Journal of Inflammation Research

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ORIGINAL RESEARCH

Identification of Immune-Related Genes in Predicting the Progression of Colitis-Associated Colorectal Cancer: An Integrated Bioinformatics and Experimental Validation Study

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Purpose: Immune infiltration plays an important role in the pathogenesis of both ulcerative colitis (UC) and colorectal cancer (CRC). Our aim is to explore the significance of immune cell-related genes in colitis-associated colorectal cancer (CAC).

Methods: Datasets related to UC and CRC were sourced from public databases. Key immune cell-related CAC genes (IC-CACs) were obtained by using WGCNA, differential expression and elastic net logistic regression analyses. Their diagnostic performance was assessed via ROC curves. Single-cell RNA sequencing (scRNA-seq) data were analyzed using the AUCell algorithm. Prognostic signatures were developed from differentially expressed genes between AUCell score-high and -low groups using Cox and LASSO regression. Moreover, the expressions of IC-CACGs were examined by RT-qPCR using clinical samples.

Results: We obtained 13 key IC-CACGs correlated with neutrophils and dendritic cells. ROC curves revealed that key IC-CACGs had the good ability to distinguish UC or CRC patients from controls. scRNA-seq analysis revealed enrichment of interferon response, inflammation, and PI3K-Akt-mTOR signaling in AUCell scorehigh groups, while glycolysis and EMT were enriched in scorelow groups. A 20-gene prognostic signature was constructed and validated, with low-risk patients showing better overall survival. Key IC-CACGs correlated strongly with prognostic genes, and high-risk patients exhibited higher TIDE scores, suggesting poorer immunotherapy response. *CD69*, *CXCR4*, and *SAMSN1* were highly expressed in T cells and natural killer T cells, and their expressions were significantly increased in CAC samples.

Conclusion: Our results provide the evidence for the promising role of immune cell-related genes in the development of CAC and new ideas for CAC prevention and treatment.

Keywords: ulcerative colitis, colorectal cancer, immune cell infiltration, prognosis, single-cell

Introduction

Ulcerative colitis (UC) is a chronic, relapsing-remitting inflammatory bowel disease characterized by nonspecific mucosal and submucosal inflammation, primarily affecting the rectum and colon, with potential extension to the sigmoid, descending, or entire colon.[1] Clinical manifestations include abdominal pain, bloody diarrhea, tenesmus, weight loss, and systemic complications.[2] Notably, the risk of colitis-associated colorectal cancer (CAC) escalates with disease duration, reaching 8% at 20 years and 18% after 30 years of active inflammation.[3] Colorectal cancer (CRC) ranks as the third most prevalent malignancy worldwide, with rising incidence rates in recent decades.[4–6] A key pathogenic driver of CRC is

https://doi.org/10.2147/JIR.S588177
Received: 25 January 2026
Accepted: 26 May 2026
Published: 11 June 2026

chronic inflammation-mediated neoplastic transformation, wherein persistent mucosal injury, aberrant repair, and immune dysregulation foster a pro-tumorigenic microenvironment.[7] The canonical inflammation-dysplasia-carcinoma sequence represents a well-established pathological continuum, underscoring the critical need to elucidate molecular mechanisms underlying this transition for early intervention and improved therapeutic strategies.[8]

The pathogenesis of CAC involves a complex interplay of chronic inflammation, genetic mutations, immune microenvironment dysregulation, and epigenetic modifications,[9,10] where persistent inflammation drives carcinogenesis through sustained activation of pro-inflammatory cytokines (IL-6/STAT3, TNF-α/NF-κB) and immunosuppressive cells (M2 macrophages, myeloid-derived suppressor cells),[9] creating a tumor-promoting microenvironment characterized by oxidative stress (ROS accumulation), DNA damage, and subsequent molecular alterations including early TP53 inactivation, late APC mutations, and epigenetic silencing of tumor suppressor genes.[10] Distinct from sporadic colorectal cancer, CAC exhibits unique molecular features such as predominant TP53 mutations, rare WNT pathway abnormalities, and a strong association with the CMS4 mesenchymal subtype,[11] while gut microbiota dysbiosis (eg., *Fusobacterium nucleatum*, colibactin-producing *E. coli*) further promotes carcinogenesis through NF-κB activation and DNA damage responses.[7] The disease progresses through defined stages from chronic inflammation with abnormal mucosal regeneration and clonal expansion, through low-grade dysplasia with accumulating mutations, to invasive carcinoma with established immunosuppressive microenvironment,[9,10] yet current research lacks a comprehensive immunological understanding of CAC,[11] particularly regarding the dynamic changes in immune cell populations during progression,[12] the precise role of innate immunity in early carcinogenesis,[13] and the complex interactions between microbial factors and host immunity,[7] highlighting the need for deeper immunological insights to develop effective preventive and therapeutic strategies.

CAC represents one of the most severe complications in patients with UC, arising from chronic inflammation-induced dysregulation of the immune microenvironment.[9,10] Despite significant advances, current research efforts face critical limitations including the absence of reliable non-invasive biomarkers for early detection,[14] poor patient compliance with conventional endoscopic surveillance methods,[15] and incomplete understanding of the dynamic immunological alterations during CAC progression,[11] particularly regarding the regulatory mechanisms of neutrophils[12] and dendritic cells.[13] To overcome these challenges, we implemented an innovative computational approach integrating multi-omics data from GEO and TCGA databases. Through comprehensive WGCNA network analysis combined with advanced machine learning algorithms, we systematically identified critical immune-related genes and delineated their functional relationships with immune cell infiltration patterns during CAC development.

Our findings provide novel molecular biomarkers for early CAC detection, offer mechanistic insights into the inflammation-to-cancer transition, and establish a foundation for developing targeted immunotherapeutic strategies for high-risk UC patients. These discoveries hold significant potential to improve clinical management and outcomes for CAC patients through enhanced diagnostic approaches and personalized treatment modalities.

Methods & Materials

Acquisition of the Ulcerative Colitis and Colorectal Cancer Datasets

The gene microarrays were acquired from the publicly available Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (TCGA, https://www.cancer.gov/ccg/research/genome-sequencing/tcga) datasets. The GSE37283 includes 4 cases of quiescent UC and 11 cases of colitis-associated CRC (CAC). A total of 640 tumor and 51 healthy control samples were enrolled from the TCGA-CRC cohort. The TCGA-CRC and GSE17537 were set as training and validation cohorts, respectively. The single-cell RNA-sequencing datasets were downloaded from the GSE132257 (5 CRC and 5 controls).

Immune Infiltration Analysis and Construction of Weighted Gene Co-Expression Networks Analysis

The CIBERSORT algorithm was used to calculate the ratios of 22 immune cells in UC and CAC samples from GSE37283, and the R package "vioplot" was used to visualize the proportions of 22 immune cells. Weighted Gene Co-

expression Network Analysis (WGCNA) is performed to identify modules correlated with differentially distributed immune cells between UC and CAC samples. Using "WGCNA" package, sample clustering is performed to identify outlier samples. An appropriate soft-threshold power was selected through "pickSoftThreshold" to build a scale-free network. Next, hierarchical clustering and the dynamic tree cut function were applied to determine modules. Finally, The relationship between immune cells and modules was determined by Pearson's correlation between module eigengenes and the abundance of immune cells. The module with the highest correlation with immune cells was selected, and genes within this module were used for downstream analysis.

Analysis of Differentially Expressed Immune Cell-Related Colitis-Associated Colorectal Cancer Genes

To determine the differentially expressed genes (DEGs) between UC and CAC, we employed the "limma" script (version 3.50.1) and applied an adjusted p-value < 0.05 as the selection criteria. Down- and up-regulated DEGs were visualized by the R package "ggplot2" for volcano plots and "ComplexHeatmap" for heatmap. To identify the candidate IC-CACGs, DEGs and WGCNA genes were intersected by "ggvenn". Subsequently, we utilized gene ontology[16] and Kyoto Encyclopedia of Genes and Genomes (KEGG) to analyze their functions through R package ClusterProfiler.

