Single-cell RNA-Seq Reveals Transcriptional Landscape and Intra-tumor Heterogeneity in Gallbladder Cancer Liver Metastasis Microenvironment

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Research

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Abstract

Background: Gallbladder cancer (GBC) is a highly aggressive biliary epithelial malignancy. Tumor invasion and metastasis contributed to the high mortality of GBC patients. However, molecular mechanisms involved in GBC metastases are still little known.

Methods: We performed single-cell RNA sequencing on GBC liver metastasis tissue and analyzed the data based on different cell types.

Results: In this study, 8 cell types, including T cells, B cells, malignant cells, fibroblasts, endothelial cells, macrophages, dendritic cells, and mast cells were identified. Malignant cells displayed a high degree of intra-tumor heterogeneity and neutrophils could promote GBC progression in vitro. Besides, cytotoxic CD8⁺ T cells became exhausted and CD4⁺ Tregs presented immunosuppressive characteristics. Macrophages played an important role in the tumor microenvironment. We identified three distinct macrophage subsets and emerged M2 polarization. We also found that cancer-associated fibroblasts exhibited heterogeneity and promoted GBC metastasis.

Conclusions: In conclusion, our work provided a landscape view at the single-cell level and may clear the way for the therapy of GBC metastases.

Background

Gallbladder cancer (GBC) is a common biliary tract tumor with extremely poor prognosis and high aggressiveness[1, 2]. The majority of GBC patients have already reached an advanced stage at the time of diagnosis and only 10% of patients could get access to curative surgery[3]. Among those who underwent surgical resection, recurrence, and metastasis rates remain high. Hepatic invasion and metastatic progression are common and represent a major cause of mortality in GBC patients[4, 5]. However, the mechanisms of GBC cell metastasis have not yet been elucidated.

The tumor microenvironment (TME) plays an important role in tumor metastasis[6]. The GBC microenvironment consists of malignant cells, stromal cells, and extracellular components. Some factors such as TGFβ, IL-10, and VEGF secreted by cellular elements in the TME contribute to desmoplasia, immunosuppression, and metastasis[7]. The TME has become an attractive target for drug therapy strategies[8].

Single-cell RNA sequencing (scRNA-seq) provided a powerful tool to reveal cellular diversity and disclose microenvironment heterogeneity at single-cell resolution[9, 10]. In recent years, scRNA-seq has been used to dissect the complicated TME of several cancers such as hepatocellular carcinoma[11, 12], intrahepatic cholangiocarcinoma[13], colorectal cancer[14, 15], glioblastoma[16], and pancreatic ductal adenocarcinoma[17]. However, microenvironment heterogeneity at the single-cell level in human GBC liver metastases is still poorly understood.

In this study, we utilized scRNA-seq to examine single cells from human GBC liver metastasis tissue and identified 8 cell types corresponding to 7788 cells. Malignant cells presented intra-tumor heterogeneity and neutrophils may play an important role in GBC liver metastases. We proved that neutrophils facilitate GBC cell proliferation, migration, and invasion in vitro. Besides, tumor-infiltrating Tregs exhibited immunosuppressive characteristics and CD8⁺ cytotoxic T cells turned to be exhausted. We also identified 3 macrophage subtypes. M2 polarization was common in the TME. Furthermore, RGS5⁺ cancer-associated fibroblasts

Together, we hope this work could help understand tumor microenvironment composition and promote GBC metastasis therapy.

Methods

Tumor Sample Collection

Human cancer tissue was obtained from a female patient who was diagnosed as squamous cell carcinoma of gallbladder with liver metastasis. The tissue sample was collected with the approval by the institutional research ethics committee of the First Affiliated Hospital of Nanjing Medical University and informed consent was also signed for the enrolled patient.

Fresh Tissue Preparation and Dissociation into Single Cells

The fresh tumor tissue was immediately washed with phosphate-buffered saline (PBS) and then conserved in MACS Tissue Storage Solution (#130-100-008) at 4 °C. Tissue digestion was performed according to the Tumor dissociation Kit user guide (Miltenyi #130-093-237). Cell suspensions were filtered using a 70 µm nylon mesh.

