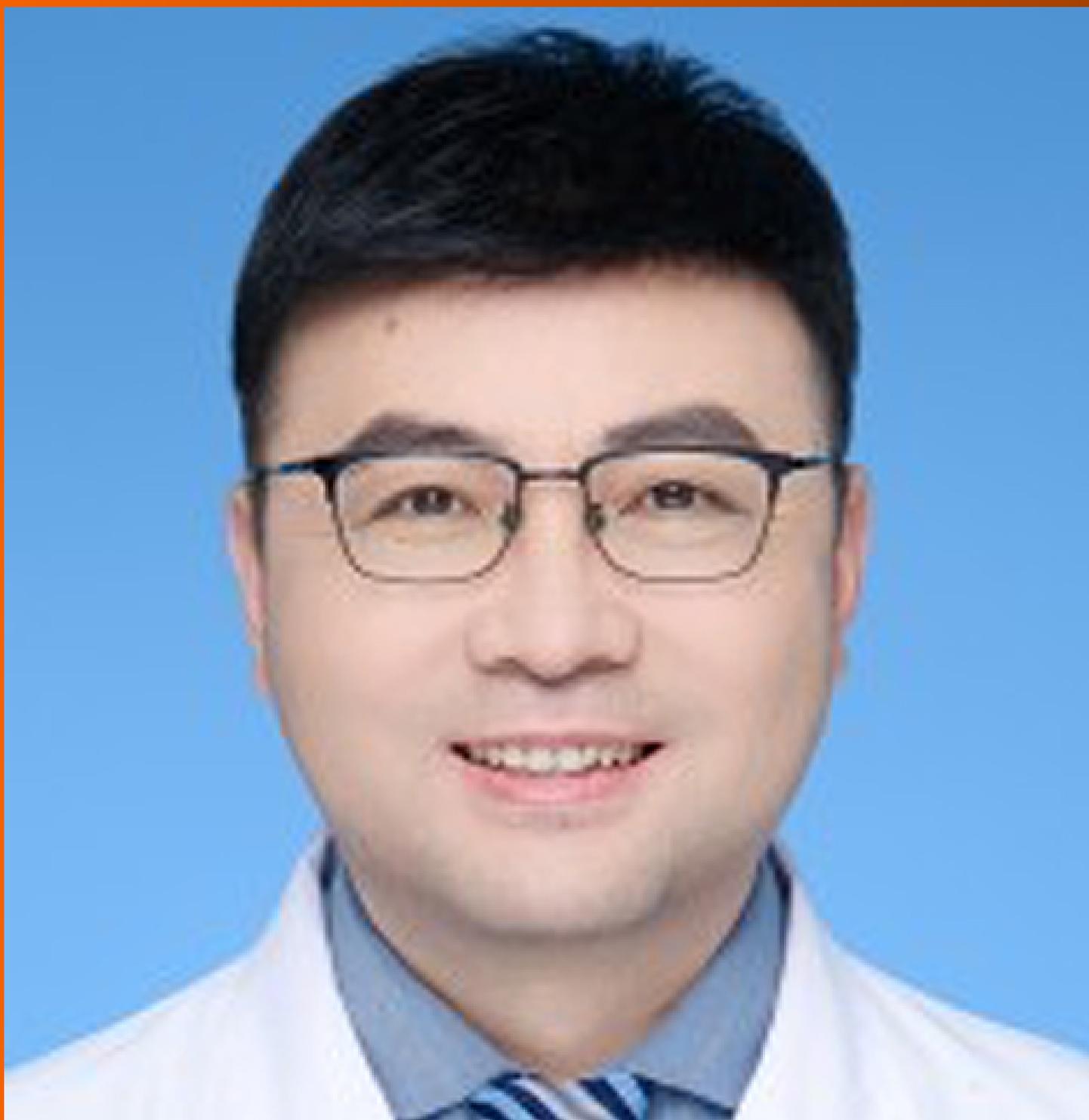


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**ABOUT COVER**

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The primary aim of *World Journal of Gastrointestinal Oncology (WJGO, World J Gastrointest Oncol)* is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

*WJGO* mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Observational Study

## Establishment of a cholangiocarcinoma risk evaluation model based on mucin expression levels

Chun-Yuan Yang, Li-Mei Guo, Yang Li, Guang-Xi Wang, Xiao-Wei Tang, Qiu-Lu Zhang, Ling-Fu Zhang, Jian-Yuan Luo

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### Abstract

#### BACKGROUND

Cholangiocarcinoma (CCA) is a highly malignant cancer, characterized by frequent mucin overexpression. *MUC1* has been identified as a critical oncogene in the progression of CCA. However, the comprehensive understanding of how the mucin family influences CCA progression and prognosis is still incomplete.

#### AIM

To investigate the functions of mucins on the progression of CCA and to establish a risk evaluation formula for stratifying CCA patients.

#### METHODS

Single-cell RNA sequencing data from 14 CCA samples were employed for elucidating the roles of mucins, complemented by bioinformatic analyses. Subsequent validations were conducted through spatial transcriptomics and immunohistochemistry. The construction of a risk evaluation model utilized the least absolute shrinkage and selection operator regression algorithm, which was further confirmed by independent cohorts and diverse data types.

#### RESULTS

CCA tumor cells with elevated levels of *MUC1* and *MUC4* showed activated nucleotide metabolic pathways and increased invasiveness. *MUC5AC*-high cells were found to promote CCA progression through WNT signaling. *MUC5B*-high cells exhibited robust cellular oxidation activities, leading to resistance against antitumoral treatments. *MUC13*-high cells were observed to secrete chemokines, recruiting and transforming macrophages into the M2-polarized state, thereby suppressing antitumor immunity. *MUC16*-high cells were found to promote tumor progression through interleukin-1/nuclear factor kappa-light-chain-enhancer of activated B cells signaling upon interaction with neutrophils. Utilizing the expression levels of these mucins, a risk factor evaluation formula for CCA was developed and validated across multiple cohorts. CCA samples with higher risk factors exhibited stronger metastatic potential, chemotherapy resistance, and poorer prognosis.

## CONCLUSION

Our study elucidates the functional mechanisms through which mucins contribute to CCA development, and provides tools for risk stratification in CCA.

**Key Words:** Mucin; Cholangiocarcinoma; Single-cell RNA sequencing; Spatial transcriptomics; Prognosis

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**Core Tip:** In this study, we have conducted a comprehensive investigation of mucins in cholangiocarcinoma (CCA) using a combination of bioinformatics analysis, including single-cell RNA sequencing and spatial transcriptomics, along with experimental validations. Our findings highlight the significant roles of *MUC1*, *MUC4*, and *MUC5B* in CCA metabolism, contributing to tumor progression and therapy resistance. Additionally, *MUC5AC* has been identified as a regulator of CCA invasiveness through the WNT signaling. *MUC13* and *MUC16* are found to play critical roles in tumor-immune interactions, regulating antitumoral immune defense. The collect impact of these mucins enables the development of a CCA prognosis evaluation model that effectively predicts tumor malignancy, treatment effectiveness, and prognosis in CCA cases.

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## INTRODUCTION

Cholangiocarcinoma (CCA) is the second most common malignant liver cancer and has exhibited a rising incidence and mortality trend over the past four decades[1,2]. CCA is categorized into intrahepatic CCA (iCCA), perihilar CCA (pCCA) and distal CCA based on the primary anatomic region, among which the former two subtypes constitute approximately 70%-80% of cases[3]. The majority of patients are diagnosed at an advanced stage, limiting treatment options[4]. For patients who are ineligible for surgical resection, chemotherapy, particularly gemcitabine and cisplatin combination, becomes their primary choice, however, the median survival period remains less than one year[5]. While molecularly targeted therapies show promising antitumor effects[6], patients suitable for these targeted therapies are relatively scarce.

