulators of cancer growth. We first dissected the gene signatures of 4 myeloid subtypes revealed in our study (Fig. S4A). Among these subclusters, monocytes (subcluster 1) were marked by APOE, C1QC and MAF, and macrophages (subcluster 2) were characterized by high

expression levels of VCAN, S100A9 and CD14 (Fig. 7A-C). The DC cells (subcluster 0 and 3) accounted for the majority of the myeloid cells (55.7%) and were enriched with HLA-DRs and low expression of CD14 (Fig. S4A and S4B), further distinguished by specific expression of CD1A/FCER1A, LAMP3/CCR7, respectively (Fig. 7A-C). We found that the subcluster 0 of DC cells were enriched with oxidative phosphorylation, while the subcluster 3 of DC cells were marked by folate biosynthesis, histidine metabolism, D-Glutamine and D-glutamate metabolism, tryptophan metabolism, and ascorbate and aldarate metabolism (Fig. 7D). The macrophages were activated with a certain set of metabolic pathways including glycosaminoglycan biosynthesis, porphyrin and chlorophyll metabolism, sphingolipid metabolism, glycosaminoglycan degradation, riboflavin metabolism, glycosphingolipid biosynthesis, ether lipid metabolism, steroid biosynthesis and degradation of aromatic compounds (Fig. 7D). Furthermore, genesets analysis showed that the macrophages in OCS tumor were mainly M2-like macrophages with immunosuppressive properties (Fig. 7E and 7F). The CD1A+/FCER1A+ DC cells were enriched with genes related to cytolytic effector pathway, cell cycle, type II interferon response and immune checkpoint suppression (Fig. 7E and 7F), while the LAMP3+/CCR7+ DC cells were characterized by increased levels of genes related to cholinergic receptors, immune checkpoint activation, CD8+ T cell activation and glutamate receptors (Fig. 7E and 7G).

OCS malignant cells broadly interact with the immune and non-immune compartments

The complex cell communication networks were primarily mediated by interactions between ligands and receptors [27, 28]. In this study, we explore the ligands-receptors pairs using intercellular interaction analyses to reveal the central cellular components shaping the OCS tissue fate in single cell maps (Fig. S5A). As shown in Fig. 8A, the ANXA1-FPR1/FPR3 pair was enriched in the interactions between epithelial cells and myeloid cells, consistent with the finding that ANXA1 signals activate tumor-associated macrophage infiltration and tumor progression [29-31]. Moreover, we verified that ANXA1 expression was mainly localized in the malignant epithelial region of OCS tissues by IHC staining, in accordance with the scRNA-seq findings that ANXA1 was highly expressed in the epithelial cells (Fig. 8B and 8C). Our results indicate that the ANXA1-FPR1/FPR3 axis is enriched in the interplay between epithelial cells and myeloid cells. Furthermore, epithelial cells showed interactions with fibroblasts through IGF2-IDE axis (Fig. 8D, Fig. S5B). In addition, myeloid cells interacted with NK, NKT and T cells through CCL5-CCR1 pair (Fig. 8E, Fig. S5C).

Discussion

OCS shows remarkable heterogeneity with aggressive tumor biology, associated with worse survival outcomes. Given the rarity of OCS, determination of the best management remains difficult. In this study, we are the first to employ scRNA-seq to comprehensively delineate a single-cell transcriptomic atlas of human OCS. Our data allowed us to identify unexpected biological features in distinct cell types and revealed novel cellular interactions between malignant cells and immune or non-immune cells.