10 µl of cell suspensions were counted by Trypan Blue to determine the concentration of live cells. Then the suspensions were centrifuged at 300 × g and 4 °C for 5 min, and the supernatant was discarded.

Library Preparation and Sequencing

We loaded viable cells that had undergone fluorescence-activated cell sorting (FACS) into a well of a microfluidic chip to generate a complementary deoxyribonucleic acid (cDNA) library using a Chromium Single-Cell Instrument (10X Genomics). Single-cell transcriptomic amplification and library preparation were performed by single-cell 3' Reagent Kit v3 (10X Genomics) according to the manufacturer's protocol. The library was loaded on an Illumina XTEN system.

Single Cell Sequencing Data Preprocessing, Quality Control and Subcluster Detection

The CellRanger software (v3.1.0) was used to process raw FASTQ files, align the sequencing reads to the GRCh38 reference, and generate a filtered feature-barcode matrix. The Seurat R package (v3.1.5)[18] was used to analyse our data. Cells with over 10000 genes and more than 25% of mitochondrial UMI counts

were removed. The remaining high-quality cells were normalized and scaled. High variable genes were calculated using FindVariableFeatures method with default parameters. The selected features were used for principal components analysis (PCA) analysis. Clusters were found using FindClusters function(resolution = 0.8). The t-Distributed Stochastic Neighbor Embedding (tSNE) analysis was used for dimension reduction and visualization of gene expression.

Cell Type Classification

We conducted tSNE dimensionality reduction with the first 50 principal components.

Cell clusters were annotated to known biological cell types using canonical marker genes and the putative CNV score.

Copy Number Variation (CNV) Inferring

We used the inferCNV (v1.2.1) R package to estimate the CNV score for each single cell. All T cells were served as the reference for inferCNV. The CNV score of each cell was calculated as quadratic sum of CNV region.

Estimate the Transcriptional Diversity of Each Cell

CytoTRACE (Cellular (Cyto) Trajectory Reconstruction Analysis using gene Counts and Expression) is a computational framework to predict the relative differentiation state of single cell[19]. We used the R software (v0.1.0) to predict the transcriptional diversity.

Pseudotime Trajectory Analysis

Trajectory analysis was performed using R package Monocle 2 (v2.14.0)[20]. The differentialGeneTest function was used to identify significant genes ($qval < 0.01$, $num_cells_expressed \geq 10$). Then cell ordering was conducted on these genes. The trajectories were visualized using DDRTree method.

Cell-Cell Communication Analysis

CellPhoneDB is a python-based tool to analysis cell-cell communication at the molecular level[21]. We used CellPhoneDB python package (v2.1.1) to analyze the potential interaction networks of the 8 major cell types.

Gene Regulatory Network Analysis

As described previously, we utilized SCENIC R package (v1.1.2-2) to infer gene regulatory networks and cell types from single-cell RNA-seq data[22]. Two gene-motif rankings including 10 kb around the transcription start site (TSS) and 500 bp upstream of the TSS were used to determine the search space around the TSS. 20-thousand motif database was downloaded for RcisTarget and GENIE3.

Functional Enrichment Analysis

DEGs were identified using FindAllMarkers function of Seurat. The cutoff threshold values were used: $adj.p.val < 0.05$, $min.pct > 0.25$ and $log\ fold\ change > 0.25$.

R package clusterProfiler[23] was used to perform the Gene Ontology (GO) enrichment analysis. Pathways which the adjusted p value < 0.05 were considered significantly enriched. Gene set variation analysis (GSVA) was performed in the GSVA R package (v1.34.0)[24] to evaluate pathway activity of 50 hallmark gene sets.

Cell Culture

The GBC cell line GBC-SD (RRID: CVCL_6903) was purchased from the Procell Life Science & Technology (Wuhan, China). The cell line has been authenticated using STR (or SNP) profiling within the last three years. The cell line was cultured at RPMI 1640 containing 2% fetal bovine serum (FBS). All experiments were performed with mycoplasma-free cells.