Histopathologically, CCA is classified into two primary subgroups: the mucin-producing subgroup and the nonmucin-producing subgroup[7]. Consequently, mucins play pivotal roles in CCA development. Mucins, a family of proteins widely distributed on the epithelial surface of various organs, serve essential functions in lubrication and defense against toxins and infections[8]. In addition to their physiological roles, mucins play important roles in pathological conditions, including cancer. Abnormal expression and distribution of mucins are found in various cancer types, such as lung cancer, breast cancer, pancreatic cancer, colorectal cancer, gastric cancer, liver cancer, and ovarian cancer[9,10]. Numerous studies have explored the mechanisms of *MUC1* in tumorigenesis, implicating multiple signaling pathways, including the Ras,  $\beta$ -catenin, TP53, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), transforming growth factor  $\beta$ , and vascular endothelial growth factor[10]. However, clinical trials investigating *MUC1* have not demonstrated significant treatment effects compared to those in control groups[11,12], highlighting the need to systematically target multiple mucins.

In recent decades, single-cell RNA sequencing (scRNA-seq) has revolutionized cancer research, leading to remarkable milestones. The application of scRNA-seq to CCA has revealed intratumor heterogeneity at the single-cell level[13], identified new markers for different CCA subtypes[14], characterized sub-clusters of cancer-associated fibroblasts[15], and elucidated intercellular crosstalk within the tumor microenvironment (TME)[16]. Nevertheless, mucin functions and the characteristics of mucin-expressing CCA cells have not been previously explored at single-cell resolution. In this study, we comprehensively analyzed the scRNA-seq data from 14 human CCA samples, including 13 iCCA patients and one pCCA patient, to determine the mechanisms of various mucins, specifically *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*, in promoting CCA progression. The mucin-based CCA patient stratification system provides a new dimension for CCA prognosis prediction, and offers potential strategies for CCA treatment.

## MATERIALS AND METHODS

### Single-cell RNA sequencing data analysis

Our scRNA-seq data of CCA samples were sourced from the gene expression omnibus database (GSE154170, GSE138709, GSE142784, and GSE125449). After selecting samples from treatment-naïve CCA patients, we integrated these data into a unified dataset using R package Harmony. Following normalization, nonlinear dimensionality reduction was applied to reduce the dimensionality of data. Subsequently, cell clustering was performed using the standard methods suggested by satijalab (<https://satijalab.org/seurat/>). This resulted in the identification of six cell types. Cells from normal tissues and hepatocytes were filtered out, leaving only CCA tumor cells for the functional evaluation of mucins. Myeloid cells were performed under the same methodology.

### Transcription factor analysis

The R package SCENIC (<http://scenic.aertslab.org>) was utilized to identify putative transcription factors and regulons of *MUC1*-high and *MUC1*-low cells[17]. Marker regulons of *MUC1*-high cells were identified by comparing different regulons between *MUC1*-high and *MUC1*-low cells using FindAllMarker function.

### Gene set enrichment analysis

Our gene set enrichment analysis (GSEA) was conducted using the R package fgsea. The oncogenic and metabolic pathways involved in our study were obtained from MSigDB (<http://www.gseamsigdb.org/gsea/msigdb>). The normalized enrichment score value was calculated by the difference enrichment score between *MUC1*-high and *MUC1*-low cells, *MUC5AC*-high vs *MUC5AC*-low cells, *MUC5B*-high vs *MUC5B*-low cells, *MUC13*-high cells vs *MUC13*-low cells, and *MUC16*-high cells vs *MUC16*-low cells.

### Gene set variation analysis

Comprehensive analyses of 70 metabolic signaling pathways were performed using gene set variation analysis (GSVA) with the R package GSVA. The 70 metabolic pathways were collected based on a previous article[18]. After calculation the rank values of each gene in each geneset, the rank value was constrained from -3 to 3 to avoid extreme values. Subsequently, the rank values of each cluster of cells were normalized to their mean value to generate the heatmap.

### Spatial transcriptomics data analysis

Spatial transcriptomics (ST) data were obtained from a published liver cancer dataset (cohort 9, [Supplementary Table 1](#)), which included hepatocellular carcinoma samples, CCA samples, and combined hepatocellular-CCA[19]. We selected the cHC-1T sample, exhibiting clear CCA histological features, for functional detection of *MUC1* and *MUC5B*. ST data analysis followed the guidelines provided by satijala ([https://satijalab.org/seurat/articles/spatial\\_vignette](https://satijalab.org/seurat/articles/spatial_vignette)).

### Gene expression correlation analyses

Expression data of mucins and *WNT7B*, *ALDH1A1*, *UCP2*, *CSF3R*, *NFKB1*, *NFKB2*, *RELA* were obtained from a previously reported dataset[20]. Correlations between mucins and these genes was evaluated using the R package ggplot2. The significance of these correlations was determined by Pearson's correlation analysis ( $P \leq 0.05$ ).

### Cell interaction analysis

Cellular interaction network analysis was performed using the R package CellChat (<http://www.cellchat.org/>)[21]. When analyzing interactions between CCA sub-cluster and other cell types in TME, we randomly selected 1000 cells in each TME cell partitions, including lymphocytes, fibroblasts, endothelial cells, and myeloid cells.

### CCA risk factor model establishment

To assess CCA prognosis based on mucins, we applied the least absolute shrinkage and selection operator (LASSO) algorithm on the RNA levels of *MUC1*, *MUC13*, *MUC16*, *MUC4*, *MUC5AC*, and *MUC5B* by R package glmnet. RNA levels were normalized using the trans per million method. The CCA risk score was then established as follows: risk score = sum (each MUC gene expression × corresponding coefficient). The coefficient for *MUC1*, *MUC13*, *MUC16*, *MUC4*, *MUC5AC*, and *MUC5B* are 0.0297, 0.0460, 0.1217, 0.0574, 0.0501, and 0.0657, respectively. Subsequently, the CCA patients were stratified into high-risk and low-risk groups.

### CCA patient survival analysis

The patient survival information, along with corresponding RNA-seq data and protein expression levels of tumor samples, were obtained from cohort 2 and cohort 10. The correlation between CCA patient survival, individual mucin expression (including *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*), and risk factors was assessed using the log-rank (Mantel-Cox) test.

### Independent CCA samples and double staining immunohistochemistry

Validation of mucin functions was conducted using primary CCA samples resected at Peking University Third Hospital. Our study was approved by the Ethics Committee of Peking University Third Hospital. All research was conducted in accordance with both the Declarations of Helsinki and Istanbul. All patients were informed and kept anonymous.

Formalin-fixed and paraffin-embedded 4- $\mu$ m tissue sections were used for immunohistochemistry (IHC) staining. Briefly, sections were dehydrated with graded concentrations of ethanol and immersed in 3% hydrogen peroxide for 15 min. Antigen retrieval was performed by heating for 2 min in a pressure cooker using 0.01 M citrate buffer (pH 6.0). Sections were then incubated with primary antibodies against *MUC13* (Abcam, Cat ab235450), *MUC16* (Origene, Cat ZM-0019), CD163 (Origene, Cat ZM-0428), and CD66b (Abcam, Cat ab300122) at 4 °C overnight. The GTVision™ Double Staining Detection System (Dako, Cat GK700110) was used for the secondary antibody and 3,3-diaminobenzidine/hydrogen peroxide was used as the chromogen. Substitution of the primary antibody with phosphate-buffered saline was used as a negative control.

### Statistics

Comparisons of gene expression levels between tumor and non-tumoral normal tissue were performed using paired or unpaired student's *t*-test. The signaling pathway enrichment analysis was performed using Fisher's exact test. The GSEA was performed using Wilcoxon-Mann-Whitney test. The GSVA was performed using Kolmogorov-Smirnov like random walk test. Patient survival analysis was performed using the log-rank (Mantel-Cox) test. Correlation analyses of mucin expression levels and CCA pathological phenotypes were subjected to Chi-square analysis. All analyses were performed by R or GraphPad Prism. Differences were regarded significant when  $P < 0.05$ .