OCS is composed of both epithelial and sarcomatous component [23]. The contribution of each element in the development and progression of the malignancy is in different extent. Thus, the preoperative diagnosis of this disease could not be reliably made by fine-needle aspiration and core biopsy. We here analyzed 2,173 cells of OCS tumor. This identified 9 cell types including epithelial cells, fibroblasts, ovarian cortex cells, endothelial cells, T cells, NK cells, NKT cells, and Myeloid cells. Each subtype showed specific marker genes and pathway activities, suggesting that they represent distinct biological entities. Among all these cell types, the epithelial cells accounted for the majority of the cell populations in our scRNA-seq data and believed to play a pivotal role in OCS. The most frequently encountered epithelial elements were serous, endometrioid and undifferentiated adenocarcinoma. However, cellular diversity of epithelial cells and how the epithelial subsets interact with OCS cells at single-cell resolution have not been well defined. We defined 6 distinct epithelial subtypes in OCS tissues, revealing the high intratumoral heterogeneity of OCS. Platinum and taxane based chemotherapy is known to be the most common adjuvant treatment for OCS [3, 32, 33]. However, OCS often recurs due to development of chemoresistance, with poor survival outcomes. Moreover, the current second line treatment options are limited. Thus, it is urgent to clarify the potential alterations to help the understanding of the tumor chemoresistance and ultimately improve the treatments of OCS. In this study, we found that four subclusters of epithelial cells expressed high levels of drug resistance genes. Of note, these epithelial subclusters also were also featured with many activated metabolic pathways, suggesting a potential association between metabolism and drug resistance of the tumor cells in OCS. Alterations of cellular metabolism could mediate the resistance of tumor cells to antitumor drugs [34]. Intervening the drug resistance genes or targeting several important metabolic pathways may restore cell sensitivity to chemotherapy. Besides the malignant epithelial cells, CNV analysis showed that the fibroblasts also displayed large chromosomal changes across the genome. Since sarcomatous is derived from stromal elements, our results suggest that the fibroblasts are the main source of malignant sarcomatous component of this OCS patient. Due to the restrictions in the number of fibroblasts analyzed, clearly discriminating fibroblast subtypes at single-cell resolution remains a challenge. We here demonstrated the presence of two fibroblast subtypes. The C0 fibroblasts were the most prevalent fibroblast subpopulation and expressed high levels of ECM signature genes and interferon-response genes, as well as growth factors, chemokines and interleukins, indicating that this fibroblast subset had the ability to potentially modulate other cell component response.

The immune component plays an important role in TME [35]. Understanding the mechanisms underlying the immune environment of OCS is of great interest. We revealed that the CD8⁺ T cells, CD4⁺ T cells and $\gamma\delta$ T cells expressed high level of exhaustion markers, suggesting that these populations may occupy important functional niches. Furthermore, the $\gamma\delta$ T cells were found to be enriched in IFN γ and IFN α response, indicating a IFN-producing $\gamma\delta$ T cell subtype in OCS tumors. $\gamma\delta$ T cells play uniquely important roles in antitumor immune responses through directly exerting cytotoxic effects to eliminate tumor cells or indirectly modulating the activities of other immune cells [36, 37]. Our results may provide a potential $\gamma\delta$ T cell-based immunotherapy. Our analyses also offer a nuanced view the myeloid lineage cells in OCS. Myeloid populations showed sharp state delineations. We found M2 associated genes frequently

expressed in macrophages. The important of understanding the DC subsets is similarly demonstrated. We revealed that the CD1A+/FCER1A+ DC cells were enriched with genes related to cytolytic effector pathway and immune checkpoint suppression. In contrast, the LAMP3+/CCR7+ DC cells were characterized by increased levels of genes related to immune checkpoint activation and CD8+ T cell activation. Additionally, our cell-cell interaction analyses predicted an unexpected interaction between the ANXA1+ epithelial population and FPR1+/FPR3 myeloid cells. A recent study revealed that ANXA1-FPR1 axis increased the aggressiveness and survival of breast cancer cells [38, 39]. IHC staining verified that epithelial cells expressed high levels of ANXA1. ANXA1 inhibition may contribute to OCS therapy.

Taken together, we have revealed a transcriptome landscape in primary OCS tumor at single-cell resolution and present a well-established resource for elucidating OCS diversity. Our data may advance the development of biomarkers and the identification of therapeutic targets of this aggressive disease.

Methods

Clinical specimens

Human ovarian carcinosarcoma tissues for scRNA-seq and IHC validation were all obtained from patients who had undergone resection in our hospital. For scRNA-seq, the patient was 61 years old with ovarian carcinosarcoma. This study was approved by the ethics committee of the Women's Hospital of Zhejiang University. Informed consent was acquired from each enrolled patient.

Isolation of single cells

After surgical resection, fresh samples were collected and washed with phosphate-buffered saline (PBS) for three times. Tissue was cut into approximately 1 mm³ pieces and enzymatically digested collagenase IV (Sigma), collagenase I (Sigma) and DNase I (Worthington) at 37°C for 30 min. After digestion, the sample was filtered out with a 35 µm cell strainer. Single cells were stained with AO/PI for viability assessment and live cells were preferentially sorted for single-cell sequencing.

