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Original Article

Single-cell RNA sequencing reveals transcriptome characteristics and clinical significance of gastric cancer-associated fibroblasts

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Abstract: Background: Cancer-associated fibroblasts (CAFs) are the most prominent components of the tumor microenvironment, which have received much attention for their role in tumorigenesis. However, most relevant studies have been based on bulk gene sequencing, and those based on the single-cell level are relatively sparse. This study aimed to reveal the transcriptomic profile of gastric CAFs and their clinical value with the help of single-cell RNA sequencing. Methods: Two patients with untreated gastric adenocarcinoma were included in this study, and comprehensive analysis of gastric and paracancerous tissues was performed by single-cell RNA sequencing to screen for markers associated with disease prognosis. Their biological functions were assessed by combining the results with data from patients with gastric cancer from The Cancer Genome Atlas (TCGA) database, screening for genes with high expression of CAFs, and identifying genes associated with survival. Results: After the quality control of single-cell sequencing data, a total of 9386 cells were obtained for further analysis. We identified seven cell type clusters: epithelium, B cells, T cells, endothelial, fibroblast, macrophage, and mast cells. KEGG pathway analysis revealed that the differential genes were enriched in the TGF-β signaling pathway, E-cadherin signaling in the nascent adherens junction, and the HIF-1-alpha transcription factor network, among other processes. Finally, we put the differential genes related to the TGF-β signaling pathway into the TCGA database for survival time analysis and found that high expression of GDF6 and GDF3 genes and low expression of BMPR2 and MAPK14 are associated with a longer survival prognosis. Conclusion: These data indicate that CAFs are highly different from those derived from paracancerous tissues. Meanwhile, as differential genes related to the TGF-β signaling pathway from CAFs, GDF6, GDF3, BMPR2, and MAPK14 can be used as markers for the duration of survival in gastric cancer.

Keywords: Cancer-associated fibroblasts, gastric cancer, single-cell RNA sequencing

Introduction

Gastric cancer (GC) is a highly heterogeneous type of tumor and one of the leading causes of cancer-related death worldwide [1]. Cancer-associated fibroblasts (CAFs), the most prominent mesenchymal cell component of the tumor microenvironment (TME), have received significant attention because of their role during tumorigenesis [2]. Most studies have shown that CAFs can promote malignant transformation through multiple pathways, including paracrine secretion of several factors, extracellular matrix proteins, and metabolic remodeling immune regulation [3, 4]. Single-cell RNA sequencing (scRNA-seq) is a technological innovation that overcomes the masking of cell subsets within the data from bulk RNA sequencing and allows for studying the transcriptome of individual cells and inferring the function of subsets of cells [5]. For example, in tumors, transcriptome analysis of single cells from patients with tumors clearly defines clusters of malignant and non-malignant cell types it can reveal stromal and immune cell interactions in the context of tumor growth, and enable more precise investigations of the TME [6, 7]. This method allows unbiased analysis of cell

characteristics in biological tissues and has been widely used to analyze tumor ecosystems, including cancer immune heterogeneity. Similarly, the recent analysis of GC using scRNA-seq is based on marker expression to classify cells into constituent cell types, including CAFs [8, 9]. However, the mechanism of CAFs and the occurrence and development of cancer still need extensive basic experimental and clinical research.

In this study, we performed a comprehensive analysis of GC tissues and paracancerous tissues by single-cell sequencing, focusing on evaluating the heterogeneity and properties of fibroblasts, screening out markers associated with disease prognosis or treatment to determine the clinical significance of CAFs. To further elucidate the signaling pathways, we analyzed these differentially expressed genes by KEGG pathway enrichment analysis, and the results showed the differentially expressed genes which were enriched in the TGF-beta pathway. We analyzed these differentially expressed genes by KEGG pathway enrichment analysis. In conclusion, with the aid of scRNA-seq strategies, our findings provide new insights into the mechanisms underlying GC progression and potential targets for therapeutic strategies.

Methods

Patients with gastric cancer

The current GC study developed here was based on samples taken from surgical remnants available after histopathological analysis of the patient's tissue that was not necessary for diagnosis. Therefore, the project does not interfere with clinical practice and does not increase the financial burden or insurance expenses. Analysis of primary tumor samples was performed following relevant national laws and accepted ethical guidelines (Declaration of Helsinki) to protect biomedical research. We have given a detailed explanation of the enrolled GC patients, stated the aim of this project, and obtained their informed consent.

The inclusion criteria for this study were initial diagnosis by pathological biopsy taken by gastroscopy, not receiving chemotherapy, biotherapy, or radiotherapy; exclusion criteria were metastatic cancer or already combined with distant metastases, combined with other tumors or autoimmune diseases, and hematologic disorders. Two patients were included in this study, both male (62 and 58 years old), one of whom had a family history of GC (his father died of GC 20 years prior).