Isolation and Preparation of Human Neutrophils

Human neutrophils were isolated from the peripheral blood of healthy donors collected at the First Affiliated Hospital of Nanjing Medical University using Human neutrophils separation kit (Solarbio, Beijing, China). All donors signed the informed consents. Neutrophils were resuspended in RPMI 1640 containing 2% fetal bovine serum (FBS). Neutrophil-conditioned medium (NCM) was obtained as previously described[25].

Cell Proliferation Assay, Cell Migration and Invasion Assays

The CCK-8 Cell Counting Kit (Vazyme Biotech Co., Ltd) was utilized to assess the abilities of cell proliferation according to the manufacturer's protocol. In brief, we planted GBC-SD cell line with or without NCM into 96-well plates (2000 cells/well) with addition of CCK-8 solution (10 µl/well). After incubation for 2 hours in the incubator described as above, the absorbance at 450 nm was measured to determine the number of variable cells in each well.

Cell migration was assessed through the scratch wound assay. We made a uniform linear scratch using a 10-µl pipet tip on the GBC-SD cell lines which have been cultured with or without NCM for 2 days. The distance between the wound edges was taken as a baseline. Then we acquired the image of the same location after 48 h. The change of the distance was measured to assess the cellular ability of migration.

The 24-well transwell chambers (Millipore, USA) were used to evaluate the invasive capabilities of the cells. Totally, 1×10^5 cells were seeded in the upper chamber. The lower chamber was filled with 100 µL RPMI 1640 with 2% FBS or NCM. After incubation for 24 hours, the cells in the upper chamber were removed using cotton swabs. Cells on the lower surface of membrane were fixed and stained with crystal violet. The GBC cells were visualized under a inverted light microscope (Olympus, Japan), photographed, and counted in five microscopic fields.

All experiments were performed three times.

Statistical Analysis

All statistical analysis was performed using R platform (v3.6.0). $P < 0.05$ was considered statistically significant. Statistical differences between multiple groups were compared using Student's t-test or analysis of variance (ANOVA).

Results

Single Cell Transcriptomic Analysis Reveals the Cellular Diversity in Gallbladder Cancer (GBC) Liver Metastasis

To explore the potential mechanisms and microenvironment of GBC liver metastasis, we generated single-cell transcriptomic profile from a GBC liver metastasis tissue using 10X Genomics (Fig. 1A). A total of 7788 cells passed quality control. 8 distinct cell types were identified (Fig. 1B and Supplemental Fig. 1) using the following conventional markers: malignant cells (925 cells, marked with *EPCAM* and *KRT19*); T cells (4258 cells, marked with *CD2*, *CD3D*, *CD3E*, and *CD3G*); B cells (907 cells, marked with *CD79A* and *MS4A1*); Macrophages (1214 cells; marked with *CD68*, *CD163*, and *CD14*); Fibroblasts (264 cells, marked with *ACTA2* and *COL1A2*); Dendritic cells (DC) (79 cells, marked with *IL3RA* and *LILRA4*); Endothelial cells (64 cells, marked with *VWF*, *ENG*, and *CD34*) and Mast cells (77 cells, marked with *KIT* and *TPSAB1*). The dot plots of marker genes were shown in Fig. 1C. Further copy number variation (CNV) analysis was conducted to distinguish malignant and non-malignant cells (Fig. 1D and Supplemental Fig. 2). The differentially expressed genes (DEGs) in 8 cell types, as shown in Fig. 1F, confirmed the accuracy of cell recognition.

Transcriptomic Intra-tumor Heterogeneity of Tumor Cells in the GBC Liver Metastasis