Identification and Diagnostic Value of Key Immune Cell Related- Colitis-Associated Colorectal Cancer Genes

The R package glmnet was employed to perform elastic net regression analysis for the selection of immune cell related-CAC genes (IC-CACG) features. Briefly, ten-fold cross-validation was applied to select the optimal lambda (λ). Then we obtained the coefficients of candidate IC-CACGs under the the optimal λ. Finally, IC-CACGs with nonzero coefficient were identified as key feature genes. Furthermore, the inflammation-cancer transformation index (transformation index) of each sample was calculated as the sum of the products of the key IC-CACGs' coefficients and their respective expression levels. The performance of key IC-CACGs in predicting the onset of CAC, UC or CRC were evaluated by plotting ROC curves through the R package "pROC". In addition, Spearman correlation analysis was used to additionally describe the correlations among key IC-CACGs and between differentially distributed immune cells and key IC-CACGs. Moreover, the inflammation cancer transformation enrichment score of UC and control samples in GSE92415 were calculated and compared based on the expressions of key IC-CACGs. Next, the UC patients were divided into low- and high-inflammation cancer transformation score groups according to the median value. The immune environment of low- and high-inflammation cancer transformation score groups were examined by CIBERSORT and ESTIMATE algorithms. Additionally, Spearman correlation analysis was used to evaluate the correlation between inflammation cancer transformation score and Mayo score. Similar analyses were also performed in the TCGA-CRC dataset.

Single-Cell Sequencing Analysis

The single-cell sequencing data were analyzed according to the Seurat pipeline. Briefly, the CreateSeuratObject function was used to filter the single-cell sequencing data, and the cells with n.features > 200, n. count > 1000 and percent.mt < 40% were retained. Besides, doublets were identified and removed using the DoubletFinder R package. UMAP algorithm was used to perform overall dimensionality reduction on the samples based on the top 50 principal components. Cell types were manually annotated by well-known markers. The "AUCell" R package was used to calculate the inflammation-cancer transformation score of each cell. Cells were divided into high- and low-score groups according to the inflammation-cancer transformation score. Subsequently, we conducted a differential study on the molecular functions between the two groups of cells by GSVA using the HALLMARK gene sets from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb) as the reference. Additionally, the DEGs between high- and low-inflammation-cancer transformation score groups were identified and analyzed for GO and Disease Ontology (DO) enrichment analyses.

Table 1 Primer Sequences

Genes	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)
CCL4	CCCAGCCAGCTGTGGTATTC	CATACACGTACTCCTGGACCC
CD69	TGCCATCAGACAGCCATGTT	ACCCTGTAACGTTGAACCAGT
CXCR4	TCCATTCCTTTGCCTCTTTTGC	ATGACAAAGAGGAGGTCGGC
SAMSN1	TCACTGGGTAGATCCAGCAC	AAACTGCTGCTTCGCCATGA
GAPDH	ATCCCATCACCATCTTCC	ATGACCCTTTTGGCTCCC

Development and Validation of the Colitis-Associated Colorectal Cancer-Related Risk Score Model Signature

Initially, genes with prognostic values in TCGA-CRC were first screened using univariate cox regression. Then these genes were input into LASSO regression analysis for feature selection. Next, risk score was calculated as follows: risk score = (exp_{gene1}×$coefficient_{gene1}$)+ (exp_{gene2}×$coefficient_{gene2}$) +…+ (exp_{genex}×$coefficient_{genex}$). Patients were divided into a high- and low-risk group using the median risk score, and the overall survival of patients in the two groups were analyzed using R "survival" package. We employed the time-dependent ROC curve using the "survivalROC" script to estimate the AUC for 1-, 2-, and 3-year survival. Spearman correlation analysis was used to evaluate the correlation between prognostic genes and key IC-CACGs. Tumor immune dysfunction and exclusion (TIDE) score was calculated via TIDE algorithm (http://tide.dfci.harvard.edu/) to infer the difference of immunotherapeutic response between high- and low-risk score groups.

Sample Collection and qRT–PCR Analysis

Five colonic mucosa tissues from UC patients and five colon cancer tissues from CAC patients were collected from the Affiliated Hospital of Shandong University of Traditional Chinese Medicine between May 8 and August 8, 2025. All experiments were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. Informed consent was obtained from all individuals involved in the study. Total RNA was isolated from tissue samples using Trizol reagent.[17] The amplification conditions involved a temperature of 42°C for 2 minutes, followed by an initial denaturation at 95°C for 3 minutes. This was followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing+extension at 60°C for 30 seconds. The results were analyzed using the 2−ΔΔCt method with GAPDH serving as the internal control reference. The primer sequences were listed in Table 1.

Statistical Analysis

R software was used to perform all analyses in this study. Differences between two groups were assessed using the Wilcoxon test. A significance level of p-value < 0.05 was considered for all comparisons between groups unless specified.

Results

Exploration of Immune Cell-Related Genes in Colitis-Associated Colorectal Cancer

CIBERSORT was used to evaluate the immune landscape by quantifying the level of immune cell infiltration in CAC. The results showed that dendritic cells resting were significantly reduced in CAC groups, while dendritic cells activated and neutrophils were significantly increased in CAC groups (Figure 1A). After removing one outlier (Supplementary Figure 1A), the optimal soft threshold power in GES37283 cohort was set as 5 with the R2 >0.85 (Figure 1B). By clustering, we identified 20 co-expression modules, among which the blue module had the strongest correlation with dendritic cells resting (Cor = 0.81, P = 4e-04), dendritic cells activated (Cor = −0.77, P = 0.001) and neutrophils (Cor = −0.68, P = 0.008) (Figure 1C and Supplementary Figure 1B). Finally, 3581 genes in the blue module were selected as immune genes related to inflammatory cancer transformation.

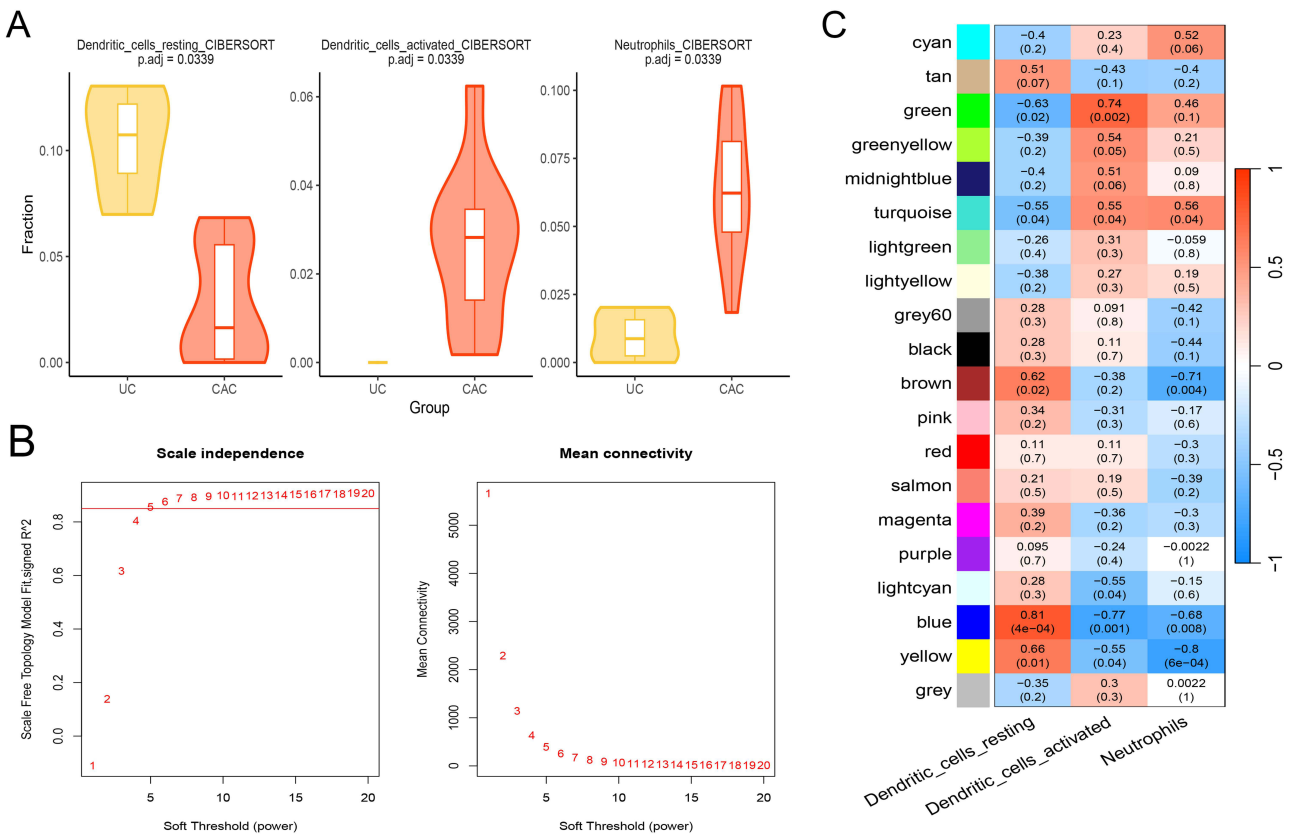

Figure 1 Identification of immune cell-related gene modules in the GEO dataset using Weighted correlation network analysis (WGCNA) and CIBERSORT. (**A**) Immune cells showing significant differences between ulcerative colitis (UC) and colorectal cancer (CRC); (**B**) Analysis of scale Independence and mean connectivity. (**C**) Correlation between the co-expression modules and immune cells. Each cell displays the correlation coefficient and corresponding p-value.