Cell isolation from gastric cancer

Fresh human gastric cancer primary tumors were collected directly from the operating room after surgical specimen macroscopic examination and selected for areas of interest by a pathologist. Samples were cut into small pieces (around 1 mm^3) and digested in CO_2-independent medium (Gibco No.18045-054) supplemented with 150 μg/mL liberase (Roche No.05401020001) and DNase I (Roche No. 11284932001) for 40 min at 37°C with shaking (180 rpm). The digested cell suspension was strained through a 100-μm cell strainer with the plunger of a plastic syringe. After centrifugation for 3 min at 300 × g, red blood cells were lysed using RBL buffer containing 0.15 M ammonium chloride and 10 mM sodium EDTA in ddH_2O for 30 s. Cells were counted after additional straining through 70-μm mesh and centrifugation at 300 × g for 3 min. Cells were then filtered through a 40-μm cell strainer (Thermo Fisher Scientific No.223635447) and resuspended in PBS + solution (PBS, Gibco No. 14190; EDTA 2 mmol/L, Gibco No. 15575; Human Serum 1%, BioWest No.S4190-100) at a final concentration between 5×10^5 and 10^6 cells in 50 μL.

Single-cell RNA-seq

Cells from each sample were labeled with sample tags (BD™ Human Single-Cell Multiplexing Kit) following the manufacturer's protocol. The viability of cells in all samples was >80%. Labeled samples were pooled equally in cold BD Sample Buffer with approximately 30,000 cells. Single cells were isolated using Single-Cell Capture and cDNA Synthesis with the BD Rhapsody Express Single-Cell Analysis System, and cDNA libraries were prepared using the BD Rhapsody™ Whole Transcriptome Analysis Amplification Kit. The final libraries were quantified using a Qubit Fluorometer with the Qubit dsDNA HS kit (ThermoFisher). Libraries were sequenced in paired-end mode on a NovaSeq 6000 in the lab of Novogene Biotech Co. Ltd (Beijing, China).

scRNA-seq and quality control

Preprocessing of raw data was initially performed using the Cell Ranger software pipeline (version 2.1.1). This step included demultiplexing of raw base call files into FASTQ files, reading alignment on the human genome assembly GRCh38 using STAR, and counting of unique molecular identifiers (UMIs). As a quality control step, low-quality cells, empty droplets, and multiplexed captures were first filtered out based on the distribution of unique genes (nonzero counts) detected in each cell in each sample. Cells with less than 200 genes and cells with more than 5000 genes were excluded. The cellular distribution of the expression-based mitochondrial gene fraction was also plotted, discarding cells with a mitochondrial gene fraction above 30% to eliminate dying cells or low mass cells with extensive mitochondrial contamination.

Clustering and data visualization

Principal component analysis (PCA) dimensionality reduction was run using default parameters. Multiple included components (PCs) were assessed using the JackStraw procedure implemented in JackStraw and ScoreJackStraw functions. A graph-based clustering approach was used to cluster the cells from the first dataset using FindNeighbours and FindClusters functions. Clusters were obtained at this resolution. For visualization of the data, the nonlinear dimensional reduction technique Uniform Manifold Approximation and Projection (UMAP) was applied using the RunUMAP function from Seurat.

Statistical analysis and gene set enrichment analysis

Differentially expressed genes (DEGs) between the cancerous and paracancerous tissues were identified with >1.5-fold change and $P < 0.05$ by two-sided t-test, and DEGs were performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Kaplan-Meier analysis was performed for survival curves, and the significance was determined by log-rank with the package's survival and survminer in R 3.6.0.

Results

Single-cell sequencing and cell-type identification

The subjects of this study were two newly diagnosed patients with gastric adenocarcinoma, which had not been diagnosed with any other cancer. Patients did not receive chemotherapy or radiotherapy prior to surgery. After removal of low-quality cells, 9386 cells were retained for biological analysis, which detected a median of 982 genes and 3016 transcripts per cell (**Figure 1A**). After normalization of gene expression and principal component analysis (PCA), we used graph-based clustering to partition the cells into seven clusters (**Figure 1B**). Using marker genes, these clusters could be assigned to seven cell types: Epithelium (n = 3216, 34.26%, marked with KRT18, EPCAM), B Cell (n = 2858, 30.45%, marked with CD79A and MS4A1), T Cell (n = 1629, 17.36%, marked with CD3D and CD3E), Endothelial (n = 1208, 12.87%, marked with, ENG and VWF), Fibroblast (n = 362, 3.86%, marked with, COL3A1 and COL1A2), Macrophage (n = 75, 0.80%, marked with CPA3 and CD68), Mast Cell (n = 38, 0.40%, marked with CD14 and CD203C). The proportion of each cell lineage varies greatly among different samples (**Figure 1C** and **1D**).