In this study, we identified 7 subclusters after the reclustering of malignant cells (Fig. 2A). To investigate the heterogeneity of tumor cells, we used CytoTRACE to evaluate the transcriptional diversity of each cell which is a hallmark of developmental potential. As shown in Fig. 2B(left), the CytoTRACE score was distributed diversely indicating that tumor cells were in different differentiation states. Monocle 2 was used to construct a trajectory. Consistent with the CytoTRACE result, we noticed that the trajectory's root was populated by the majority of M0 cells (high CytoTRACE score). M3 and M4 cells (low CytoTRACE score) were present at the end of two differentiation branches (Fig. 2B right and Supplemental Fig. 3A). In addition, pathway analysis by gene set variation analysis (GSVA) revealed that hallmark pathways were partially activated in subpopulations M0, M1, or M2 with higher CytoTRACE scores, respectively. Particularly, the M4 subcluster had the lowest GSVA score (Fig. 2C). We conducted gene function enrichment analysis on DEGs of M0, M3, and M4 subclusters. As shown in Fig. 2D and Supplemental Fig. 3B, G2M/S cell cycle-related genes were enriched in M0. In M3, neutrophils-associated immune response and IFN-gamma signaling were commonly observed. Besides, the M4 cluster was mainly correlated with nucleocytoplasmic transport pathways. Not only in M3 but also in M2 and M5, neutrophils-related gene functions were enriched suggesting that there is a lot of crosstalk between

neutrophils and cancer cells during the metastasis of gallbladder cancer (Supplemental Fig. 3C-F). Different functional patterns of tumor cells may reflect diverse processes of the metastatic seeds adapted to the soil. In conclusion, all these results showed a high degree of intra-tumor heterogeneity in malignant cells of GBC metastasis.

Neutrophils promote proliferation, migration and invasion of GBC cells in vitro

Significant neutrophils infiltration has been observed in human cancer metastatic sites. To examine the effect of neutrophils on GBC cells, we incubated the GBC cell line (GBC-SD) with NCM. CCK-8 proliferation analysis showed that NCM promoted GBC cell growth in vitro (Fig. 3A). Wound-healing migration assays and cell invasion assays also demonstrated that NCM significantly enhanced the migration and invasion abilities of GBC cells (Fig. 3B and C).

Immunosuppressive Tumor-infiltrating Tregs and Exhausted CD8⁺ T cells are Enriched in the Metastatic Tumor

T cells were found to be the most prevalent cell type in the tumor microenvironment (TME) of liver metastasis tumor. The tumor-infiltrating immune cells were considered to play an important role in response to immunotherapy. As shown in Fig. 4A, T cells were clustered into 9 distinct subgroups. According to the known markers of T cells, 9 subclusters were annotated as T0-CD8T-GZMB(*CD8A⁺GZMB⁺*, cluster 0, 4), T1-CD8T-GZMK(*CD8A⁺GZMK⁺*, cluster 1), T2-Treg-FOXP3(*CD4⁺FOXP3⁺*, cluster 2), T3-CD4T-naïve (*CD4⁺IL7R⁺*, cluster 3, 7), T4-CD8T-proliferation (*CD8A⁺MKI67⁺*, cluster 5, 8), T5-CD8T- naïve(*CD8A⁺IL7R⁺*,cluster 6;Figure 4B and 4D). In this study, we found that CD8⁺ T cells (cluster 0,1,4,5,6,8) expressed different levels of cytotoxic markers like *GZMK*, *IFNG*, and *PRF1*. In proliferating CD8⁺ T cell populations (cluster 5 and 8), a set of T cell exhaustion-related immune checkpoints including *TIGIT*, *LAG3*, *HAVCR2*(*TIM3*), and *CTLA4* tend to be upregulated indicating that the CD8⁺ T cells became exhausted. We utilized Monocle 2 to perform pseudotime analysis on CD8⁺ T cells. The trajectory was visualized as a DDRTree plot (Fig. 4E). We can notice that naïve T cells tend to convert into proliferating CD8⁺ T cell subgroups which have been become exhausted suggesting that cytotoxic CD8⁺ T cells were turning to be exhausted. In addition, Tregs (cluster 2) expressed a certain number of immunosuppression markers such as *TIGIT*, *CTLA4*, and *TNFRSF18*(*GITR*). Interestingly, the expression *LAG3* and *HAVCR2* were found to be highly correlated with the activity of CD8⁺ T cells which was measured by the average expression of granzymes (GZMA, GZMB and GZMH) (Fig. 4F). Taken together, our analysis showed that in the GBC metastatic tumor environment, cytotoxic T cells tend to be exhausted and Tregs present a highly immunosuppressive phenotype. *LAG3* and *HAVCR2* may be a potential treatment target for patients with GBC liver metastasis.