## RESULTS

### Clinical significance and heterogeneity of mucins in CCA

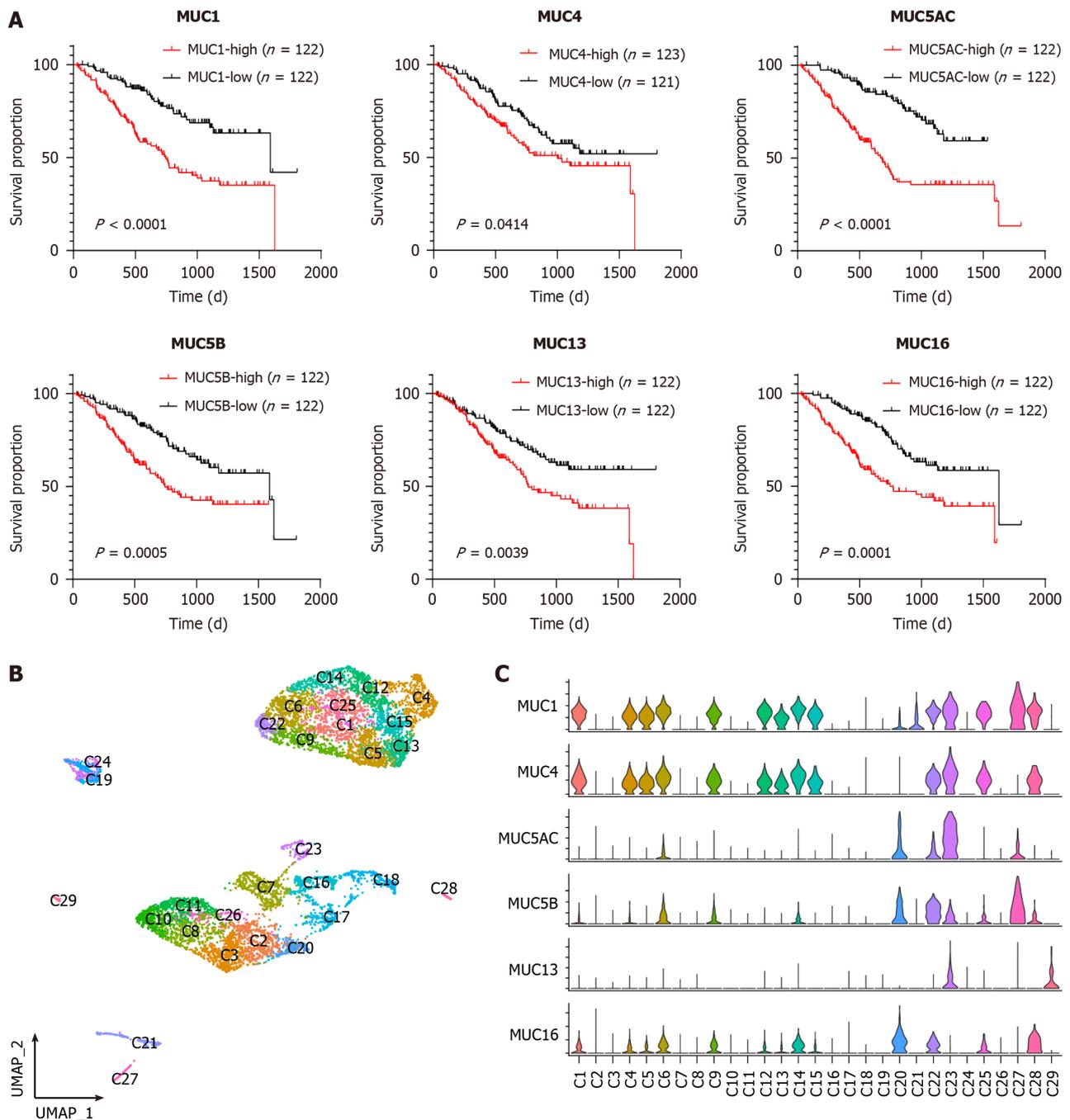
Despite previous reports suggesting the potential diagnostic roles of mucins in CCA [10,22,23], systematic evaluation of all mucins in CCA is lacking. This study introduced multiple clinical cohorts to assess the clinical relevance of mucins in CCA. Cohort 1 (Supplementary Table 1) was sourced from the CCA dataset in The Cancer Genome Atlas database. Among the 20 mucin-encoding genes, many mucins exhibited significantly higher expression levels in tumor tissues than in normal tissues (Supplementary Figure 1A), emphasizing the critical functions of mucins in CCA development. Notably, elevated expression levels of *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16* were found to predict poor CCA prognosis in cohort 2 (Supplementary Table 1) [24] (Figure 1A). Therefore, we focused on the functional evaluation of these mucins in this study. Two additional independent CCA cohorts (cohorts 3 and 4, Supplementary Table 1) further validated the upregulation of the abovementioned mucins in both unpaired [25] (Supplementary Figure 1B) and paired [26] (Supplementary Figure 1C) CCA samples. However, given the heterogeneity of CCA, a detailed investigation of mucins at the single-cell level is imperative.

To evaluate mucin heterogeneity at the single-cell level, we combined the scRNA-seq data from four independent datasets (cohorts 5, 6, 7, and 8 in Supplementary Table 1) [15,16,27], and reanalyzed the integrated data. The integrated dataset comprised 51810 single cells from 14 patients (Supplementary Table 2), and was classified into six cell types, namely cycling cells, dying cells, epithelial cells, lymphocytes, myeloid cells, and stromal cells (Supplementary Figure 2A). The marker gene expression levels and proportions further confirmed the annotation of these cell types (Supplementary Figure 2B). For quality control, we examined the batch effect of our data integration by evaluating the distribution features of cells from different patients. The result indicated a fine mixture of cells from different patients, suggesting low batch effects (Supplementary Figure 2C). Additionally, the observed differences in gene expression between cells from normal regions and tumor regions (Supplementary Figure 2D) suggested the occurrence of transcriptomic remodeling during CCA development.

To evaluate the functions of mucins (*MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*) in CCA at the single-cell level, we extracted tumor cells from the integrated data. Re-clustering of the tumor cells resulted in the formation of 29 sub-clusters, designated C1 to C29 (Figure 1B). The marker gene expression for each sub-cluster is shown in Supplementary Figure 3. Notably, the detection of mucin expression revealed many sub-clusters expressing high levels of the *MUC1* and *MUC4* genes, including C1, C4, C5, C6, C9, C12, C13, C14, C15, C22, C23, C25, C27 and C28 (Figure 1C). Therefore, we examined *MUC1* and *MUC4* collectively afterward. In contrast, *MUC5AC*, *MUC5B*, *MUC13* and *MUC16* exhibited relatively unique distribution patterns (Figure 1C). *MUC5AC* was enriched in C23. *MUC5B* was enriched in C22 and C27. *MUC13* was enriched in C23 and C29. *MUC16* was enriched in C20 and C28. Subsequently, functional analyses were performed on *MUC1*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*, independently.

### Selective activation of nucleotide metabolism in *MUC1*-high CCA cells

To elucidate the functions of *MUC1* in CCA, we integrated sub-clusters C1, C4, C5, C6, C9, C12, C13, C14, C15, C22, C23, C25, C27, and C28 into *MUC1*-high subgroup, and integrated the remaining sub-clusters into *MUC1*-low subgroup. Next, we compared the gene expression profiles between *MUC1*-high cells and *MUC1*-low cells, and identified differentially expressed genes (DEGs). Pathway enrichment analysis of these DEGs revealed pronounced activation of metabolic pathways in *MUC1*-high cells (Supplementary Figure 4A). To determine the metabolic characteristics of *MUC1*-high cells, we employed a 70-pathway metabolic analysis panel, which demonstrated heightened activation of pathways related to nucleotide, energy, amino acid, and detoxification metabolism in *MUC1*-high cells (Figure 2A). Among these metabolic processes, nucleotide metabolism exhibited a prominent role, as indicated by the identification of numerous pathways (Figure 2A, highlighted in red). Independent analyses of nucleotide metabolic pathways (Supplementary Figure 4B-D) and nucleotide excision repair (Supplementary Figure 4E and F) further validated the distinctive features of *MUC1*-high cells. Moreover, compared with *MUC1*-low cells, *MUC1*-high cells displayed elevated expression levels of RNA polymerase components and DNA repair regulators (Supplementary Figure 4G).