Eighteen Candidate Inflammatory Cancer Transformation Genes Were Identified

Using the bulk sequencing data of UC and CAC, we identified 363 DEGs, comprising 210 up-regulated and 153 down-regulated genes (Figure 2A). Among them, the top 34 DEGs with |log2FC| > 2 were selected in the following analyses. As shown in the heatmap, there were 30 up-regulated (*S100A8*, *SERPINA3*, *SAMSN1*, *FCGR2C*, *LY96*, *BCAT1*, *CXCR4*, *STEAP4*, *NCF2*, *FCGR3B*, *GPR171*, *CXCL8*, *CD69*, *MNDA*, *REG3A*, *REG1A*, *RGS18, FPR1*, *MGP*, *CFI*, *HOPX*, *CLC*, *CCL4*, *SELL*, *CLEC4E*, *SFRP2*, *LYVE1*, *PDE4B*, *MMP3*, and *BCL2A1*) and 4 down-regulated genes (*TPH1*, *EXPH5*, *VSIG10*, and *SLC26A2*) in the CAC group (Supplementary Figure 2). Finally, we identified 18 candidate genes by overlapping 34 DEGs and 3581 genes (Figure 2B). These candidate genes were mainly involved in immune-related GO terms, such as immune response, T cell activation and differentiation, external side of plasma membrane, apical part of cell, and immune receptor activity (Figure 2C). Additionally, the KEGG result demonstrated that the candidate CACs were significantly enriched in staphylococcus aureus infection (Figure 2D). These findings further demonstrate the importance of immune infiltration in the progression of CAC.

Key Genes Had Good Performance in Predicting the Onset of Colitis-Associated Colorectal Cancer

To obtain key CACs, elastic net logistic regression was carried out on the candidate CACs. In the optimal model (λ = 0.048) (Supplementary Figure 3A), we obtained 13 key CACs [Fc gamma receptor IIc (*FCGR2C*), lymphocyte antigen 96 (*LY96*), C-X-C motif chemokine receptor 4 (CXCR4), serpin family a member 3 (*SERPINA3*), CD69 molecule (*CD69*), SAM domain, SH3 domain and nuclear localization signals 1 (*SAMSN1*), Regenerating family member 3 alpha (*REG3A*), matrix metallopeptidase 3 (*MMP3*), regenerating family member 1 alpha (*REG1A*), C-C motif chemokine ligand 4 (*CCL4*), matrix gla protein (*MGP*), exophilin 5 (*EXPH5*), tryptophan hydroxylase 1 (*TPH1*)] and their

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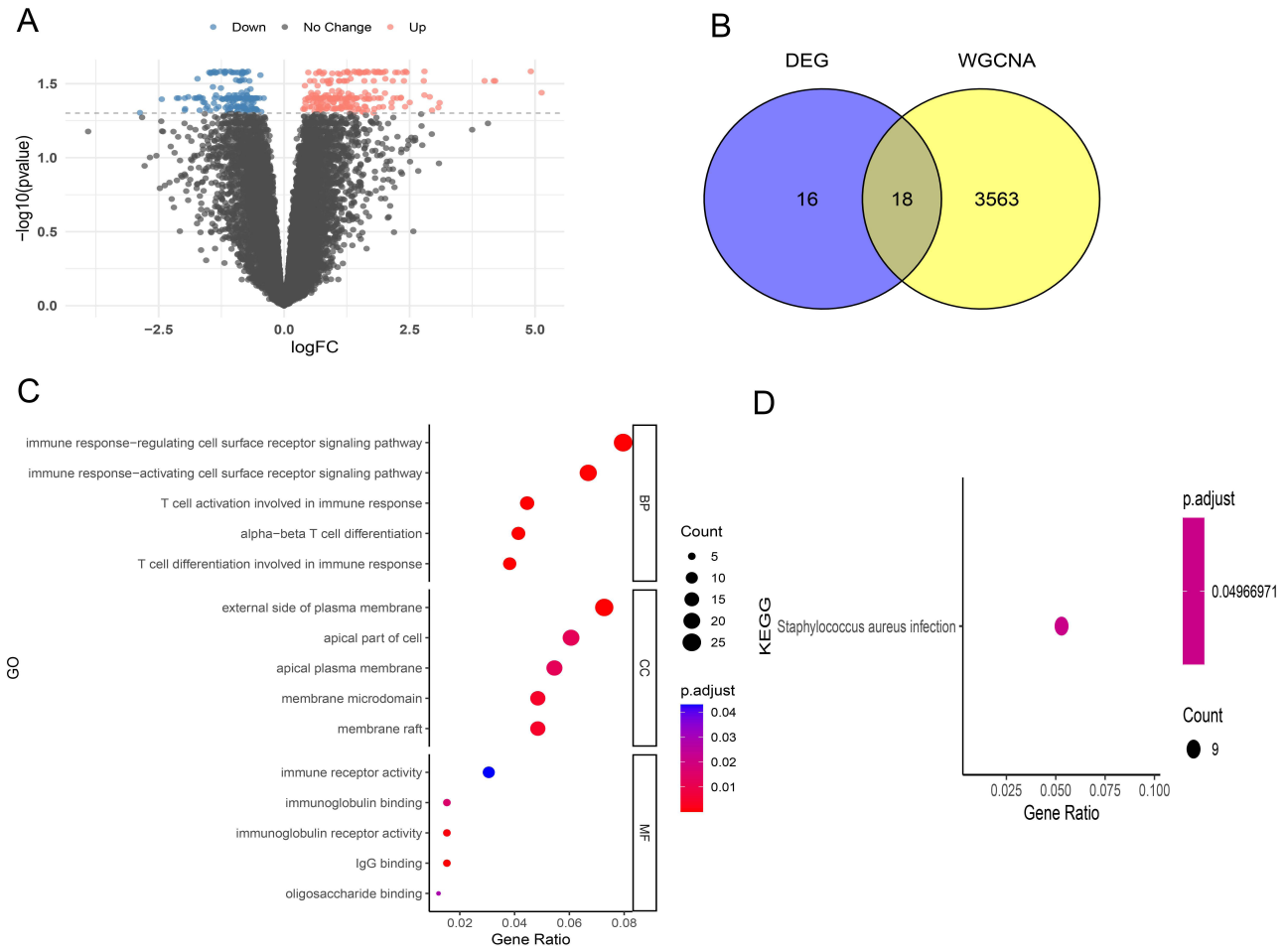

Figure 2 Identification of candidate genes and functional enrichment analysis. (**A**) Volcano plot of differential expressed genes (DEGs) between UC and colitis-associated CRC (CAC); (**B**) Venn diagram illustrating the overlap between DEGs and module genes from WGCNA; The Gene Ontology (**C**) and Kyoto Encyclopedia of Genes and Genomes (**D**) enrichment analyses using candidate genes.

coefficients (Figure 3A). The expressions of *EXPH5* and *TPH1* were significantly decreased, while the expressions of other 11 key IC-CACGs were remarkably enhanced in CAC compared with UC (Figure 3B). These key genes were highly correlated with each other, for example, the correlation coefficients among *CCL4*, *CD69*, *EPH5*, *FCGR2C*, *LY69*, *REG1A*, and *SAMSN1* were greater than 0.7. It was worth noting that EXPH5 and TPH1 were positively correlated (cor = 0.8), but these two genes were negatively correlated with the other 11 key IC-CACGs (Supplementary Figure 3B). Additionally, we provided a supplementary description of the correlations between genes and immune cells. *EXPH5* and *TPH1* were positively correlated with the resting dendritic cells (cor =0.79 for *EXPH5*, cor = 0.61 for *TPH1*), but negatively correlated with activated dendritic cells (cor = −0.85 for *EXPH5*, cor = −0.79 for *TPH1*) and neutrophils (cor = −0.75 for *EXPH5*, cor = −0.71 for *TPH1*). Other key CACs (*CCL4*, *CD69*, *CXCR4*, *FCGR2C*, *LY96*, *MGP*, *MMP3*, *REG1A*, *REG3A*, *SAMSN1*, and *SERPINA3*) were negatively correlated with resting dendritic cells and positively correlated with activated dendritic cells and neutrophils (Supplementary Figure 3C). Moreover, ROC curves revealed that these key genes served as a candidate signature associated with CAC classification (AUC > 0.9, Figure 3C).