Single-cell RNA sequencing reveals molecular and functional characteristics of gastric cancer-associated fibroblasts

CAFs coexist as a heterogeneous population, and several CAF subtypes with distinct molecular profiles have been identified in various cancers. We believe fibroblasts derived from tumor tissues differ greatly from those of fibroblasts derived from paracancerous tissues or normal tissues at the RNA level. Through single-cell sequencing data analysis, we screened out relatively high expression genes in CAFs, including COL3A1, RARRES2, IGF1, SFRP4, MMP14, and MFAP5 (**Figure 2B**). Among them, COL3A1 and MMP14 are characteristic genes of CAFs. In this study, RNA from fibroblasts derived from cancerous and paracancerous tissues was compared to screen out differential genes. Compared with the fibroblasts in paracancerous tissues, RORA, PDGFRA, FBLN5, LAMA2, and COL5A1 were overexpressed in CAFs, and MYH11, ABI3BP, GPM6B, MFAP4,

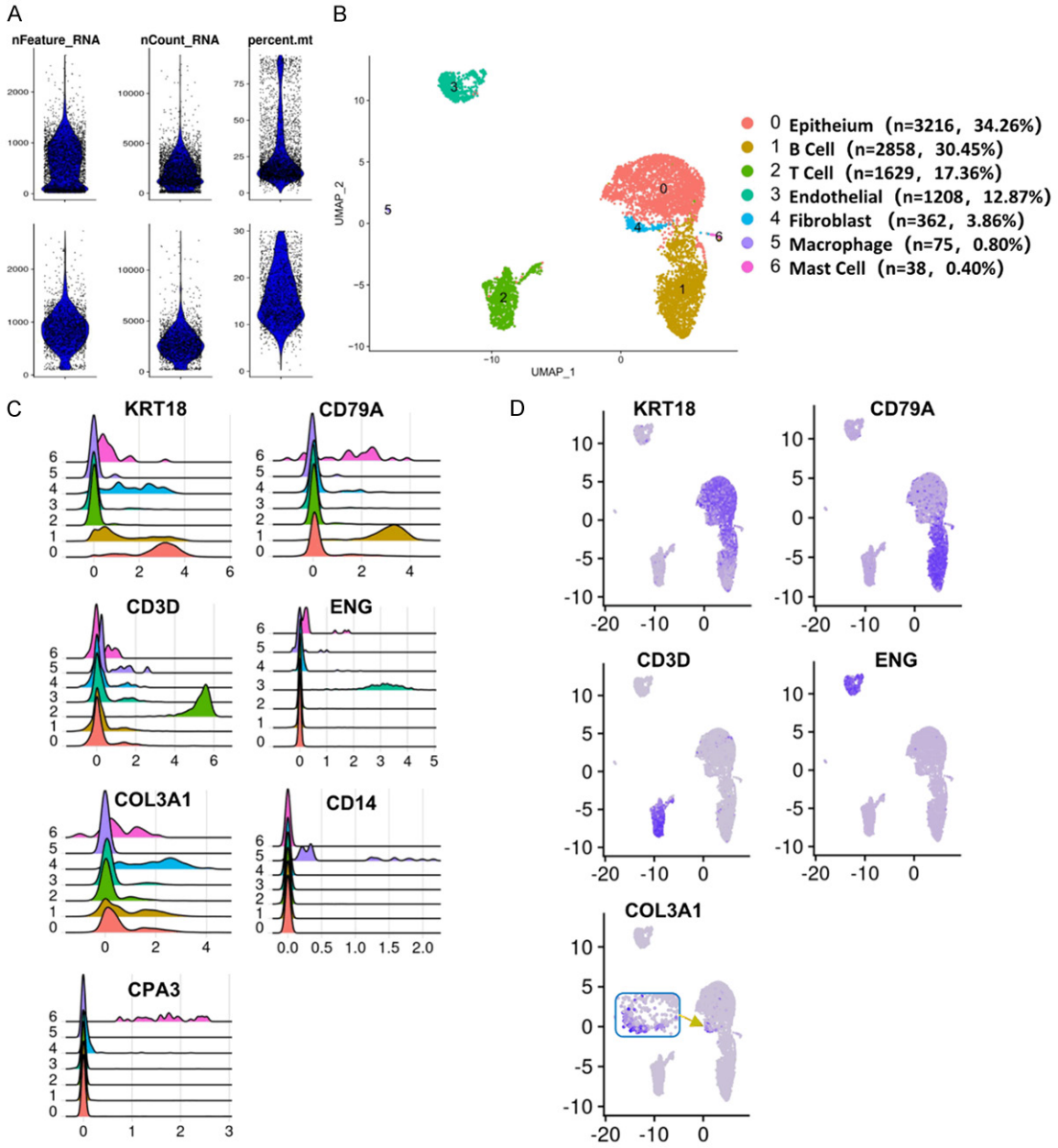
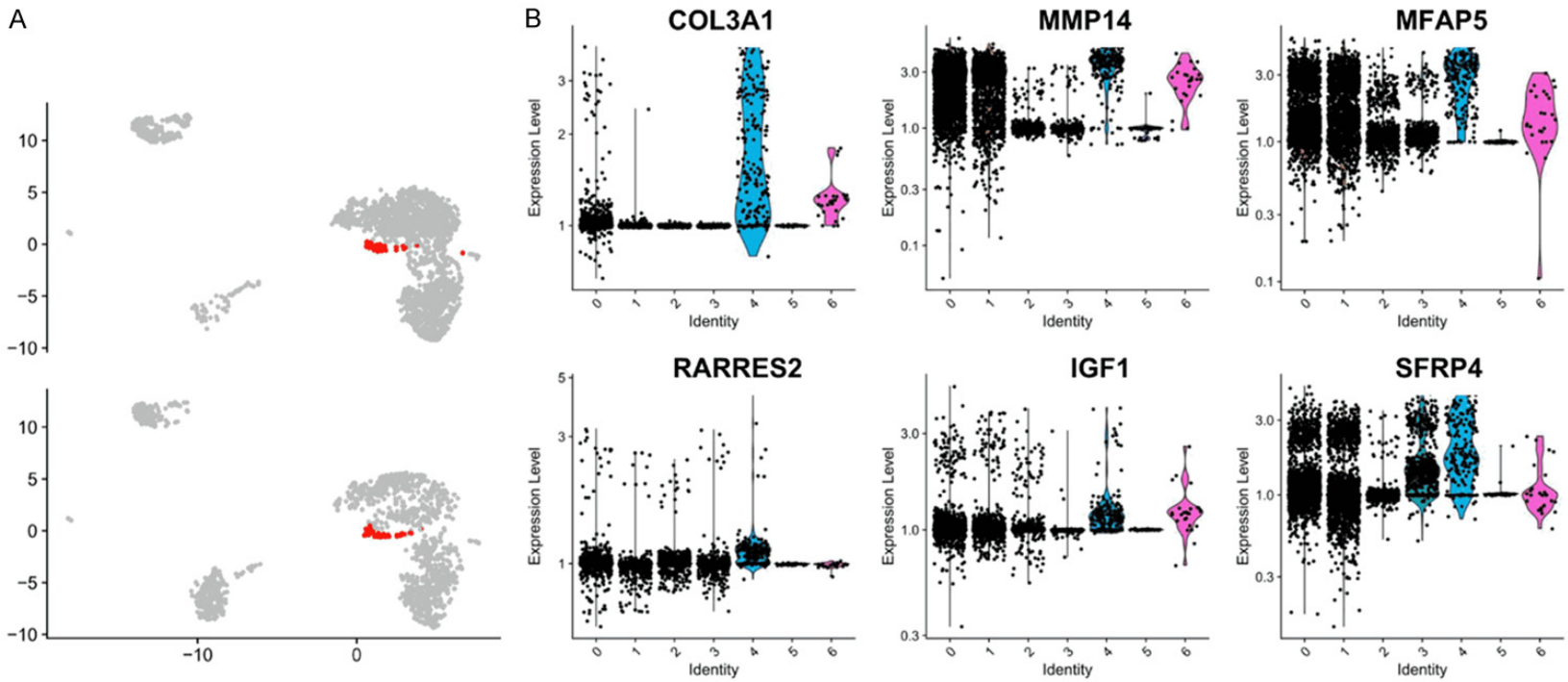