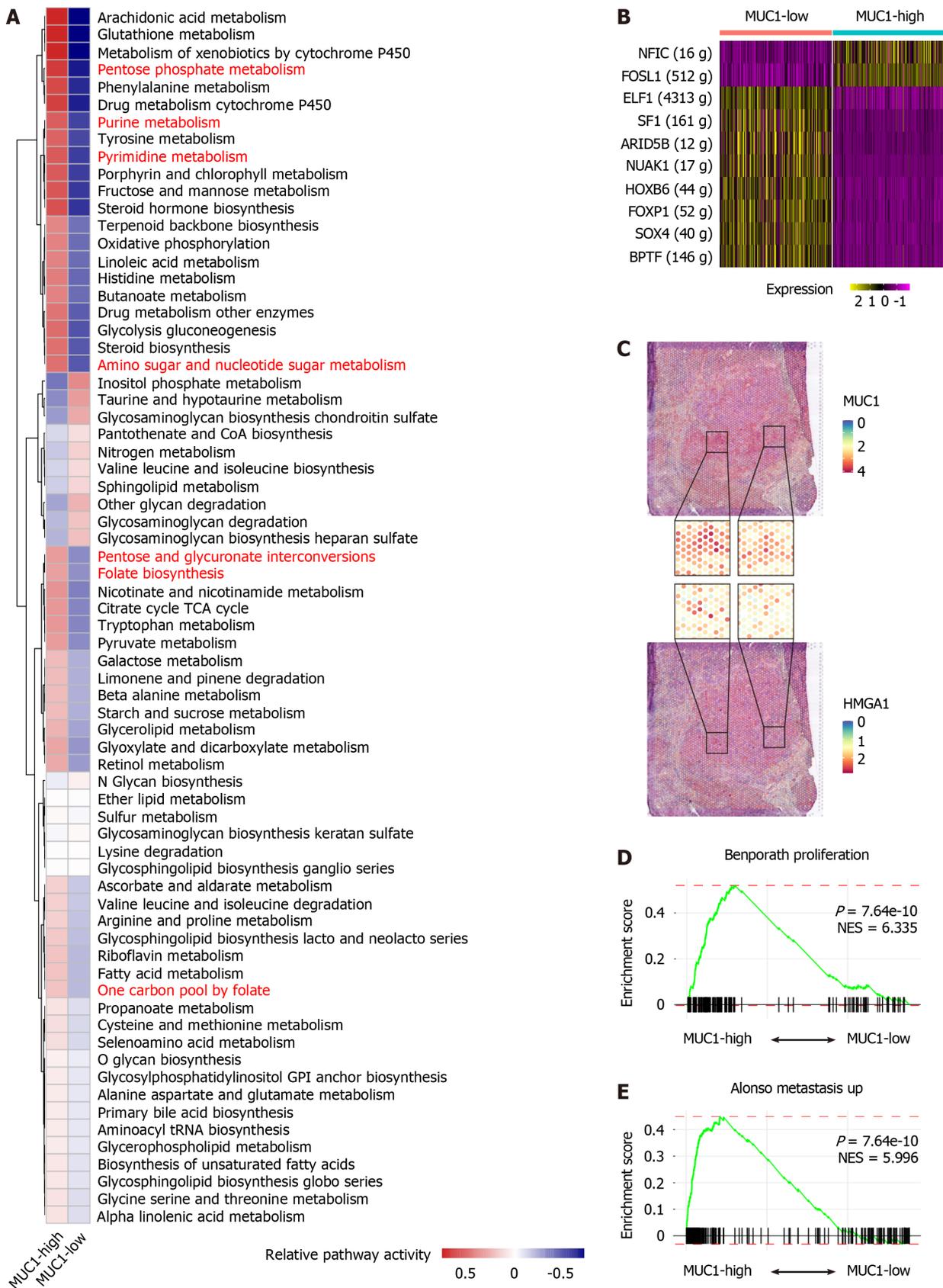


**Figure 1 Clinical significance and heterogeneity of mucins in cholangiocarcinoma.** A: Survival curves generated using cohort 2 data illustrating the relationship between cholangiocarcinoma (CCA) patient survival and the mRNA levels of mucins; B: Uniform manifold approximation and projection plot visually representing the tumor cell sub-clusters within the integrated CCA dataset; C: Violin plots depicting of the relative gene expression levels of *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16* in CCA tumor sub-clusters.

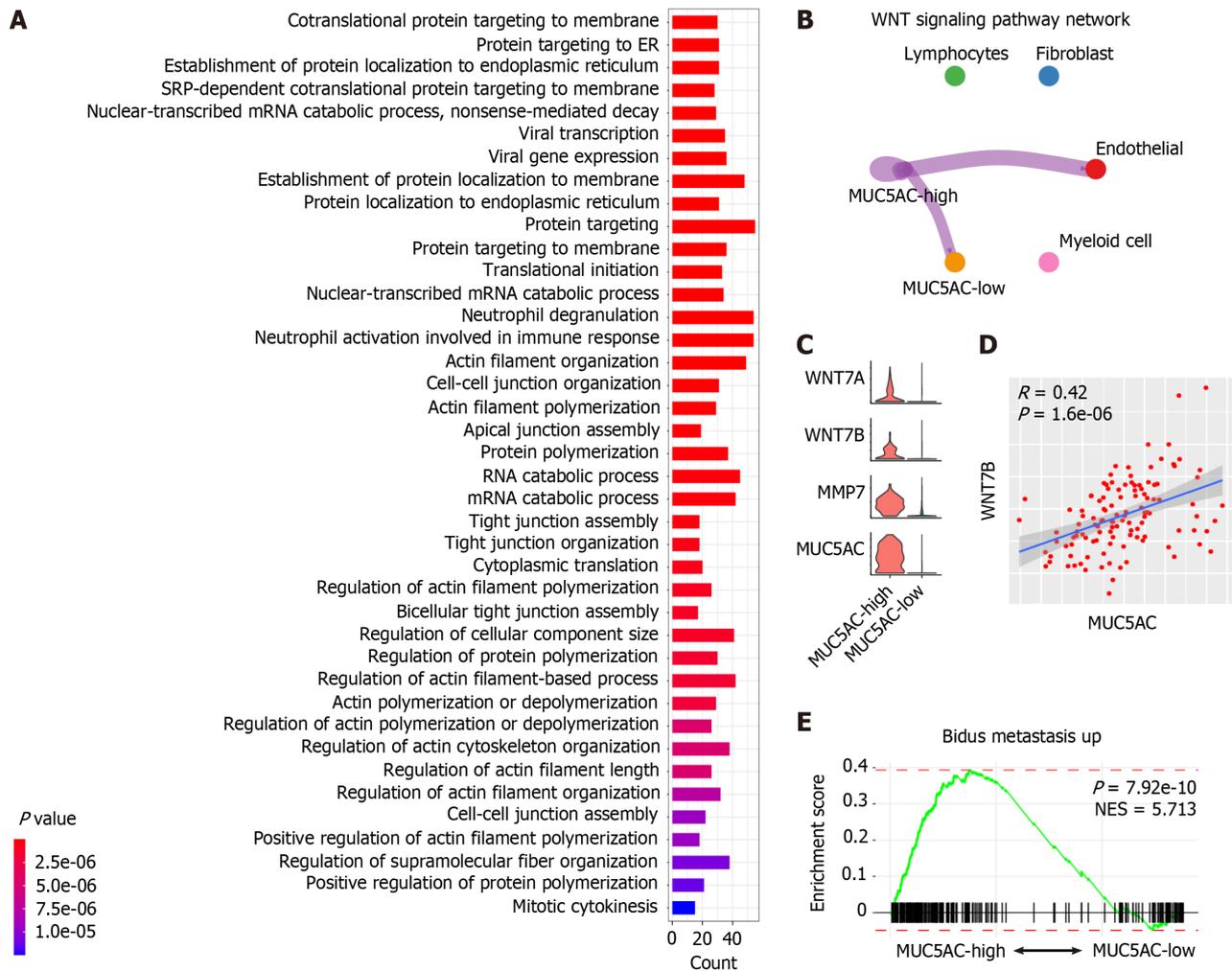
To unravel the mechanisms underlying the observed metabolic characteristics of *MUC1*-high cells, we analyzed the transcription factor expression in these cells. Compared with *MUC1*-low cells, *MUC1*-high cells exhibited uniquely higher expression levels of *NFIC* and *FOSL1* (Figure 2B). *FOSL1* is known to regulate the expression of another transcription factor, *HMGA1*[28], which was found to play important roles in nucleotide metabolism[29,30]. Examination of the co-expression patterns of *FOSL1*, *HMGA1*, and *MUC1* in both scRNA-seq (Supplementary Figure 4H) and ST (cohort 9, Supplementary Table 1) (Figure 2C) data indicated significant co-expression. Importantly, both *FOSL1* and *HMGA1* are implicated tumor progression[30,31]. GSEA confirmed the enhanced proliferation and metastasis characteristics of *MUC1*-high cells compared to *MUC1*-low cells (Figure 2D and E). Taken together, our analyses reveal the activation of nucleotide metabolism and high invasion status of *MUC1*-high cells.