Macrophages Have More Crosstalk with Malignant Cells and Tend to generate M2-type polarization

We used CellPhoneDB to infer intercellular communication in the TME. Macrophages were found to have more interactions with tumor cells, revealing an important role in the microenvironment (Fig. 5A). To investigate the heterogeneity of macrophages, we clustered macrophages into 3 subclusters (Fig. 5B). As shown in Fig. 5C, subcluster 0 cells highly expressed M2-type macrophage markers including *CD163*, *APOE*, and *MAF*. Cells from cluster 1 exhibited the M1-like phenotype. *SPP1* also known as secreted phosphoprotein 1 was also expressed in subcluster 1. It has been reported that pro-inflammatory molecules such as interferon- gamma and interleukin-1 β could induce *SPP1* expression which plays an important role in colon cancer liver metastasis in previous studies[26]. Figure 5D shows the expression of *SPP1* in liver hepatocellular carcinoma (LIHC) and cholangiocarcinoma (CHOL) for the lack of GBC expression data. *SPP1* was highly expressed in tumor tissues and correlated with overall survival in LIHC (Supplemental Fig. 4A) and disease-free survival in CHOL (Supplemental Fig. 4B). In addition, *MKI67* and *STMN1* which correlated with cell cycle were expressed by cells from subcluster 2. GSVA revealed that interferon (IFN) pathways were enriched in subcluster 0, however, the inflammatory response was also activated (Fig. 5F). SCENIC analysis showed that genes regulated by FOS, JUN, STAT1, ERG1, and RUNX1 were upregulated in subcluster 0 (Fig. 5E). Fos/Jun has been reported to be able to enhance inflammatory responses in macrophages. IRF7, which was activated in Macrophage subcluster 1, participates in the regulation of the M1-to-M2 phenotype switch[27]. In conclusion, M2-like macrophages remained some pro-inflammatory functions. Furthermore, trajectory analysis showed that M2-like macrophages were present at the end of the differentiation trajectory (Fig. 5G). All evidence supported the M2 polarization phenomenon in GBC metastasis.

Fibroblasts Exhibit Heterogeneity and promote GBC progression

As shown in Fig. 6A and B, cancer-associate fibroblasts (CAFs) from three subgroups were almost positive for α -SMA (*ACTA2*) which is a classical marker of fibroblasts. These three subgroups expressed different markers (cluster 0: *MAFB*⁺, *GPB5*⁺; cluster 1: *RGS5*⁺, *SMOC2*⁺; cluster 2: *ACTG2*⁺, *CCL11*⁺; Fig. 6C). The GO analysis results of DEGs in three distinct subgroups were shown in Supplemental Fig. 5. *RGS5*, a marker for vascular CAFs (vCAFs), has been reported to be related to invasion and metastasis of cancers such as liver cancer and lung cancer by inducing EMT [28, 29]. The SCENIC analysis revealed that genes regulated by TF NR2F2 and NFIL3 were upregulated in cells from the *RGS5*⁺ fibroblasts subgroup (Fig. 6D). Previous studies have proved that NR2F2 (COUP-TFII) is a transcription factor closely associated with tumorigenesis, invasion and metastasis in several digestive cancer such as pancreatic[30], colorectal[31], and gastric cancer[32]. NFIL3 also known as E4-binding protein 4 (E4BP4) is a leucine zipper transcription factor in the immune system. Lin et al. found that cellular prion protein transcriptionally regulated by NFIL3 promotes lung cancer invasion and metastasis[33]. In conclusion, all

these data suggested that RGS5⁺ fibroblasts may contribute to the tumorigenesis, invasion, and metastasis of GBC.

Discussion

GBC is a highly aggressive and lethal tumor[34]. Currently, the therapy of GBC, especially metastatic GBC, is still a challenge[2, 3, 34]. Only a few patients could benefit from curative resection, however, the prognosis is poor. The mechanisms underlying GBC metastasis are little known. Several studies showed that TME participates in tumor progression and metastasis. Disrupting the pro-tumorigenic TME is a promising therapeutic target for cancer patients. In this study, we employed scRNA-seq to delineate the transcriptomic heterogeneity of the TME in GBC liver metastasis at single-cell resolution.