Key Genes are Also Involved in the Development of Ulcerative Colitis

We first observed the expression patterns of key IC-CACGs in UC and healthy control samples. The results showed that *EXPH5* and *TPH1* were down-regulated, while other key CACs were up-regulated in UC group compared with those in the healthy controls (Figure 4A), and the UC group had a significantly higher transformation index (Figure 4B). Subsequently, according to the median of transformation index, UC patients were divided into high- and low-index

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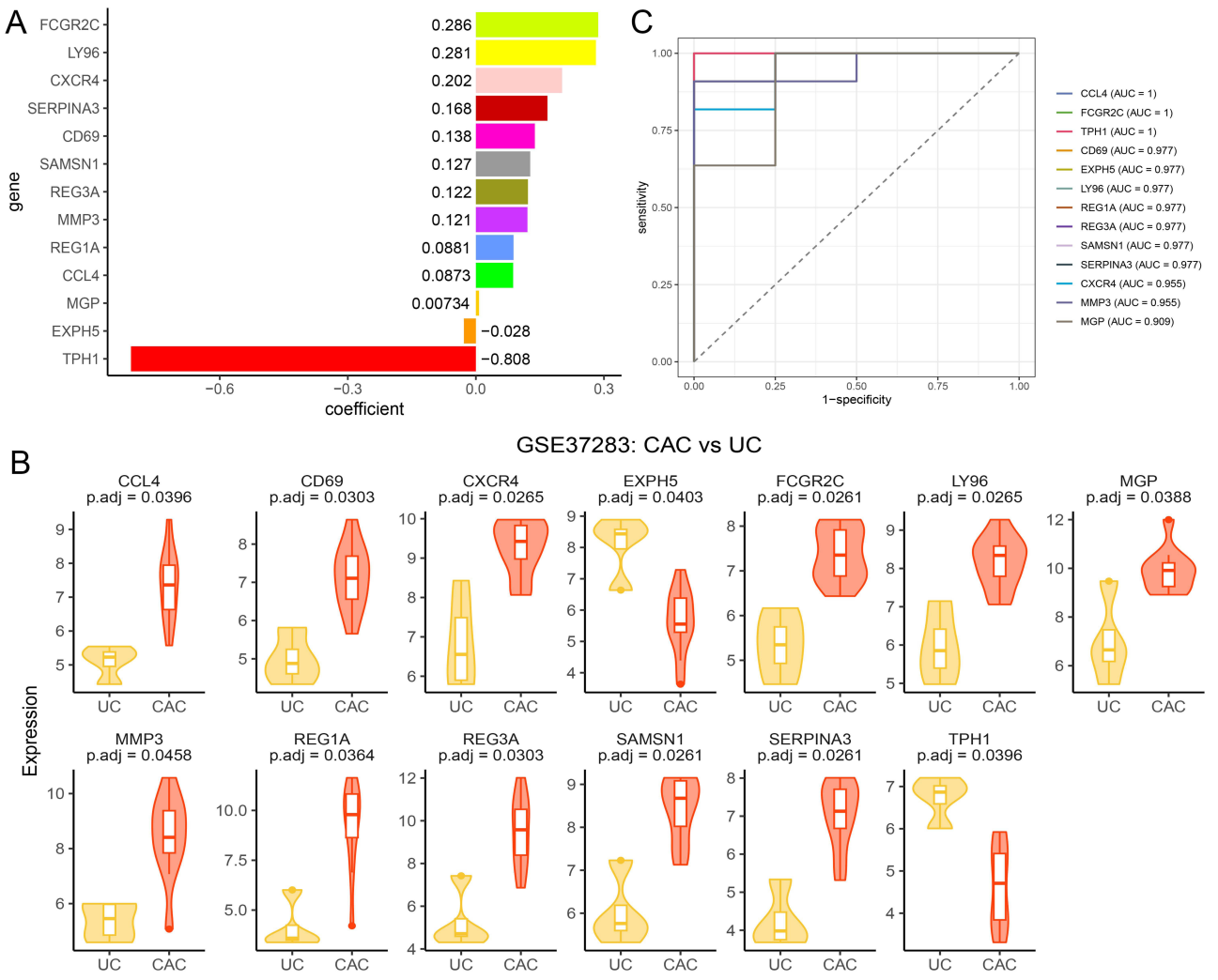

Figure 3 Identification of key immune cell related- CAC genes (IC-CACGs) via LASSO regression. (**A**) Bar plot showing key IC-CACGs coefficients; (**B**) The violin plot showing the difference expression of key IC-CACGs between UC and CAC; (**C**) The receiver operating characteristic (ROC) curves showing the area under curve (AUC) value of key IC-CACGs.

groups. The high-index group had more neutrophils, resting memory CD4 T cells and higher immune score compared to the low-index group (Figure 4C and D). In addition, the transformation index was positively correlated with the Mayo score (cor = 0.36, p-value <0.0001, Figure 4E). Also, we evaluated the diagnostic value of key IC-CACGs in UC, and found that key IC-CACGs except TPH1 might serve as candidate markers to distinguish UC patients from healthy controls (AUC > 0.8, Figure 4F).

The Transformation Index is Associated with Tumor Microenvironment of CRC Patients

Meanwhile, we investigated the role of key IC-CACGs in the TCGA-CRC cohort. We found that *CXCR4* and *SERPINA3* had no significant difference between CRC and control samples, *CXCR4*, *MMP3*, *REG1A*, and *REG3A* were up-regulated, while *CD69*, *EXPH5*, *LY96*, *MGP*, *SAMSN1* and *TPH1* were down-regulated in CRC group (Figure 5A). We observed that most key IC-CACGs, except *EXPH5*, *CCL4*, *CXCR4*, and *SERPINA3*, might serve as candidate markers to distinguish CRC samples from controls, as evidenced by the ROC curve (AUC > 0.7, Figure 5B). Also, we observed a higher transformation index in the CRC group (Figure 5C). In the high-index group, there is a higher proportion of neutrophils, M1 macrophages, CD4+ memory activated T cells, M2 macrophages, and eosinophils, whereas