**Activation of the WNT signaling pathway in *MUC5AC*-high CCA cells**

Given the substantial expression of *MUC5AC* in cluster C23 (Figure 1C), we defined C23 cells as *MUC5AC*-high cells, and the remaining sub-clusters as *MUC5AC*-low cells. Analysis of DEGs in the *MUC5AC*-high cluster *vs* the *MUC5AC*-low



**Figure 2 Activated nucleotide metabolic signaling and higher malignant characteristics in *MUC1*-high tumor cells of cholangiocarcinoma.** A: Heatmap showing the activity of metabolic signaling pathways in *MUC1*-high vs *MUC1*-low cells; B: Heatmap showing the different expression levels of transcription factors in *MUC1*-high cells vs *MUC1*-low cells; C: Distribution of *MUC1* and *HMGA1* in the spatial transcriptomics (ST) slide. Zoomed in portions of the ST chip (middle) showing multiple areas of co-localization; D and E: Gene set enrichment analysis plots showing the enrichment of genes from *MUC1*-high cells (left) vs *MUC1*-low cells (right) in cell proliferation and metastasis signaling pathways. The *P* values and normalized enrichment score are indicated on the plots.

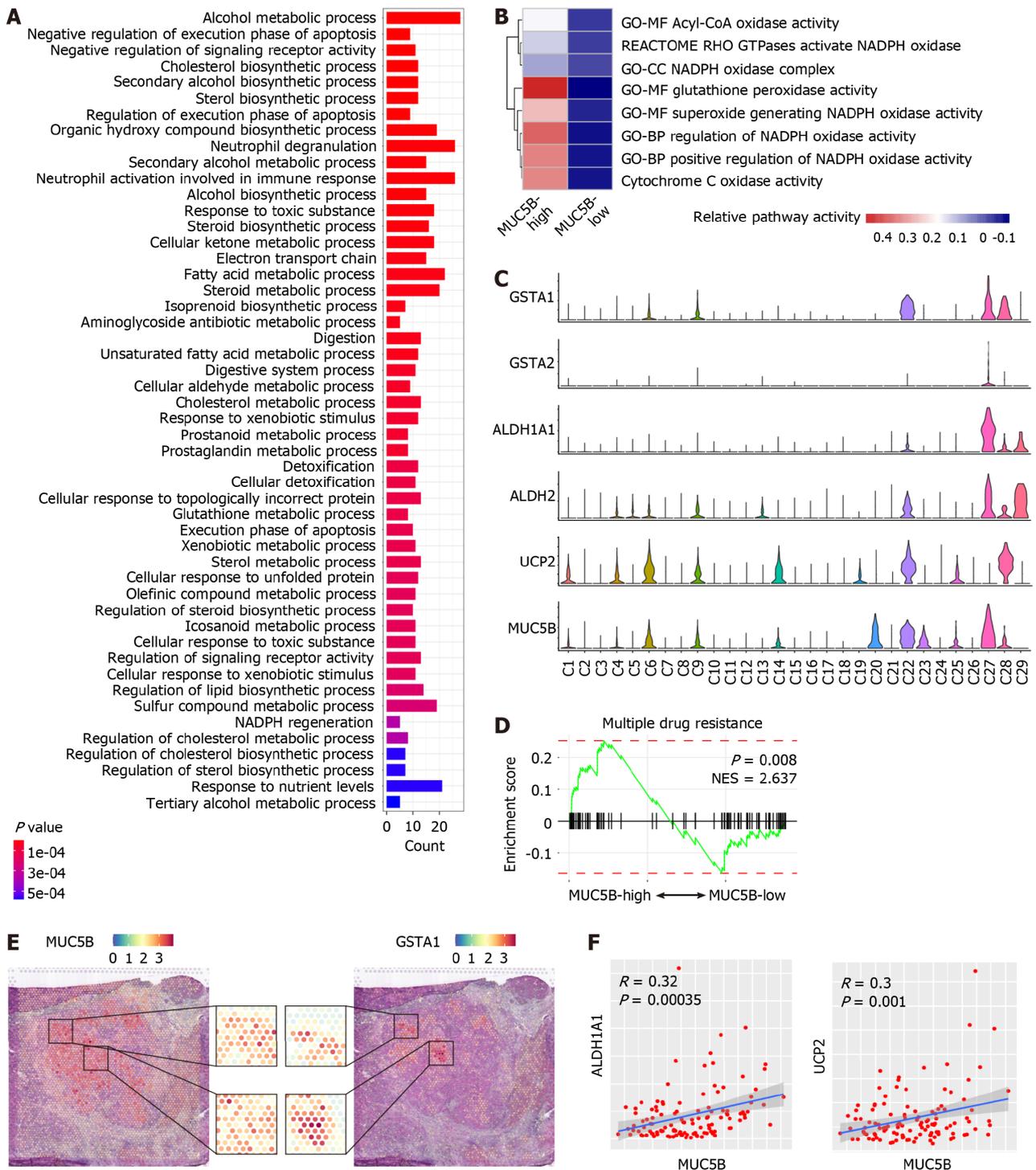


**Figure 3** WNT signaling and metabolic phenotypes in *MUC5AC*-high tumor cells of cholangiocarcinoma. **A**: Top 40 enriched signaling pathways of the differentially expressed genes in *MUC5AC*-high cells; **B**: Relative activity of the WNT signaling pathway among *MUC5AC*-high, *MUC5AC*-low, myeloid cells, endothelial cells, fibroblasts, and lymphocytes; **C**: Violin plots showing relative expression levels of *WNT7A*, *WNT7B*, and *MMP7* in *MUC5AC*-high vs *MUC5AC*-low cholangiocarcinoma (CCA) cells; **D**: Correlation analysis between the expression of *MUC5AC* and *WNT7B* in CCA tumor samples from cohort 10; **E**: Gene set enrichment analysis plot showing the enrichment of genes from the *MUC5AC*-high (left) and *MUC5AC*-low (right) cells in tumor metastasis signaling pathway. The *P* value and normalized enrichment score are indicated on the plot.

cluster revealed enrichment of cell-cell junctions and actin-related cellular mobility pathways (Figure 3A), implicating *MUC5AC* in CCA metastasis. Cellular interaction analysis using CellChat demonstrated activation of the WNT signaling pathway in *MUC5AC*-high cells (Figure 3B). Given the tumor-promoting functions of the WNT pathway[32], we examined the expression of WNT ligands, including *WNT7A* and *WNT7B*, as well as the WNT target gene *MMP7*[33], in *MUC5AC*-high and *MUC5AC*-low cells (Figure 3C). Co-expression analysis of *MUC5B* and *WNT7B* in an independent CCA cohort (cohort 10, Supplementary Table 1) confirmed a significant correlation (Figure 3D), supporting the activation of the WNT signaling pathway in *MUC5AC*-high CCA cells. Subsequent GSEA further verified the metastasis-promoting role of *MUC5AC*-high cells (Figure 3E and Supplementary Figure 5).