In this work, several observations were found by describing intra-tumoral heterogeneity of the TME in GBC liver metastasis. First, GBC cells exhibit high heterogeneity at the metastatic site. The existence of cell subpopulations in different differentiation states fully reflects the complexity and diversity of GBC in its evolution. In previous studies, neutrophil-to-lymphocyte ratio (NLR) showed prognostic significance in GBC patients[35] and increasing neutrophil levels have a significant influence on the TME by inducing various cytokines and chemokines which could accelerate the proliferation and promote metastasis of tumor cells[36, 37]. Targeting neutrophils might become a new direction for the treatment of GBC metastasis. By pseudotime analysis and GO analysis, neutrophils-associated gene ontology was enriched in the intermediate stage of differentiation trajectory indicating that tumor cells communicate extensively with immune cells at the intermediate stage of metastasis. We also validate the influence of neutrophils on GBC cells in vitro. All evidence proved that targeting tumor-associated neutrophils is a promising method to antitumor metastasis for GBC patients.

Second, the high expression of *TIGIT*, *CTLA4*, *LAG3*, and *HAVCR2* in different T cells indicated a tumor-suppressive TME in GBC metastasis. Tregs are immunosuppressive T cells that participate in immune escape and block the antitumor immune response for cancer patients[38]. A set number of Tregs in GBC liver metastasis were observed. The infiltrating level of Tregs is correlated with the disease progression of GBC patients[39, 40]. Besides, *LAG3* and *HAVCR2*, known as exhausted markers, were found to be correlated with CD8⁺ T cells activity suggesting that *LAG3* and *HAVCR2* are the most prominent immune checkpoint molecules in GBC metastasis.

Third, three types of macrophages were identified from the TME. M1-like macrophages exhibit CD68⁺CD163⁺SPP1⁺ phenotype while M2-like macrophages exhibit CD68⁺IL1B⁺S100A8⁺ phenotype. M2 polarization was common in the TME.

By GSVA pathway analysis and SCENIC analysis, we revealed some underlying TFs and potential mechanisms involved in the M2 polarization phenomenon. Besides, transcription factor IRF8 was also found activated in the proliferative macrophage population. IRF8 could repress macrophage-derived extracellular matrix protein osteopontin and make it become a novel immunosuppressive checkpoint[41].

Osteopontin controls immunosuppression in the tumor microenvironment[42]. Interestingly, M1-like macrophages highly expressed secreted phosphoprotein 1 indicating that SPP1⁺ M1-like macrophages are correlated with immunosuppression and may be associated with immunotherapy outcomes for patients with GBC metastasis.

Finally, several TFs related to metastasis were found to be activated in RGS5⁺ fibroblasts via SCENIC analysis. A previous study indicated that RGS5⁺ CAFs (vCAFs) contribute to tumor progression in intrahepatic cholangiocarcinoma[13]. The data supported that RGS5⁺ CAFs is also a key factor in GBC progression.

Conclusions

Our findings provide a landscape of single-cell expression profiles in GBC liver metastasis and confirmed characteristics of cell subpopulations. We hope this study help address microenvironment-mediated GBC progression and drug resistance while identify novel therapeutic targets for GBC metastasis.

Abbreviations

GBC

Gallbladder cancer

scRNA-seq

Single-cell RNA sequencing

TME

tumor microenvironment

CNV

copy number variation

TF

transcription factors

DEGs

differentially expressed genes

GSVA

Gene set variation analysis

FBS

fetal bovine serum

PBS

phosphate-buffered saline

NCM

Neutrophil-conditioned medium

LIHC

liver hepatocellular carcinoma

CHOL

cholangiocarcinoma

CAFs

cancer-associated fibroblasts

NLR

neutrophil-to-lymphocyte ratio

Declarations

Ethics approval and consent to participate

The ethic approval has been accepted from the institutional research ethics committee of the First Affiliated Hospital of Nanjing Medical University.

Consent for publication

Not applicable.

Availability of data and materials

All data that obtained and analyzed during our study are available from the corresponding author once reasonably requested.

Competing interests

All authors of this study confirm that they have absolutely no conflicts of interest concerning this manuscript.