Single-cell RNA sequencing and statistical analysis

The cellular suspension was loaded on a 10X Genomics Chromium Controller instrument to generate single-cell Gel Beads-in-emulsions. Then, scRNA-seq library was estimated by using Chromium Single-Cell 3' V3 Reagent Kits (10x Genomics). Sequencing was performed on the Illumina sequencer (Illumina, San Diego, CA) on a 150 bp paired-end run and the data analysis was performed by NovelBio Bio-Pharm Technology Co., Ltd.. Fastp was first used with default parameter to filter the adaptor sequence and remove the low quality reads to achieve the clean data [40]. Then, Cell Ranger (version 3.1.0) was used to obtain feature-barcode matrices by aligning to the human genome (GRCh38 Ensemble: version 91). Cells with less than 200 expressed genes and mitochondria unique molecular identifier (UMI) rate over 30% were removed. Subsequently, Seurat (version: 3.1.4) was used for the procession QC to obtain the scaled data. PCA was constructed based on the scaled data with top 2000 high variable genes. The top 10

principals were used for tSNE construction. We acquired the unsupervised cell cluster results by utilizing graph-based cluster method.

CNV estimation

To distinguish malignant cells from non-malignant cells in human OCS sample, we identify somatic copy number variations (CNVs) with the R package infercnv (v0.8.2). Cells with CNV signal above 0.05 and CNV correlation above 0.5 were identified as putative malignant cells.

SCENIC analysis

Single-cell regulatory network inference and clustering (pySCENIC, v0.9.5) was used to assess transcription factor regulation strength [41].

Gene enrichment analysis

QuSAGE (2.16.1) analysis was used to characterize the relative activation of a given gene set such as pathway activation [42].

Cell communication analysis

CellphoneDB was used to analyze the ligands, receptors and their interactions [43]. Significant mean and cell communication significance (P-value < 0.05) was determined based on the interaction and the normalized cell matrix achieved by Seurat normalization.

Immunohistochemistry (IHC)

All specimens were acquired from individuals with OCS. 4 µm thick formalin-fixed paraffin embedded (FFPE) human tissue sections were processed as previously described [44]. Briefly, slides were deparaffinized in xylenes and rehydrated in graded dilutions of aqueous ethanol (100% EtOH; 85% EtOH; 85% EtOH). Slides were placed in an antigen target retrieval solution (Dako) and pressure cooked for 15 min for antigen retrieval. Slides were incubated for 1 hour in a protein blocking buffer solution and then incubated overnight at 4C with either CK (#ZM-0308, ZSGB-BIO, 1:100 dilution), E-cadherin (#ZM-0092, ZSGB-BIO, 1:500 dilution), Vimentin (#ZM-0260, ZSGB-BIO, 1:200 dilution), Desmin (#ZA-0610, ZSGB-BIO, 1:150 dilution), Ki-67 (#ZM-0116, ZSGB-BIO, 1:200 dilution), p16 (#ZM-0205, ZSGB-BIO, 1:100 dilution), p53 (#ZM0408, ZSGB-BIO, 1:50 dilution), PD1 (#ZM-0381, ZSGB-BIO), EPCAM (#21050-1-AP, Proteintech, 1:200 dilution), KRT7 (#17513-1-AP, Proteintech, 1:2000 dilution), DCN (#14667-1-AP, Proteintech, 1:400 dilution), COL3A (#22734-1-AP, Proteintech, 1:1000 dilution), or ANXA1 rabbit polyclonal antibody (#21990-1-AP, Proteintech, 1:200 dilution). The following morning, the slides were washed and incubated with anti-rabbit and anti-mouse labeled polymer-HRP solution (Dako) for 1 hour. Slides were incubated with freshly prepared 3,3-diaminobenzidine chromogen solution and then counterstained in hematoxylin. Stained slides were imaged using an digital scanning microscopic imaging system (OCUS) using a 20X objective.

Declarations

Acknowledgments

None.

Authors' contributions

J.X. and W.L. conceived and designed the study. J.X. performed experiments and analyzed the scRNA-seq data. Y.C. contributed sample information collection and IHC analysis. J.X. and W.L. wrote the manuscript. All authors reviewed the manuscript and consented for publication.

Data availability

The scRNA-seq data of OCS has been deposited in Gene Expression Omnibus (GEO) with the accession number: GSE185014.