### Cellular oxidation and detoxification characteristics of *MUC5B*-high cells

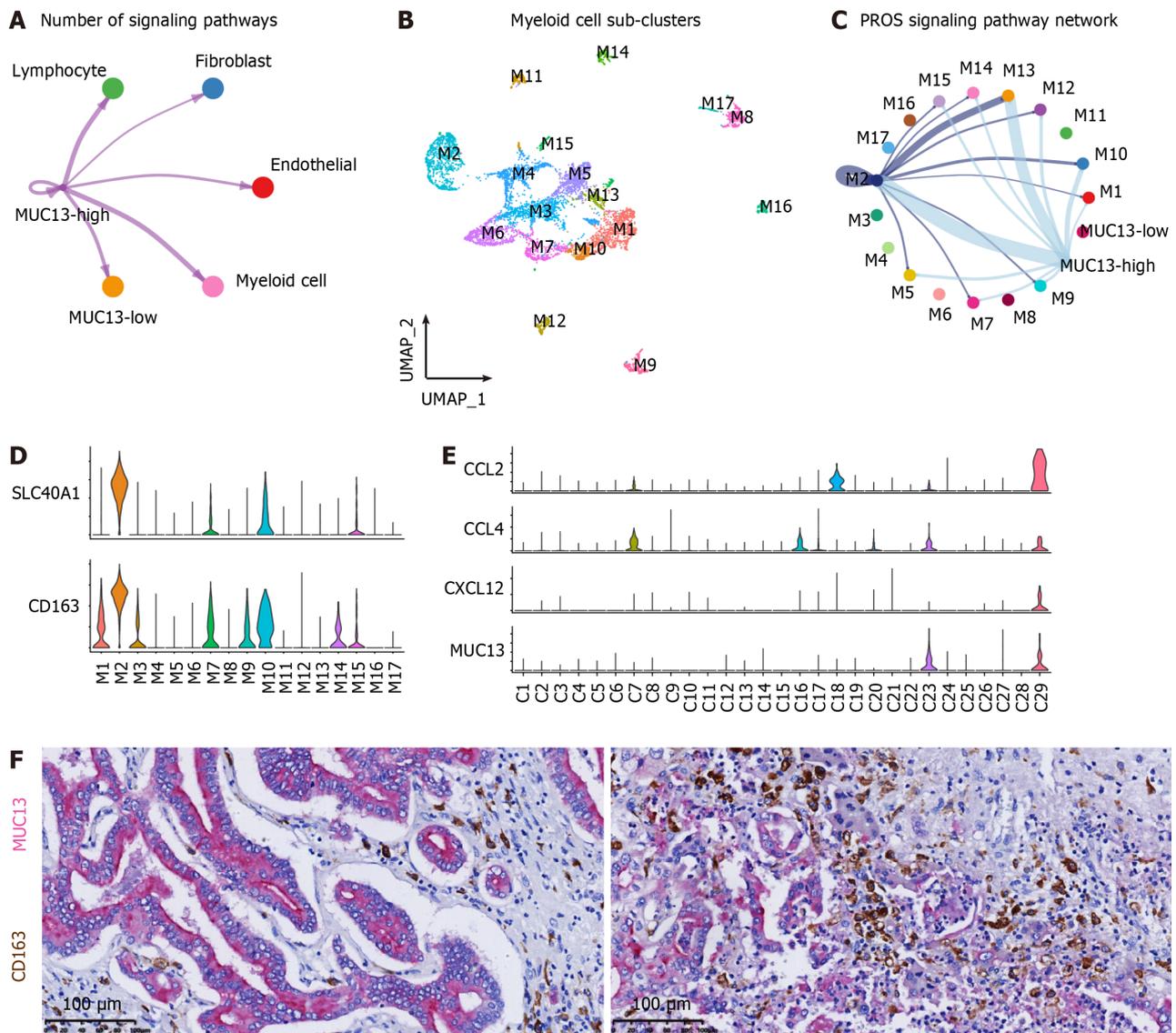
To unravel the functions of *MUC5B* in CCA, we stratified CCA cells based on *MUC5B* expression level in the scRNA-seq data. Combining sub-clusters C22 and C27 as the *MUC5B*-high subgroup (Figure 1C), the remaining sub-clusters formed the *MUC5B*-low subgroup. Comparative analysis revealed that the DEGs of *MUC5B*-high cells were highly enriched in cellular oxidation and detoxification pathways (Figure 4A). Additional cellular oxidation pathways from independent datasets further confirmed the active oxidation status of *MUC5B*-high cells (Figure 4B). Furthermore, the scRNA-seq data revealed that *GSTA1*, *GSTA2*, *ALDH1A1*, *ALDH2*, and *UCP2* were highly co-expressed with *MUC5B* (Figure 4C). These genes not only participate in detoxification processes, but also indicate antitumor treatment resistance and poor prognosis in cancer patients[34-37]. Chemotherapy sensitivity analysis of *MUC5B*-high cells demonstrated that *MUC5B*-high cells exhibited significantly greater resistance to antitumor drugs than *MUC5B*-low cells did (Figure 4D, and Supplementary Figure 6). Furthermore, *in-situ* co-expression of *MUC5B* and *GSTA1* was observed in the ST data (Figure 4E). Co-expression of *MUC5B* with *ALDH1A1* or *UCP2* was further confirmed in cohort 10 (Figure 4F). Collectively, these data reveal the active oxidation state and chemotherapy resistance characteristics of *MUC5B*-high cells.



**Figure 4 Detoxification function of *MUC5B*-high tumor cells of cholangiocarcinoma.** A: Top 50 enriched signaling pathways of the differentially expressed genes in *MUC5B*-high cells; B: Heatmap showing the relative activities of oxidation-related metabolic signaling pathways in *MUC5B*-high vs *MUC5B*-low cells; C: Violin plots showing relative expression levels of *GSTA1*, *GSTA2*, *ALDH1A1*, *ALDH2*, *UCP2*, and *MUC5B* in cholangiocarcinoma (CCA) tumor sub-clusters; D: Gene set enrichment analysis plot showing the enrichment of genes from *MUC5B*-high (left) and *MUC5B*-low (right) cells in the drug resistance signaling pathway. The *P* value and normalized enrichment score are indicated on the plot; E: Distribution of *MUC5B* and *GSTA1* in the spatial transcriptomics (ST) slide. Zoomed in portions of the ST chip (middle) showing multiple areas of co-localization; F: Correlation analysis between the expression of *MUC5B* and *ALDH1A1* (left), *MUC5B* and *UCP2* (right) in CCA tumor samples from cohort 10.

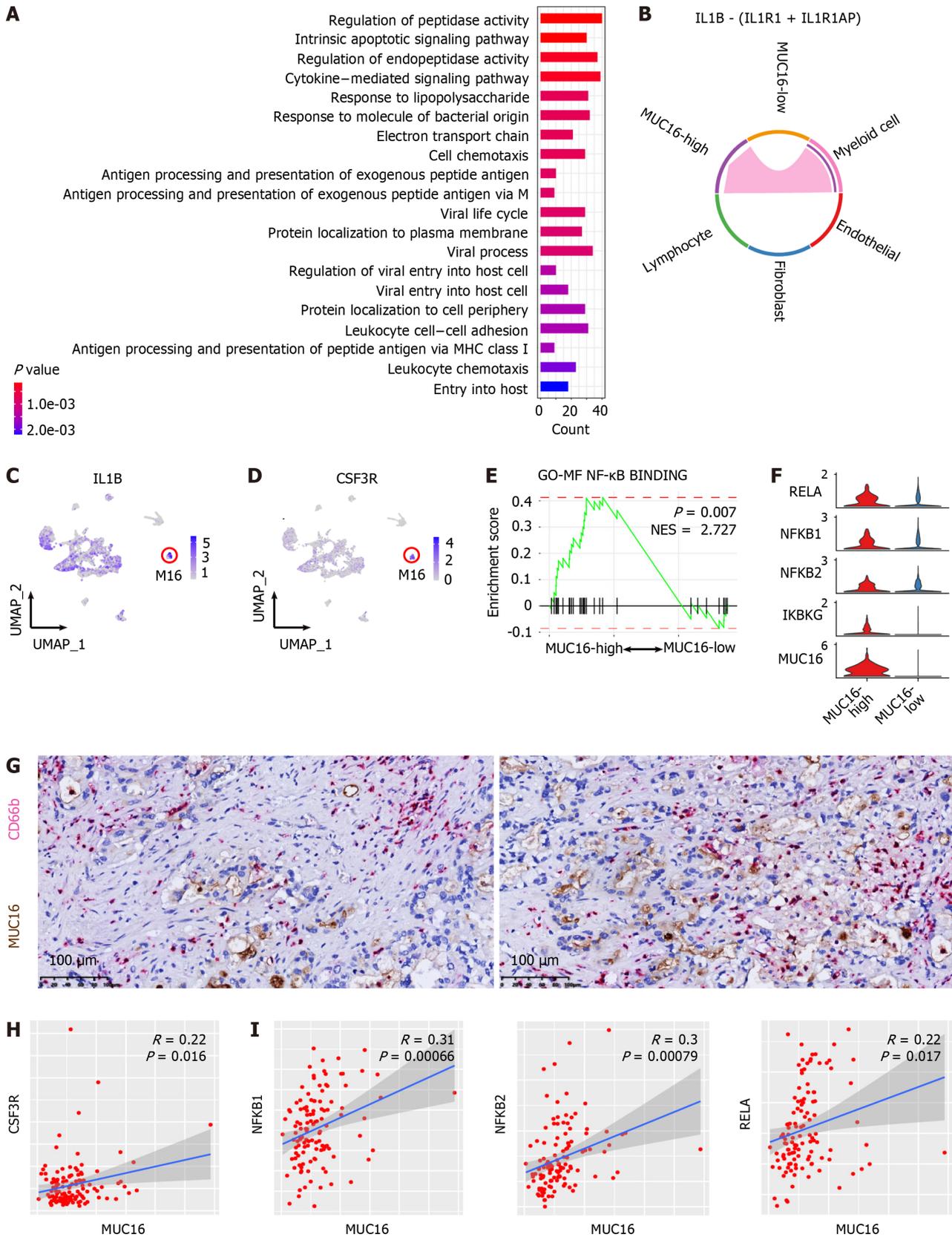
### M2-polarization of macrophages induced by *MUC13*-high CCA cells