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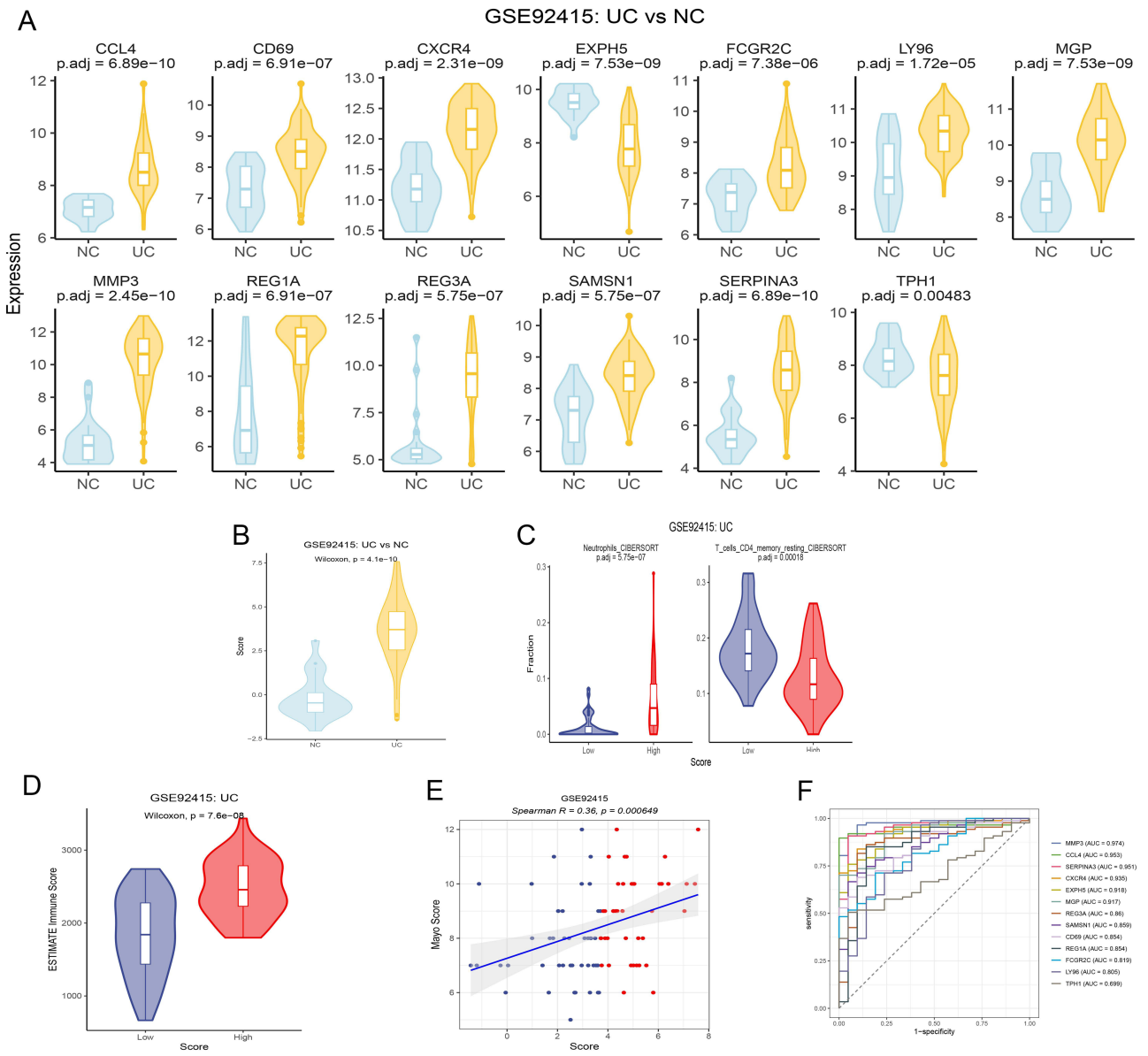

Figure 4 Characterization of Key IC-CACGs in UC. (**A**) The violin plot showing the difference expression of key genes between UC and NC; (**B**) Evaluation of a transformation index between UC and NC groups; (**C**) Comparison of immune cell activity between the high- and low-index groups; (**D**) Distribution of ESTIMATE scores between the high- and low-index groups; (**E**) Correlation analysis between the transformation index and the Mayo endoscopic score; (**F**) The ROC curves showing the diagnostic value of key IC-CACGs in UC.

the low-score group had a higher abundance of regulatory Tregs T cells and M0 macrophages (Figure 5D). Additionally, the high-index group had higher immune score compared to low-index group (Figure 5E). Furthermore, we analyzed the differences in the expression of immune activation genes between two transformation index groups (Figure 5F), and the results showed that most immune activation genes were up-regulated in high-index groups.

A Reliable Transformation Index-Related Risk Model Was Developed and Validated in Colorectal Cancer Cohort

After quality control and annotation, we identified ten cell types (Figure 6A), including myeloid cells, fibroblasts, natural killer T cells, plasma cells, plasmacytoid dendritic cells, endothelial cells, epithelial cells, B cells, and T cells, the markers of which were displayed in the bubble plot (Figure 6B). Based on the key IC-CACGs, the transformation index of each cell was calculated, and cells were divided into high- and low-index groups by AUCell.

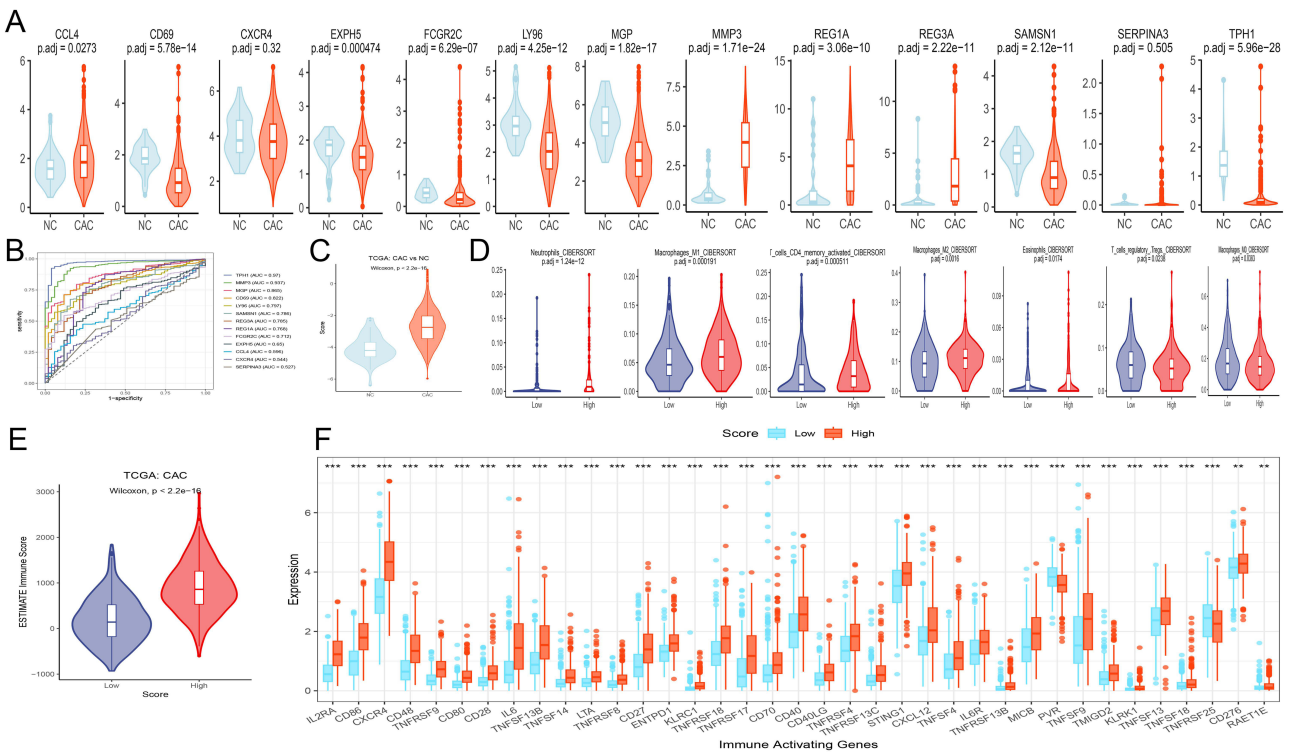

Figure 5 Characterization of key IC-CACGs in CAC. (**A**) The violin plot showing the difference expression of key genes between CAC and NC; (**B**) The ROC curves showing the diagnostic value of key IC-CACGs in CAC; (**C**) Evaluation of a transformation index between CAC and NC groups; (**D**) Comparison of immune cell activity between the high- and low-index groups in the TCGA-CRC cohort; (**E**) Distribution of ESTIMATE scores between the high- and low-index groups in the TCGA-CRC cohort; (**F**) Comparison of immune activity genes between the high- and low-index groups in the TCGA-CRC cohort. ** $p < 0.01$; *** $p < 0.001$.

We found that most B cells, natural killer T (NKT) cells, and T cells, as well as a subset of epithelial cells, were assigned to the high-index group. (Figure 6C). Moreover, in order to explore the differential molecular function between high- and low-index group, we performed GSVA. As shown in Figure 6D, allograft rejection, interferon gamma/ alpha response, PI3K/Akt/ mTOR signaling, and inflammatory response were enriched in high-index group, while apical junction, hedgehog signaling, angiogenesis, and estrogen response late were enriched in the low-index group. Moreover, we identified a total of 713 CAC-DEGs between low- and high-index group. GO enrichment analysis suggested that the CAC-DEGs might be involved in immune-related biological functions, such as leukocyte cell-cell adhesion, external side of plasma membrane, immune receptor activity (Figure 6E). The DO result demonstrated that the CAC-DEGs were enriched in intestinal cancer, colorectal cancer, inflammatory bowel disease, and intestinal disease (Figure 6F).