Figure 1. Single-cell RNA sequencing and cell-type identification. A. Process of raw data preprocessing. The upper part of the graph is unprocessed data, and the lower part of the graph is the result of removing the low-quality data. B. After normalization of gene expression, the clusters were identified as seven cell types with the marker genes. C and D. The most significant characteristic genes of each cluster.

and LAMA2 were downregulated in CAFs (**Figure 2C**). Next, we performed KEGG pathway analysis, which showed that the differential genes were enriched in the TGF-β signaling pathway, E-cadherin signaling in the nascent adherens junction, HIF-1α transcription factor network, RAC1 signaling pathway, validated transcriptional targets of AP1 family members FRA1 and FRA2, cAMP signaling pathway, AP-1 transcription factor network, VEGFR1 specific signals, validated targets of C-MYC transcriptional repression, enhanced monocyte differentiation, and protumoral tumor-associated macrophage activation (**Figure 3A**).

TGF-β signaling pathway in cancer-associated fibroblasts

The TGF-β signal pathway contributes to the maintenance of tissue homeostasis and the


A
B
COL3A1
MMP14
MFAP5
RARRES2
IGF1
SFRP4
Expression Level
Identity

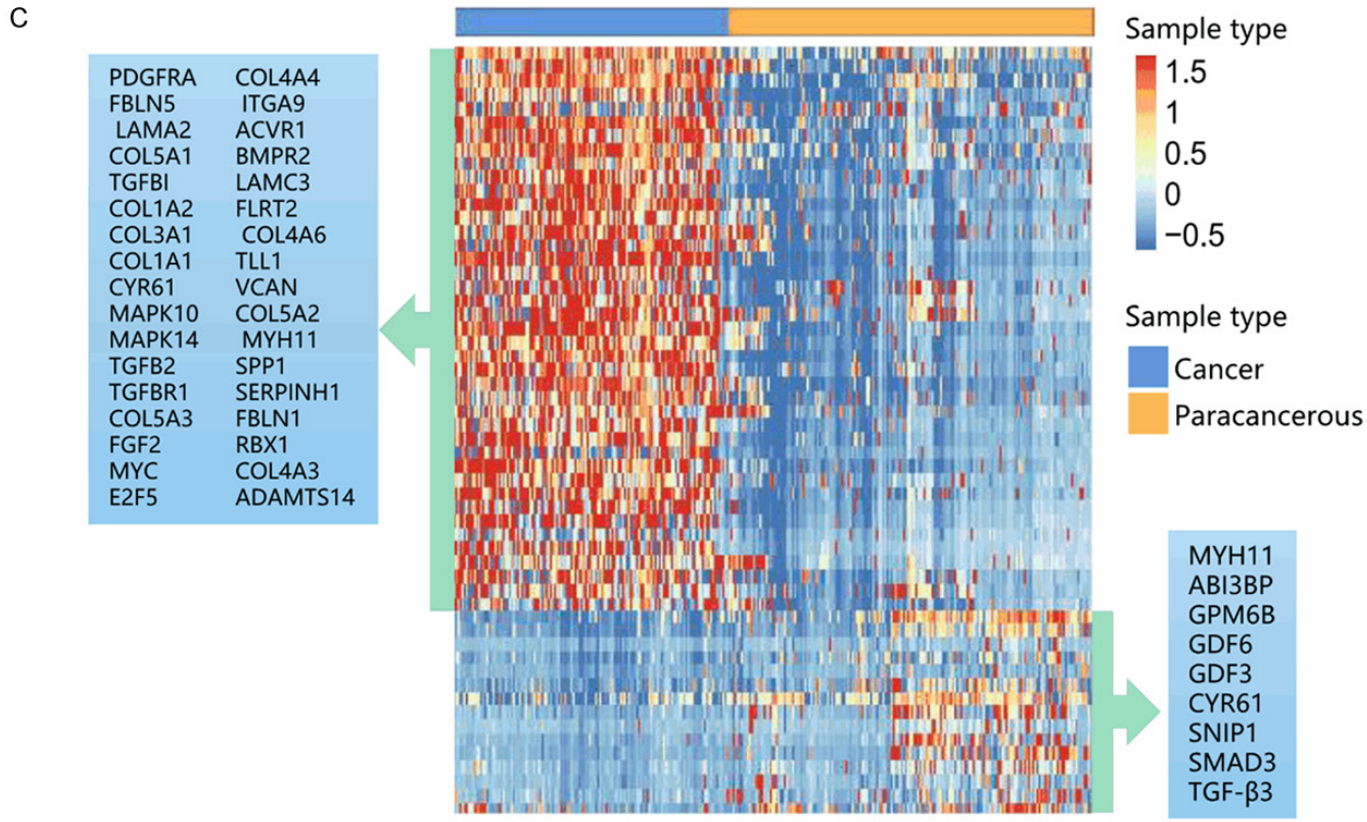
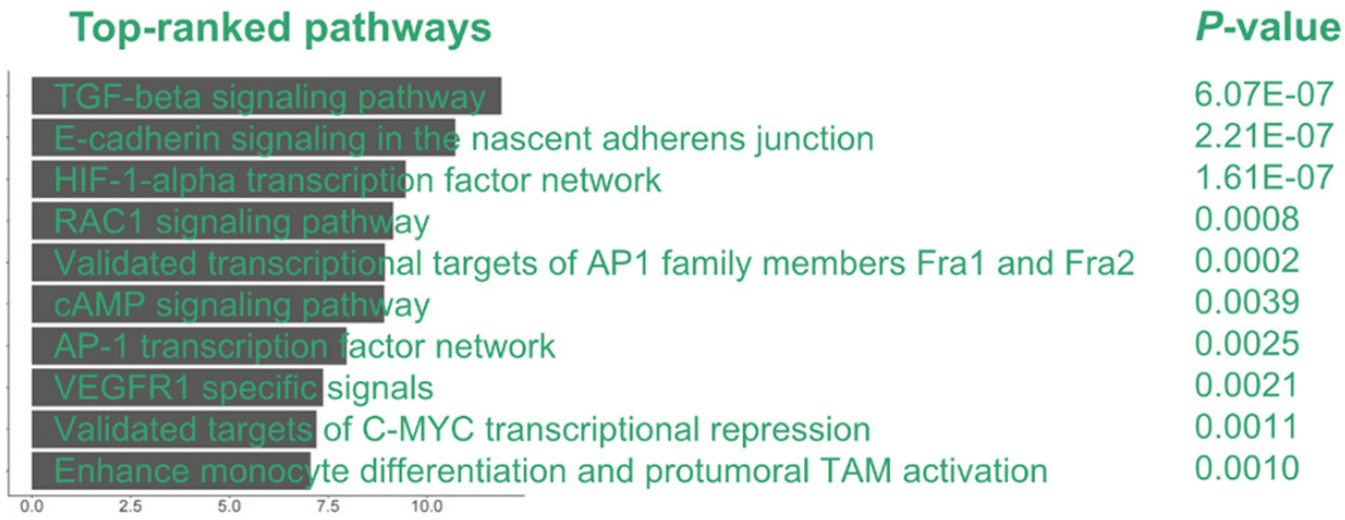
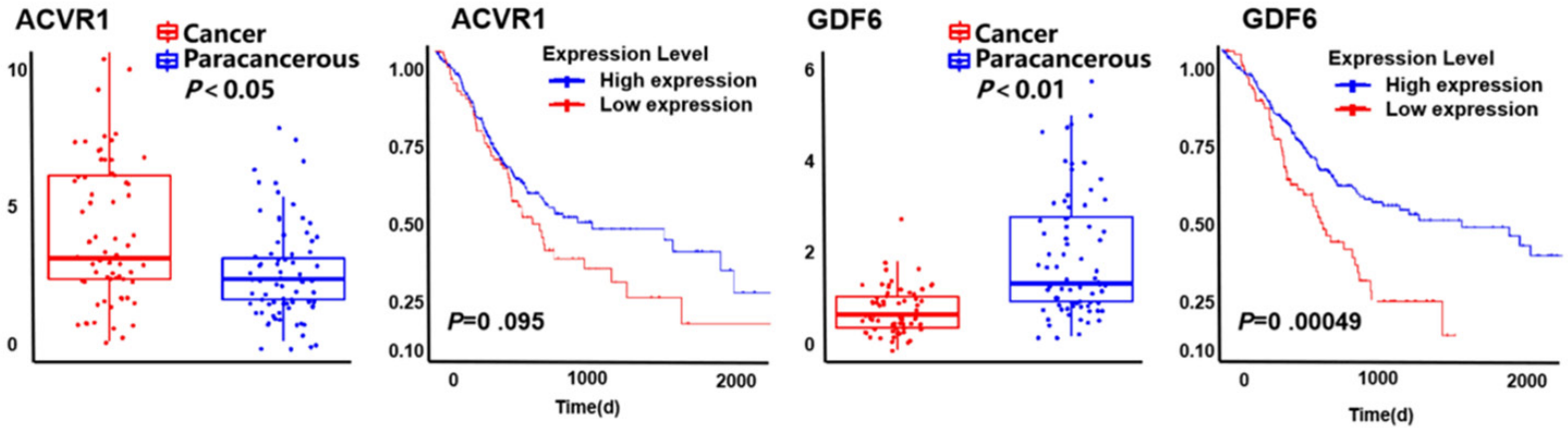