Although *MUC13* has been reported to play important roles in the progression of various tumors[38], the interaction between *MUC13*-high cells and the TME has not been fully elucidated. Considering the distinctive expression of *MUC13* in C23 and C29 (Figure 1C), we integrated C23 and C29 to generate *MUC13*-high CCA cells, while the remaining sub-clusters were categorized as *MUC13*-low cells. Interaction analyses between *MUC13*-high cells and TME components, namely *MUC13*-low cells, myeloid cells, endothelial cells, fibroblasts, and lymphocytes, revealed significant interactions



**Figure 5 Chemotaxis and M2-polarization of macrophage induced by *MUC13*-high tumor cells of cholangiocarcinoma.** A: Number of signaling pathways among *MUC13*-high, *MUC13*-low, myeloid cells, endothelial cells, fibroblasts, and lymphocytes; B: Uniform manifold approximation and projection plot showing the identification of 17 myeloid cell sub-clusters; C: PROS signaling pathway activity among the *MUC13*-high sub-cluster, *MUC13*-low sub-cluster, and myeloid cell sub-clusters; D: Violin plots showing relative expression levels of M2-polarized macrophage marker genes, including *SLC40A1* and *CD163*, in myeloid cell sub-clusters; E: Violin plots showing relative expression levels of *CCL2*, *CCL4*, and *CXCL12* in tumor cell sub-clusters; F: Immunohistochemistry double staining showing the adjacent distribution of *MUC13*-high cells (*MUC13*-positive, purple) and M2-polarized macrophages (*CD163*-positive, brown) in cholangiocarcinoma. Scale bar, 100  $\mu$ m.

between *MUC13*-high cells and myeloid cells (Figure 5A). To comprehensively evaluate the transcriptomic characteristics of the myeloid cell sub-populations interacting with *MUC13*-high cells, we isolated myeloid cell components and re-clustered them into 17 sub-populations (Figure 5B). In comparison with *MUC13*-low cells, *MUC13*-high cells exhibited a specific interaction with the M2 sub-cluster of myeloid cells in the PROS1-AXL signaling pathway (Figure 5C). The PROS signaling pathway, particularly the AXL receptor on the macrophage surface, has been shown to induce macrophage M2-polarization and tumor progression[39]. Therefore, the communication between *MUC13*-high cells and the M2 sub-cluster suggested that M2-polarization was induced by *MUC13*-high cells. Moreover, the M2 sub-cluster was characterized by high expression of *SLC40A1* (Supplementary Figure 7, red frame), which was reported to be a marker gene of macrophage M2-polarization[40]. Subsequently, we examined the expression of *SLC40A1* and another M2-polarization marker, *CD163*, in the myeloid sub-clusters of our data. The results indicated significantly elevated expression levels of both *SLC40A1* and *CD163* in the M2 sub-cluster (Figure 5D), confirming the M2-polarization of these cells. We next explored the mechanisms of these interactions, and identified uniquely expressed *CCL2*, *CCL4*, and *CXCL12* in *MUC13*-high clusters (Figure 5E). The chemokine activities of *CCL2* and *CCL4* in macrophage recruitment and *CXCL12* in macrophage M2-polarization suggest that *MUC13*-high cells recruit macrophages into the TME and induce M2 polarization[41]. Additionally, double staining IHC was used to confirm the histologically adjacent distribution between *MUC13*-high cells and M2-polarized macrophages in multiple CCA samples (Figure 5F). Taken together, these findings suggest that *MUC13*-high cells promote CCA progression by inducing M2-polarization of macrophages through the



**Figure 6** Interaction with neutrophils and activated nuclear factor kappa-light-chain-enhancer of activated B cells signaling in *MUC16*-high tumor cells of cholangiocarcinoma. A: Top 20 enriched signaling pathways of the differentially expressed genes of *MUC16*-high cells; B: Interleukin-1 signaling pathway activity among *MUC16*-high cells, *MUC16*-low cells, and cells in tumor microenvironment; C and D: Feature-plots showing the expression level and distribution of *IL1B* and *CSF3R* in myeloid cell sub-clusters. Red circles showing the enrichment of *IL1B* and *CSF3R* in M16 sub-cluster; E: Gene set enrichment analysis plots showing the enrichment of genes from *MUC16*-high cells (left) and *MUC16*-low cells (right) in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. The *P* value and normalized enrichment score are indicated on the plot; F: Violin plots showing relative expression levels of key

regulatory genes (*RELA*, *NFKB1*, *NFKB2*, *IKBKKG*) of the NF- $\kappa$ B pathway in *MUC16*-high vs *MUC16*-low cells; G: Immunohistochemistry double staining showing the adjacent distribution of *MUC16*-high cells (*MUC16*-positive, brown) and neutrophils (CD66b-positive, purple) in cholangiocarcinoma (CCA). Scale bar, 100  $\mu$ m; H: Correlation analysis between the expression of *MUC16* and *CSF3R* in CCA tumor samples from cohort 10; I: Correlation analysis between the expression of *MUC16* and *NFKB1*, *NFKB2*, and *RELA* in CCA tumor samples from cohort 10.

PROS1-AXL signaling pathway.