Next, we conducted univariate Cox regression and LASSO analyses to screen prognostic signatures from CAC-DEGs. Specifically, we first identified 45 prognostic factors with $P < 0.05$, including 11 favorable factors and 34 risk factors (Figure 7A). We further conducted LASSO analysis and selected 20 important variables from 45 prognostic genes (Figure 7B). Then we calculated the risk score of CRC patients and divided them into low-or high-risk score queues in both training and validation cohorts (Supplementary Figure 4). We observed that CRC patients with low-risk scores had significantly better prognosis than those with high-risk scores (Figure 7C and D). The AUC of 1-year, 2-year and 3-year ROC curves were 0.715, 0.722 and 0.732 in the training cohort (Figure 7E), and were 0.732, 0.771 and 0.673 in the validation cohort (Figure 7F). These findings showed that the risk score model could accurately classify CAC samples into different risk subtypes and are related to clinical survival results. The TIDE algorithm is a developed tool for determining the efficacy of tumor immune checkpoint therapy. In the present study, we found exclusion score and dysfunction score in high-risk group were significantly increased (Figure 7G), indicating that the immune escape potential of patients in high-risk group was increased.

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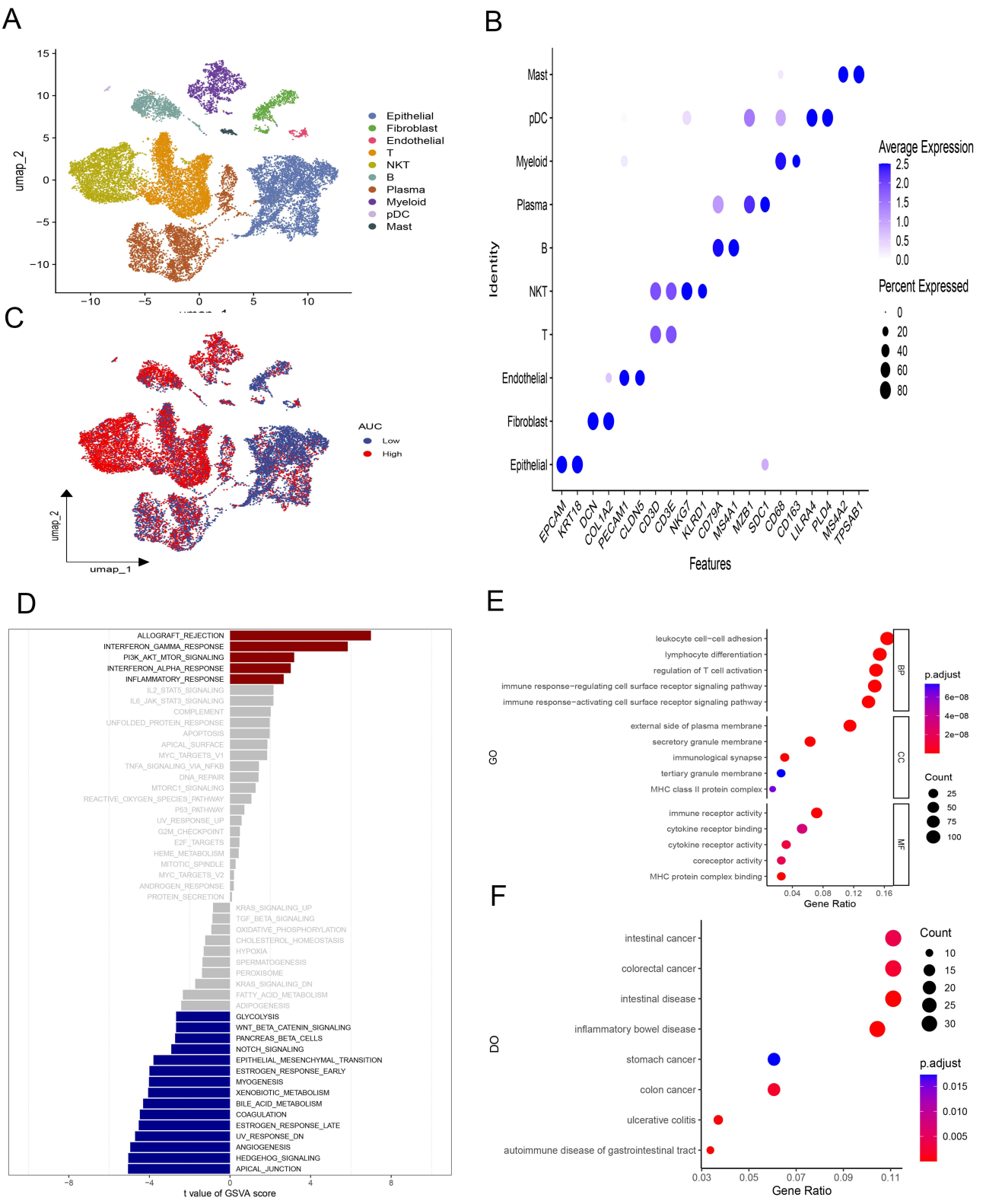

Figure 6 Single-cell sequencing analysis. (**A**) The UMAP plots of different subclusters of cells in CRC samples; (**B**) The marker genes of different subclusters in CRC samples; (**C**) The UMAP showing the distribution of the transformation index in CRC scRNA-seq; (**D**) Differential molecular function between high- and low-index group using Gene set variation analysis (GSVA); The Gene Ontology (**E**) and Disease Ontology (**F**) enrichment analyses using the DEGs between high- and low- transformation index groups.

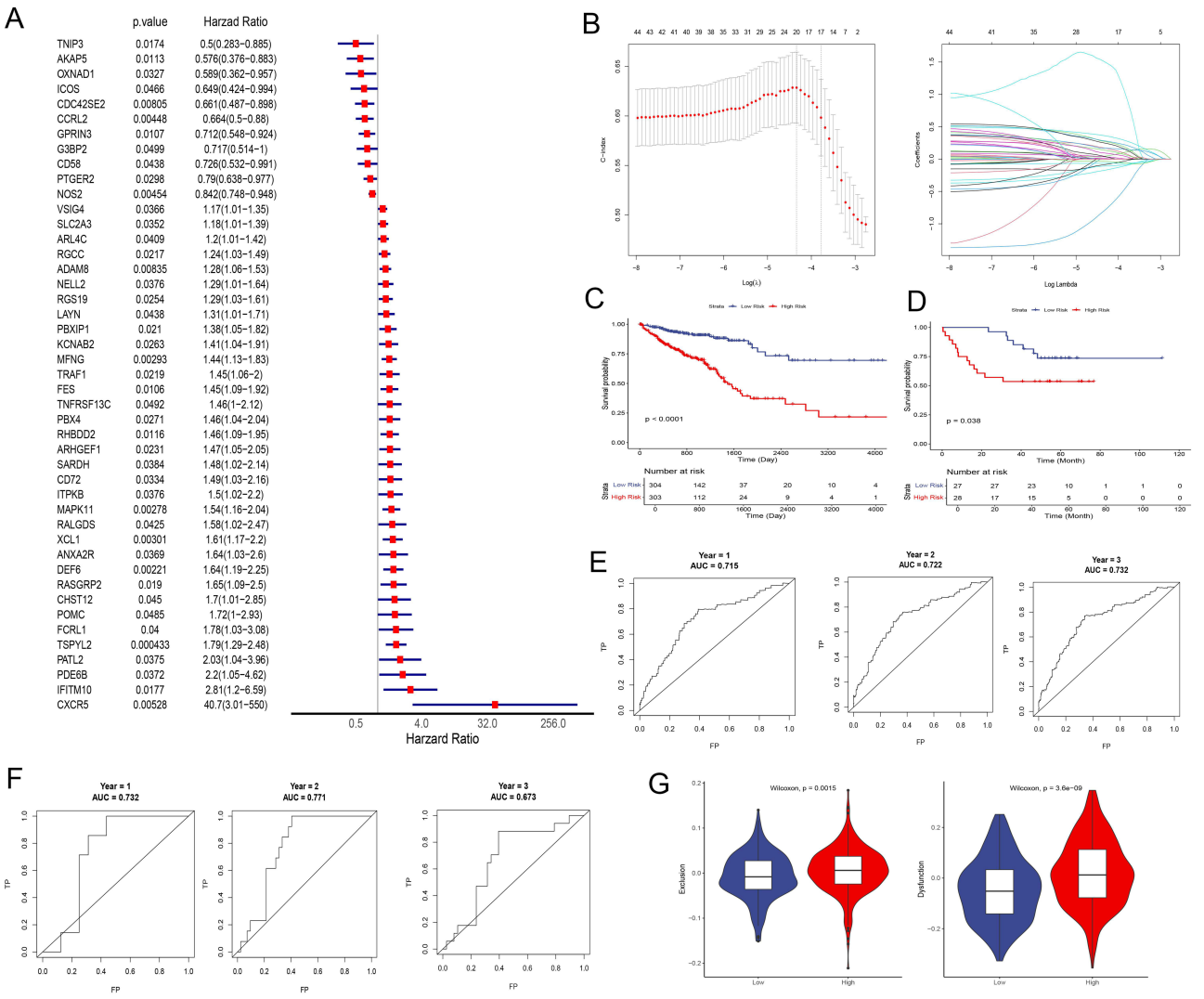

Figure 7 Construction and validation of risk model based on the CAC-related prognostic signature. (a) The univariate Cox analysis of CAC-related DEGs; (**B**) Identification of feature prognostic variables via LASSO analysis; Clinical survival curve analysis of CRC samples in transformation index groups in the (**C**) training and (**D**) validation cohorts; One-, two- and three-year's AUC in the (**E**) training and (**F**) validation cohorts; (**G**) The distribution of exclusion score and dysfunction score in transformation index groups.