Figure 2. scRNA-seq reveals the characteristics of CAFs. A. The fibroblasts (CAFs) from cancer tissue (up) and paracancerous tissue (down). B. The marker genes of fibroblasts. C. The differential genes of fibroblasts in cancer tissues and paracancerous tissue.

A


Top-ranked pathways
P-value
TGF-beta signaling pathway
E-cadherin signaling in the nascent adherens junction
HIF-1-alpha transcription factor network
RAC1 signaling pathway
Validated transcriptional targets of AP1 family members Fra1 and Fra2
cAMP signaling pathway
AP-1 transcription factor network
VEGFR1 specific signals
Validated targets of C-MYC transcriptional repression
Enhance monocyte differentiation and protumoral TAM activation
6.07E-07
2.21E-07
1.61E-07
0.0008
0.0002
0.0039
0.0025
0.0021
0.0011
0.0010
0.0
2.5
5.0
7.5
10.0

B


ACVR1
Cancer
Paracancerous
P< 0.05
ACVR1
Expression Level
High expression
Low expression
P=0 .095
Time(d)
GDF6
Cancer
Paracancerous
P< 0.01
GDF6
Expression Level
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Low expression
P=0 .00049
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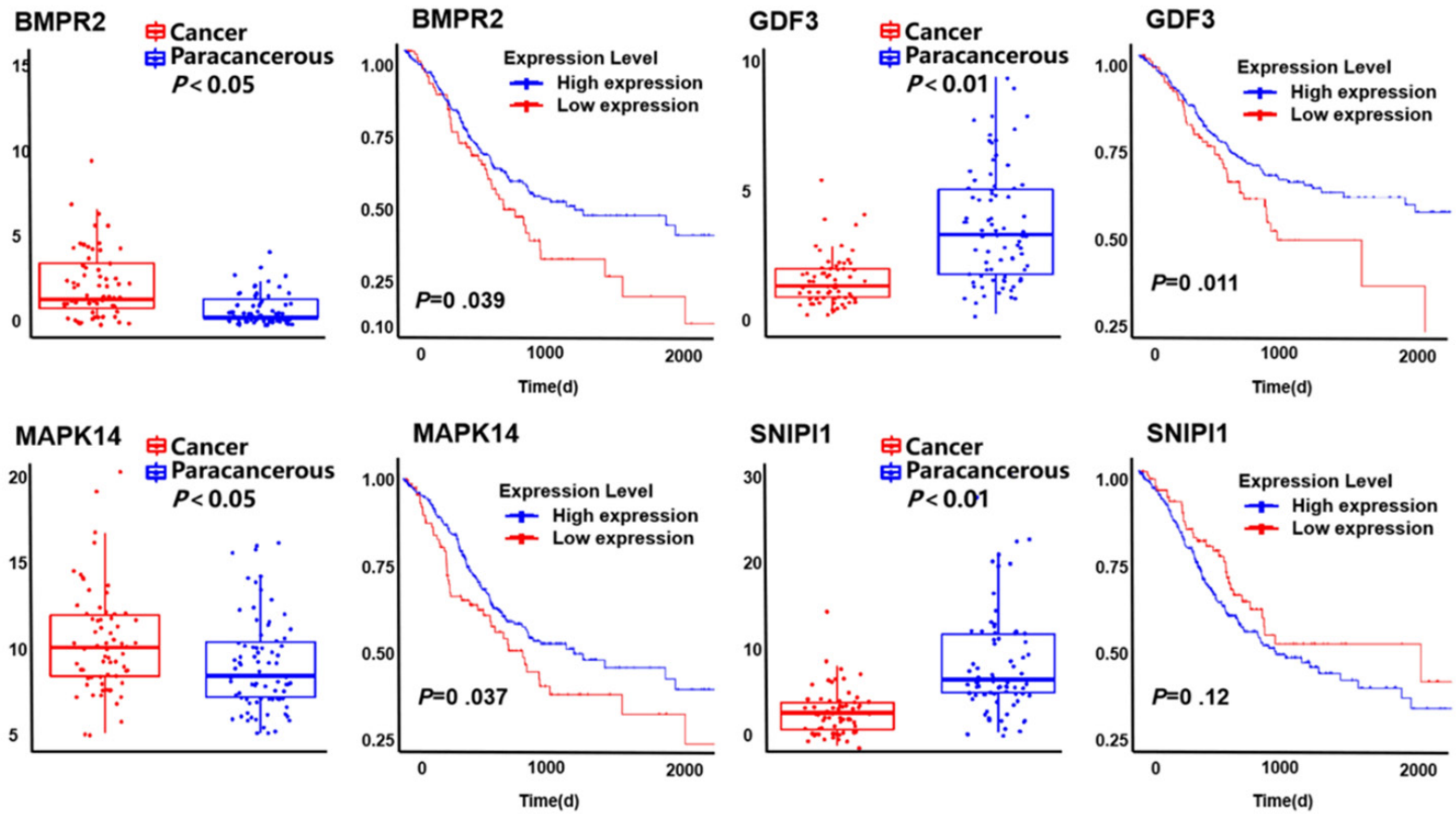

Figure 3. Fibroblasts pathway enrichment analysis of the differential genes in cancer tissues and paracancerous tissue. A. KEGG pathway analysis showed the top 10 enriched pathways. B. Survival analysis of TGF-β signal pathway-related genes, according to TCGA database.

prevention of incipient malignancies by regulating cell adhesion, differentiation, proliferation, and modulating the microenvironment [10, 11]. Conversely, abnormalities in the TGF-β signal pathway lead to tumor growth and invasion, escape from immune surveillance, and metastasis, including cancer cell dissemination. We found that the TGF-β signal pathway was one of the top-ranked enriched signaling pathways; further, TGF-β signal pathway-related genes were analyzed, and the results showed ACVR1, BMPR2, and MAPK14 were highly expressed in CAFs, while SNIP1, GDF6, and GDF3 were lowly expressed in CAFs. Finally, we put the differential genes into The Cancer Genome Atlas (TCGA) database for survival time analysis. We found that those with relatively high expression of GDF6 and GDF3 and low expression of BMPR2 and MAPK14 have a longer survival prognosis (**Figure 3B**).