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Authors' Contributions

WY, XL, PW and BS contributed to study concept and design, perform experiment and data analysis, literature search, make figures and writing of the manuscript. YL, JZ, JQ and HH contributed to data search and analysis. LL and FC contributed to study design, literature search and review of the manuscript. All authors have approved the final version of this paper.

Acknowledgements

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Figures

Figure 1

The overview of cell types distribution in GBC liver metastasis. (a) The workflow of this scRNA-seq study. Metastatic liver tissue was dissociated into single cells and sequenced with 10X platform. (b) The tSNE plot of 8 cell types in this study and each color indicating an associated cell type. (c) Dot plots showing the expression of marker genes in 8 distinct cell types. (d) Box plots of CNV score for each cell type. (e) Heatmap showing the top 5 DEGs in each subtype.

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Figure 2

Transcriptomic heterogeneity of malignant cells in metastatic liver tissue. (a) tSNE map showing 7 cancer cell clusters. (b) Boxplot showing transcriptional diversity of cancer cells estimated by CytoTRACE (left) and differentiation trajectory of cancer cells in GBC liver metastasis (right). (c) Differences in 50 hallmark pathway activity scored by GSVA software. t values were calculated by a linear model. (d) GO analysis of DEGs for cluster 0 (top), cluster 3 (middle) and cluster 4 (bottom). FDR<0.05 was considered as significantly enriched.

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Figure 3

Neutrophils promote proliferation, migration and invasion of GBC cells in vitro. (a) Cell proliferation measured by CCK8 assay. NCM significantly promoted GBC proliferation. (b) Wound-healing migration assays showed that there was a significant increase in the wound closure rate of ICC cells co-cultured with NCM compared with the controls. (c) In vitro invasive assays showed that the number of invasive ICC cells was higher when cells were cultured with NCM than the control.

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correlation between the activity of CD8+ T cells, as measured by the mean granzyme expression (GZMA, GZMB and GZMH) and known immune checkpoint molecules.

Figure 4

Distinct subpopulations of infiltrating T cells in the metastatic TME. (a) tSNE plot for 9 T cell subclusters. (b) t-SNE plots showing the expression of marker genes for each cell type. (c) Violin plots of cytotoxic, proliferating, and suppressive genes in distinct T cell subclusters. (d) Heatmap showing the expression of

macrophages. (c) Average expression of canonical marker genes for macrophage subpopulations. (d) SPP1 expression in TCGA LIHC cohort and CHOL cohort. (e) Heatmap showing the area under the curve (AUC) scores of expression regulation by transcription factors estimated by SCENIC. (f) Heatmap of differences in 50 hallmark pathways scored per cell by GSVA. (g) Differentiation trajectory of macrophages with each color coded for subclusters (left) and pseudotime (right).

Figure 5

Macrophages emerge M2 polarization in the TME. (a) Interaction network of 8 cell types in the TME constructed by CellPhoneDB. Thicker line means more interaction. (b) tSNE plot of three subclusters of macrophages. (c) Average expression of canonical marker genes for macrophage subpopulations. (d) SPP1 expression in TCGA LIHC cohort and CHOL cohort. (e) Heatmap showing the area under the curve (AUC) scores of expression regulation by transcription factors estimated by SCENIC. (f) Heatmap of differences in 50 hallmark pathways scored per cell by GSVA. (G) Differentiation trajectory of macrophages with each color coded for subclusters (left) and pseudotime (right).

Figure 6

Three distinct fibroblast subpopulations detected in GBC liver metastasis. (a and b) tSNE plot of three fibroblast subpopulations(a) and the expression levels of α -SMA (ACTA2) (b). (c) Violin plots showing marker genes of three subclusters. (d) Differences of AUC scores in expression regulated by transcription factors (TFs).

Figure 6

Three distinct fibroblast subpopulations detected in GBC liver metastasis. (a and b) tSNE plot of three fibroblast subpopulations(a) and the expression levels of α -SMA (ACTA2) (b). (c) Violin plots showing marker genes of three subclusters. (d) Differences of AUC scores in expression regulated by transcription factors (TFs).

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