The Expressions of Key Genes Were Altered

Subsequently, we observed the expression of IC-CACGs in CRC scRNA-seq datasets (Supplementary Figure 5A). Notably, *CCL4*, *CD69*, *CXCR4*, and *SAMSN1* were significantly expressed in T cells and NKT cells, while *REG1A* and *REG3A* were expressed in epithelial cells. Furthermore, the most of prognostic genes were significantly expressed in T cells and NKT cells. However, CXCR5 and TNFRSF13C were higher expressed in B cells, and *VSIG4* was highly expressed in myeloid cells (Supplementary Figure 5B). Moreover, we analyzed the correlation between the prognosis gene signatures and IC-CACGs. The results showed that the prognostic gene signatures, especially *VSIG4*, *TNIP3*, *SLC2A3*, *LAYN*, *ICOS*, were strongly correlated with key IC-CACGs (Cor > 0.6, P < 0.05) (Supplementary Figure 5C). At last, qPCR was utilized for preliminary experimental verification of the expressions of the *CCL4*, *CD69*, *CXCR4*, and *SAMSN1* in UC and CAC samples. We found that the abundances of *CCL4*, *CD69*, *CXCR4*, and *SAMSN1* were significantly higher in CAC group compared to those with UC group (Figure 8A–D). These results were consistent with the RNA sequencing results, indicating that *CCL4*, *CD69*, *CXCR4*, and *SAMSN1* play an important role in the development and progression of colitis-associated colorectal cancer.

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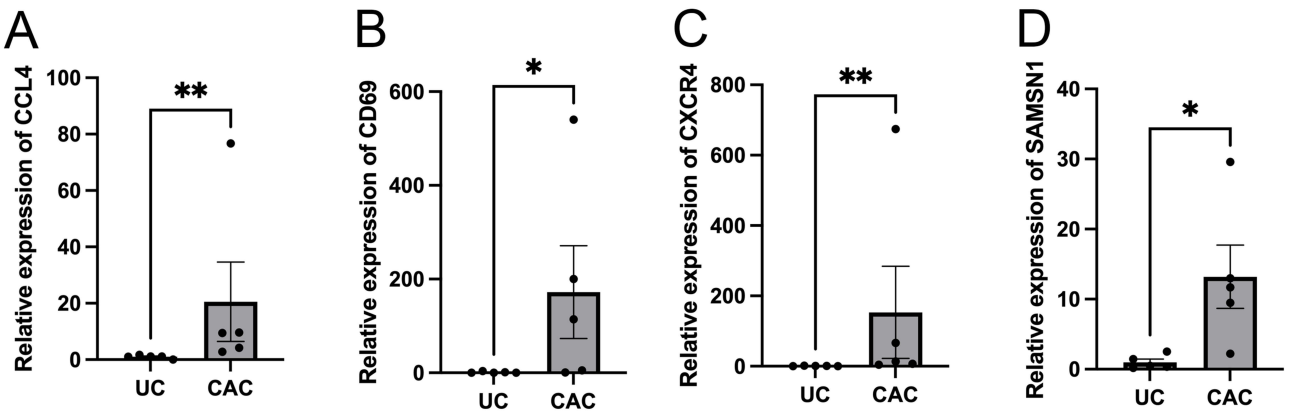

Figure 8 Validation expression level on colonic mucosa tissues and colon cancer tissues from clinical samples. The levels of (**A**) *CCL4*, (**B**) *CD69*, (**C**) *CXCR4*, and (**D**) *SAMSN1* were detected by qPCR in clinical tissues samples. * $p < 0.05$; ** $p < 0.01$.

Discussion

CAC represents a significant complication of UC. Notably, it carries a mortality rate of approximately 15%, which underscores the critical importance of early detection.[14] Current understanding suggests that sustained intestinal inflammation drives CAC pathogenesis by establishing an immune microenvironment that promotes tumorigenesis.[18] Given that immune dysregulation plays a pivotal role in cancer development, it becomes imperative to elucidate the specific contributions of immune cells in UC-associated carcinogenesis and CAC progression. Therefore, in this study, we systematically investigate the key molecular and immunological mechanisms underlying the malignant transformation from UC to CRC, with particular emphasis on characterizing the tumor-immune microenvironment. Ultimately, our findings may provide valuable insights for developing more effective strategies for early diagnosis and preventive interventions for CAC.

CIBERSORT analysis revealed significant differences in the immune landscape between UC and CAC. Specifically, DCs were downregulated in CAC, whereas activated DCs and neutrophils were markedly upregulated. These shifts in immune cell infiltration patterns may critically contribute to the inflammatory-to-cancerous transformation of UC into CRC.[12,16,19] Notably, neutrophil accumulation is a well-documented feature in both UC lesions and CRC.[20,21] Although neutrophils are traditionally recognized as key effectors of acute inflammation and pathogen clearance.[22] Consistent with these findings, our data demonstrate that neutrophil upregulation may drive the malignant transformation of UC to CAC.

Similarly, DC activation status showed significant changes during this transition. Activated DCs were elevated in CAC, while resting DCs were reduced. Given that DCs are crucial regulators of innate and adaptive immunity in Inflammatory bowel disease (IBD),[13,23] their activation may promote inflammation-associated carcinogenesis. Intriguingly, our results align with previous reports showing heightened DC activation in CAC patients compared to UC cases,[24] suggesting their potential role in modulating tumor immunity during CAC development. Collectively, although the present study focused on CAC, the key inflammatory and immune signatures identified here are also consistent with the findings of sporadic CRC. This indicates that CAC and sporadic CRC may share common core inflammatory pathways despite their distinct etiologies. Sustained chronic inflammation, aberrant activation of neutrophils and DCs, as well as the downstream dysregulation of chemotactic and matrix-remodeling signals, may serve as convergent mechanisms driving both inflammation-driven and sporadic colorectal tumorigenesis.

Through machine learning algorithms, we identified 13 key genes associated with CAC, including *EXPH5*, *TPH1*, *CCL4*, *CD69*, *CXCR4*, *FCGR2C*, *LY96*, *MGP*, *MMP3*, *REG1A*, *REG3A*, *SAMSN1*, and *SERPINA3*. Among these, *CXCR4*, *MMP3*, *REG1A*, and *REG3A* were upregulated in the CRC group, whereas *CD69*, *EXPH5*, *LY96*, *MGP*, *SAMSN1*, and *TPH1* were downregulated. Subsequently, we observed that *EXPH5* and *TPH1* were downregulated in UC patients compared to healthy controls, while the remaining key CAC-related genes were upregulated. Notably, *CXCR4*, *MMP3*, *REG1A*, and *REG3A* exhibited progressive upregulation from healthy controls to UC and further to CAC, suggesting their potential role in disease progression. CXCR4, initially identified as a chemokine receptor linked to

lung metastasis in breast cancer,[25] is widely expressed in human malignancies but minimally in normal tissues.[17,26] Accumulating evidence indicates that the CXCL12-CXCR4 axis promotes tumor growth, invasion, angiogenesis, and metastasis in various cancers, including CRC and pancreatic cancer.[27–29] Mechanistically, CXCR4 activation by CXCL12 triggers G protein dissociation into Gα (regulating RAS/RAF) and Gβγ (activating PI3K/Akt/mTOR and MEK/ERK pathways).[30] Similarly, MMP3, a zinc-dependent protease, is overexpressed in CRC and activates the PI3K/AKT pathway.[31] Specifically, MMP3 facilitates cancer invasion and metastasis by cleaving E-cadherin and disrupting its interaction with β-catenin.[32,33] In line with our immune profiling results, *CXCR4* is highly expressed in neutrophils and DCs to regulate their recruitment and activation,[34] while MMP3 is predominantly secreted by these activated immune cells, thereby linking immune cell dysfunction directly to extracellular matrix remodeling and malignant progression. In addition, *REG1A*, a member of the REG family, is upregulated in CRC patients with poor prognosis.[35–37] Recent studies suggest that *REG1A* is a potential therapeutic target in IBD, regulated by IL-6/IL-22-JAK-STAT3 signaling.[38] Furthermore, both *REG3A* and *REG1A* serve as serum biomarkers for CRC, offering a non-invasive screening tool.[36] These findings highlight the critical roles of *CXCR4*, *MMP3*, *REG1A*, and *REG3A* in the transition from UC to CAC, providing insights into their potential as diagnostic markers and therapeutic targets.