Ethical approval and consent to participate

This study was approved by the ethics committee of the Women's Hospital of Zhejiang University (IRB-20200346-R). Informed consent was acquired from each enrolled patient.

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Consent for publication

Not applicable.

Conflict of interest

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

Author details

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Figures

Figure 1

Feature of OCS. A CT scan showing the location and volume of OCS. B H&E staining in OCS tissue. C-F IHC staining of CK (C), E-cadherin (C), vimentin (D), desmin (D), Ki-67 (E), p16 (E), p53 (F) and PD1 (F) in OCS tumor.

Figure 2

Identification of OCS cell populations. A Schematic diagram of single-cell RNA sequencing (scRNA-seq) analysis workflow. B The t-distributed stochastic neighbor embedding (t-SNE) plots for the cell type identification of 2,173 high-quality single cells in OCS. C Heatmap showing the expression levels of specific markers in each cell type. D Expression levels of representative well-known marker genes across the cell types in OCS. Color key from green to orange indicates relative expression levels from low to high.

Figure 3

Single cell CNV inference analysis of OCS tumor. A and C The copy number variation (CNV) score and correlation for each cell in the indicated cells. Grey, CNA score > 0.05 and CNA correlation > 0.5. B and D Inferred large-scale CNVs help identify malignant and non-malignant cells. Amplifications (red) or deletions (blue) were inferred by averaging expression over 100-gene stretches on the respective

chromosomes. E IHC staining of EPCAM and KRT for epithelial cells in OCS tumors. F IHC staining of DCN and COL3A for fibroblasts in OCS tumors.

Figure 4

Transcriptomic heterogeneity of epithelial cells in OCS. A tSNE plots for 6 distinct epithelial cell subclusters. B Differences in pathway activity in epithelial cell subclusters. C Violin plots showing the expression of specific transcription factors in distinct epithelial cell subclusters. D Heatmap showing the

distinct activated metabolic pathways in 6 epithelial cell subclusters. E Distinct drug resistance scores in each single cell. Color key from blue to red indicates relative mean value from low to high. F Heatmap showing the different drug resistance-related pathways in 6 epithelial cell subclusters. G Violin plots showing the expression of certain drug resistant genes in distinct epithelial cell subclusters.

Figure 5

Distinct subtypes of fibroblasts in OCS tumor. A tSNE plots for fibroblast cells, color-coded for 2 subclusters. B Violin plots of the expression of marker genes for each cell subtype, as indicated. C Top 3 GO terms for each cell subtype. D Heatmap showing the differences in metabolic pathway activities in each cell subcluster. E and F Gene bubble plots showing different expression levels of type I (E) and type II (F) IFN-response genes in each cell subtype. G Gene bubble plots showing expression levels of growth factors in each cell subtype.

Figure 6

Distinct subtypes of infiltrating T and NK cells in OCS tumor. A tSNE plots for T cells and NK cells, color-coded for 9 subclusters. B tSNE plots, color-coded for the expression (gray to orange) of marker genes for each cell subtype. C-E Dot plots showing expression levels of specific cytotoxicity (C), exhaustion (D), treg (E) genes in each immune cell subcluster. F Violin plots of selected exhaustion markers in distinct T cell and NK cell subclusters. G Heatmap showing hallmark pathways in each cell subcluster.

Figure 7

Distinct myeloid subpopulations detected in human OCS. A tSNE plots of total myeloid cells. B tSNE plots, color-coded for the expression (gray to orange) of marker genes for each cell subtype. C Heatmap showing expression levels for marker genes with subpopulation-specific patterns in myeloid cells. D Heatmap showing the differences in metabolic pathway activities in each cell subcluster. E Heatmap showing the differences of genesets in each cell subcluster. F and G Dot plots showing expression levels of selected immune checkpoint suppressive (F) and active (G) genes in each cell subcluster.

Figure 8

Cell-cell interactions in OCS. A Dot plot showing receptor-ligand pair analysis of the interactions between epithelial cells and distinct cell types. B tSNE plots, color-coded for the expression (green to orange) of ANXA1-FPR1/FPR3 axis in OCS tissue. C IHC staining of ANXA1 in OCS tissues. D Dot plot showing receptor-ligand pair analysis of the interactions between fibroblasts and distinct cell types. E Dot plot showing receptor-ligand pair analysis of the interactions between myeloid cells and distinct cell types.

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