Discussion

Previous studies on biomarkers have mainly been based on the bulk gene expression of tissues and cells. In fact, the conclusions drawn by this method are often inaccurate. At present, the technology of scRNA-seq provided a channel to find diagnostic markers and survival prediction markers at the single-cell level. However, studies derived from the scRNA-seq data are always without clinical data, so the TCGA database with clinical survival information is an essential supplement. This study performed scRNA-seq of cancer and paracancerous tissues, identifying and characterizing fibroblasts. Three-hundred-sixty-two CAFs were identified with COL3A1 and COL1A2 as marker genes, accounting for 3.86% of the total cells. Indeed, the primary cells in tissues are epithelial, immune cells, and endothelial cells. GC is a very heterogeneous tissue constituted of cancer cells and an abnormal TME, such as tumor vessels, an extracellular matrix (ECM), and non-cancer stromal cells represented by endothelial cells, pericytes, immune cells, CAFs, activated adipocytes, and mesenchymal stem cells (MSCs) [12, 13]. Studies have divided CAFs into different subtypes, and some of the subtypes exhibit inhibitory and tumor-promoting effects; in addition, some CAFs subtypes can affect tumor immunity, angiogenesis, and proliferation of cancer cells or have the ability to reconstruct the extracellular matrix [14, 15].

In addition to the marker genes, we also screened out the high expression genes of CAFs, such as RARRES2, IGF1, MMP14, SFRP4, and MFAP5. IGF1 is structurally and functionally related to insulin but has a much higher growth-promoting activity. As a ligand for IGF1R, IGF1 binds to the alpha subunit of IGF1R, leading to the activation of the intrinsic tyrosine kinase activity, which autophosphorylates tyrosine residues in the β subunit, thus initiating a cascade of down-stream signaling events leading to activation of the PI3K-AKT/PKB and the Ras-MAPK pathways [16, 17]. Also, the high expression of IGF1 in fibroblasts is related to the inflammation of TME, which involves the PI3K-AKT/PKB signaling pathway [18]. The aberrant activation of the TGF-β signaling pathway was closely related to the occurrence and development of cancers, and it has been shown that the activation of the TGF-β signaling pathway promotes GC metastasis by regulating the epithelial-mesenchymal transition (EMT) [19, 20]. In this study, we screened and analyzed the genes related to this TGF-β signaling pathway and found that GDF6, GDF3, BMPR2, and MAPK14 in CAFs were associated with survival in the TCGA database, suggesting that they can be used as survival markers of GC. GDF6 and GDF3 belong to the TGF-β superfamily [21]. It is reported in the literature that GDF6 is specifically and highly expressed in melanoma, and patients with low GDF6 expression have a lower risk of metastasis and a higher chance of survival [22]. A study has shown that the expression of GDF3 is significantly reduced in breast cancer tissues, and the recombination of GDF3 may be a potential therapeutic approach to inhibit invasive breast cancer [23]. However, our data analysis shows that the highly expressed GDF6 and GDF3 have a longer survival time.

Studies have shown that inhibiting BMPR2 can induce chondrosarcoma cell apoptosis and autophagy, and the expression of BMPR2 is related to the clinicopathological characteristics of chondrosarcoma, which can be used as an important treatment index of chondrosarcoma [24]. Differently from GDF3 and GDF6, MAPK14 has been proven to be associated with the prognosis of many cancers, including gastric cancer [25, 26]. Our study found that the expression of MAPK14 in CAFs was higher than that in fibroblasts derived from paracan-

cerous tissues at single-cell levels. The result of this study showed that the high expression of MAPK14 indicated a longer survival time. Still, the mechanism is not clear, and further cellular experiments and *in vitro* experiments are needed. CAFs have been proposed in metabolic reprogramming to perform aerobic glycolysis and participate in the tumor cell-stimulating tumor lactate shuttle [27]. In this study, we also found signaling pathways involved in the metabolic reprogramming of CAFs, which were not shown in the results.

A limitation of this project is the insufficient sample size; we collected only two patients for a total of four samples. Also, the present study included two patients with differentiated gastric adenocarcinoma, which might not be representative of all patients with GC.

Conclusion

In conclusion, with the aid of single-cell sequencing, we identified CAFs and found that the screened TGF-β signaling pathway-related genes GDF6, GDF3, BMPR2, and MAPK14 were associated with survival. In addition, through single-cell sequencing technology, it is possible to discover some so-called non-key genes previously neglected in the field of tumor research.

Acknowledgements

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Disclosure of conflict of interest

None.

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