Compared to NC, patients with UC exhibited significantly elevated transformation indices. Using the median transformation index as a cutoff value, we stratified UC patients into distinct high- and low-index subgroups. Notably, the high-index group demonstrated markedly increased infiltration levels of neutrophils and resting memory CD4+ T cells. Through integrative analysis of gene expression profiles and ROC curves, we identified nine key discriminative markers (*TPH1*, *CD69*, *FCGR2C*, *LY96*, *MGP*, *MMP3*, *REG1A*, *REG3A*, and *SAMSN1*) that effectively distinguished CRC from control samples. CD69+ tissue-resident memory T (TRM) cells enhance intestinal immunity by secreting higher levels of pro-inflammatory cytokines, including TNF-α and IFN-γ.[39] SAMSN1 has been reported to be a tumor suppressor gene in multiple myeloma, hepatocellular carcinoma (HCC), gastric cancer;[40–42] Wang et al[41] reported that *SAMSN1* can inhibit the activation and proliferation of natural killer T cells, and it plays a role as a targetable checkpoint for natural killer cells. It has direct therapeutic significance for immunotherapy of HCC.

Furthermore, the high-index subgroup showed significantly elevated proportions of several immune cell populations, including neutrophils, M1 macrophages, activated CD4+ memory T cells, M2 macrophages, and eosinophils. Although our PCR experiments confirmed the expression trends of these candidate genes, future studies should evaluate the proposed candidate signature in prospectively collected, large-scale multicenter cohorts (including patients with UC, CAC, and healthy controls) using standardized protocols. Furthermore, it will be necessary to perform loss-of-function and gain-of-function experiments in cellular models (such as colitis-associated cancer cell lines or organoids), as well as in vivo studies using established animal models (eg., the AOM/DSS-induced CAC mouse model), to investigate whether these candidate genes are directly involved in the pathogenesis of CAC.

Single-cell RNA sequencing analysis revealed significantly greater infiltration of epithelial cells, B cells, NKT cells, and T cells in high-index patients. GSVA further demonstrated significant enrichment of PI3K-Akt-mTOR signaling and inflammatory response pathways in this subgroup. In dextran sulfate sodium/azoxymethane (DSS/AOM)-induced colitis models, the PI3K-Akt-mTOR signaling pathway is activated, indicating that this signaling axis represents an important mechanism in the pathogenesis of CAC.[43,44] In addition, the PI3K/Akt signaling pathway is also a key signaling pathway in the development of colorectal cancer, and inhibition of PI3K and Akt phosphorylation can alleviate the progression of CRC.[45,46] Through systematic prognostic modeling, we identified five signature genes (*VSIG4*, *TNIP3*, *SLC2A3*, *LAYN*, and *ICOS*) that showed strong correlations with key immune-related CAC genes (IC-CACGs; $r>0.7$, $p<0.001$). While these molecular markers demonstrate promising prognostic value, their specific functional roles in CAC pathogenesis remain to be elucidated and represent important targets for future investigation.

This study systematically characterized the dynamic evolution of the immune microenvironment during the progression from healthy controls to UC and ultimately to CAC. Our findings revealed that activated dendritic cells and neutrophils were significantly upregulated in CAC compared to UC, while stratification of UC patients by transformation indices demonstrated markedly elevated proportions of neutrophils, M1/M2 macrophages, activated CD4+ memory T cells, and eosinophils in the high-index group. Molecular profiling identified progressive upregulation of *CXCR4*, *MMP3*, *REG1A*, and *REG3A* across the disease continuum, with single-cell RNA sequencing further confirming

activation of PI3K/Akt/mTOR signaling pathways in high-index patients. However, this study has several limitations. First, the identification of key genes was primarily based on bioinformatics analyses and exploratory research, and systematic biological function experiments—such as in vitro validation using gene knockdown or overexpression techniques, as well as in vivo functional studies using animal models—have not yet been conducted. Therefore, the precise biological roles of these genes in the development and progression of CAC remain to be elucidated. Second, the sample size for preliminary experimental validation was relatively small, including only five UC and five CAC tissue samples, which may limit the statistical power and generalizability of the findings. Third, the screening of key IC-CACGs was performed using a relatively small discovery cohort (GSE37283, n = 15), and the proposed diagnostic and prognostic signatures lack validation in independent external cohorts. Fourth, the mechanisms by which these genes regulate neutrophil and dendritic cell functions, as well as the causal relationship between the PI3K/Akt/mTOR signaling pathway and malignant transformation, have not been experimentally confirmed. Future research should involve validation in larger independent cohorts, combined with CAC animal models and in vitro loss-of-function and gain-of-function experiments, to further explore the biological functions and underlying mechanisms of these candidate genes. In summary, this study provides novel insights into the immune microenvironment remodeling underlying the malignant transformation of CAC.

Conclusion

This study identified 13 immune-related genes closely associated with neutrophils and dendritic cells (IC-CACGs), which may have discriminative value in distinguishing UC from CAC. Single-cell sequencing analysis revealed significant enrichment of the PI3K/Akt/mTOR signaling pathway and inflammatory responses in patients with a high transformation index. The 20-gene prognostic model developed based on these findings may help predict survival outcomes in patients with CAC, and the association between high-risk scores and poor response to immunotherapy warrants further investigation. Experimental validation further demonstrated that the expression levels of *CCL4*, *CD69*, *CXCR4*, and *SAMSN1* were significantly elevated in CAC tissues. Collectively, these findings suggest that immune-related genes may participate in the development and progression of CAC, and provide potential molecular targets and research directions for early diagnosis, risk stratification, and exploration of immunotherapeutic strategies for this disease.

Data Sharing Statement

The data supporting the findings of this study are publicly available from publicly accessible repositories. The gene microarray data were obtained from the Gene Expression Omnibus (GEO) under accession numbers GSE37283, GSE17537, and the single-cell RNA-sequencing dataset under GSE132257. These datasets are accessible at https://www.ncbi.nlm.nih.gov/geo/. The transcriptomic and clinical data of the TCGA-CRC cohort (640 tumor and 51 healthy control samples) were obtained from The Cancer Genome Atlas (TCGA) database at https://www.cancer.gov/ccg/research/genome-sequencing/tcga. No new or unpublished data were generated in this study. Researchers may directly access all datasets from the original public repositories using the provided accession numbers. Data are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The Affiliated Hospital of Shandong University of Traditional Chinese Medicine (2025-070-01-KY). The patients/participants provided their written informed consent to participate in this study.

Acknowledgments

We thank all the patients who donated tissue in this study.

Author Contributions

Chunhua Chi: Conceptualization, Investigation, Data curation, Formal analysis, Methodology, Writing – original draft, Supervision, Project administration. Yunjie Zhang: Conceptualization, Funding acquisition, Methodology, Writing –

review & editing, Supervision. Shanyu Gao: Investigation, Methodology, Supervision, Validation, Writing – review & editing. Congcong Liu: Investigation, Methodology, Software, Data curation, Formal analysis, Writing – review & editing. Benjun Wang: Investigation, Methodology, Software, Data curation, Formal analysis,Writing – review & editing. Jian Chen: Investigation, Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – review & editing. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by Project supported by the Natural Science Foundation of Shandong Province, China (Grant No. ZR2021MH399).

Disclosure

The authors have no conflicts of interest to declare.

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