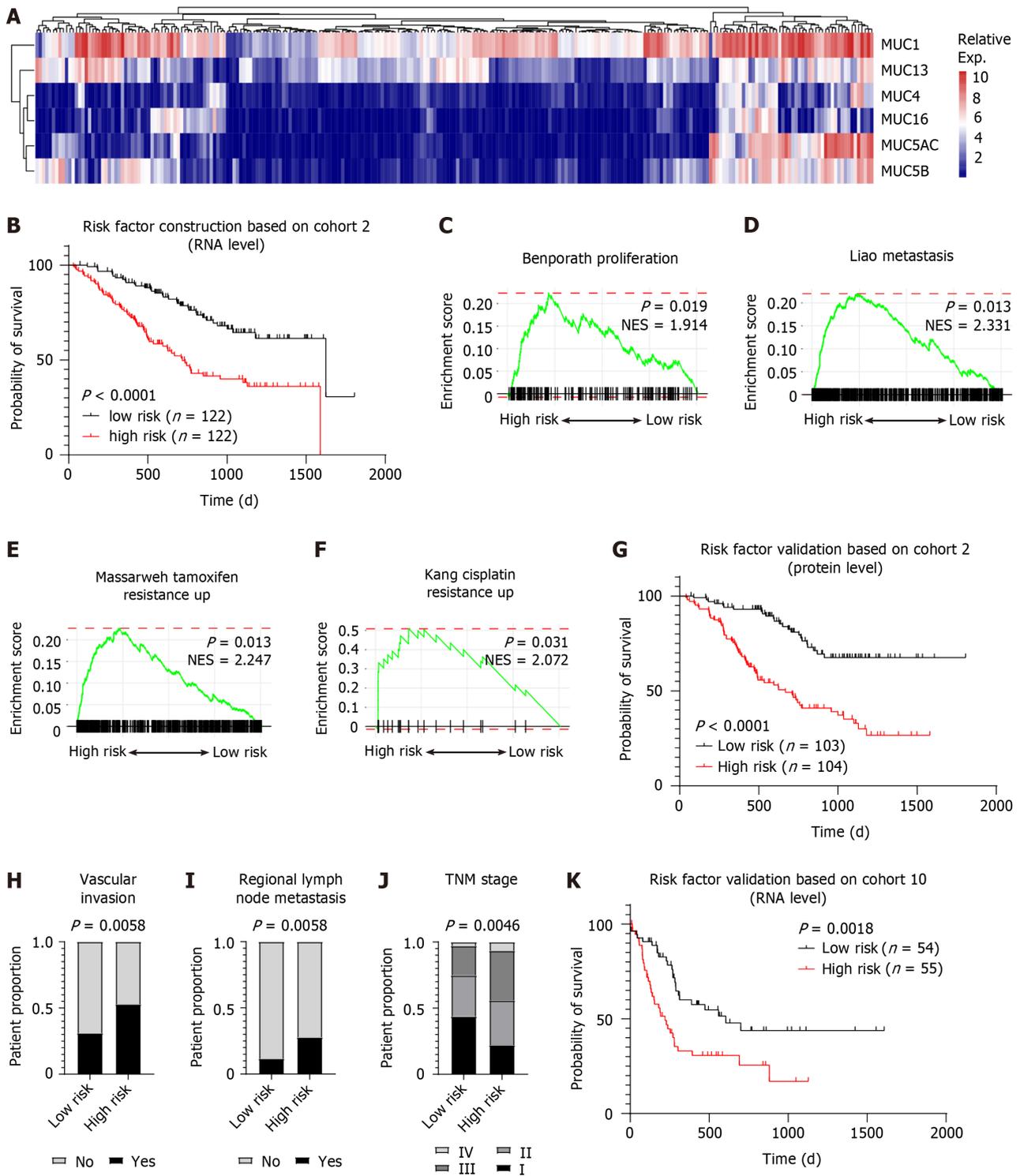
### Activation of the NF- $\kappa$ B signaling pathway in *MUC16*-high CCA cells

*MUC16*, also known as CA125, serves as a widely used biomarker for ovarian cancer[42]. Nevertheless, the impact of *MUC16* on CCA progression and the underlying mechanisms remain elusive. Given the abundant and relatively unique expression of *MUC16* in the C20 cluster (Figure 1C), we designated the C20 cluster as *MUC16*-high cells, and the remaining sub-clusters were termed *MUC16*-low cells. To unravel the functions of *MUC16*, we first compared the transcriptomics of *MUC16*-high and *MUC16*-low cells. The enrichment of DEGs in *MUC16*-high cells revealed multiple pathways involved in immunoregulatory processes (Figure 6A). Therefore, we hypothesized interactions between *MUC16*-high cells and immune cells. Through an analysis of the interaction network of *MUC16*-high cells with cells in the TME, we identified unique interactions between myeloid cells and *MUC16*-high cells in the IL-1 signaling pathway, whereas *MUC16*-low cells did not show such interactions (Figure 6B). To further specify the sub-cluster of myeloid cells interacting with *MUC16*-high cells in the IL-1 signaling pathway, we examined the expression level of IL-1B in myeloid sub-clusters and found that the highest expression of IL-1B was in the M16 sub-cluster (Figure 6C). Intriguingly, M16 was characterized by high expression levels of *CXCL8*, *FCGR3B*, *NAMPT*, and *PTGS2* (Supplementary Figure 7, blue frame), indicating that M16 cells are a cluster of neutrophils. Consistently, *CSF3R*, a neutrophil marker[43], was found to be uniquely highly expressed in the M16 cluster (Figure 6D), supporting the interaction between *MUC16*-high cells and neutrophils through the IL1 signaling pathway. The activity of the IL-1 signaling pathway has been reported to be one of the most potent triggers for NF- $\kappa$ B signaling[44]. Therefore, we analyzed the activity of the NF- $\kappa$ B signaling in *MUC16*-high cells, and found significant enrichment of NF- $\kappa$ B signaling pathway genes in *MUC16*-high cells compared with *MUC16*-low cells (Figure 6E). To further confirm the activation of the NF- $\kappa$ B pathway in *MUC16*-high cells, we compared the expression levels of critical molecules in the NF- $\kappa$ B pathway, including *RELA*, *NFKB1*, *NFKB2*, and *IKBKKG*, and found that the expression levels of these molecules were greater in *MUC16*-high cells than in *MUC16*-low cells (Figure 6F). Double staining IHC was further employed to confirm the histological adjacent distribution between *MUC16*-high cells and neutrophils in multiple CCA samples (Figure 6G). Moreover, gene co-expression pattern detection in cohort 10 further validated the interaction between *MUC16*-high cells and neutrophils (Figure 6H) and the activation of NF- $\kappa$ B signaling in *MUC16*-high cells (Figure 6I). Taken together, these findings suggest that *MUC16*-high cells interact with neutrophils, and induce CCA progression through activation of the NF- $\kappa$ B signaling pathway.

### CCA prognosis prediction model based on mucin levels

Given the intricate yet pivotal roles of mucins in CCA progression, we aimed to establish a comprehensive prognostic assessment strategy for CCA using mucins systematically. The RNA levels of mucins in different CCA patients exhibited mosaic distribution features (Figure 7A). Therefore, we used the LASSO-Cox regression strategy to construct a CCA risk factor evaluation system based on the RNA levels of *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*. This resulted in the following formula: Risk factor =  $0.0297 \times \text{Expr}_{\text{MUC1}} + 0.0574 \times \text{Expr}_{\text{MUC4}} + 0.0501 \times \text{Expr}_{\text{MUC5AC}} + 0.0657 \times \text{Expr}_{\text{MUC5B}} + 0.0460 \times \text{Expr}_{\text{MUC13}} + 0.1217 \times \text{Expr}_{\text{MUC16}}$ . Survival analysis of high-risk CCA patients and low-risk CCA patients revealed a significant difference (Figure 7B), suggesting the efficacy of our CCA risk assessment system. The superior performance of the risk factor compared with that of the individual mucins was further confirmed by area under curve analysis (Supplementary Figure 8A). Notably, compared to low-risk CCA patients, high-risk CCA patients exhibited markedly greater cell proliferation (Figure 7C), tumor metastasis (Figure 7D), and antitumor drug resistance (Figure 7E and F), thereby providing additional confirmation of the mechanisms involving mucins as we established. Intriguingly, we then tested the applicability of the risk factor evaluation formula to other types of carcinoma, and found that this formula could effectively predict the prognosis of pancreatic carcinoma and pulmonary adenocarcinoma (Supplementary Figure 8B).

To assess the universality of the CCA risk evaluation system, we expanded the input of the formula from RNA levels to protein levels. The protein levels of mucins in CCA patients showed mosaic distribution features similar to those of the RNA levels (Supplementary Figure 8C). By applying mucin proteins to the risk factor formula, we stratified the CCA patients into two groups, namely the high-risk group and low-risk group. As a result, high-risk CCA patients showed significantly shorter survival than low-risk CCA patients (Figure 7G), consistent with the result obtained from mucin RNA. Additionally, CCA patients in the high-risk group showed a higher vascular invasion rate (Figure 7H), regional lymph node metastasis rate (Figure 7I), and TNM stage (Figure 7J), further verifying the worse prognosis of high-risk patients. Notably, we further confirmed the reliability of our risk factor evaluation system using data from cohort 10, which included 83 iCCA patients and 29 pCCA patients, and found that patients with a higher risk factor showed a significantly worse prognosis than those with a lower risk factor (Figure 7K). In conclusion, we construct a CCA risk assessment tool that can assess both the RNA and protein levels of mucins.



**Figure 7 Establishment of cholangiocarcinoma risk evaluation model based on mucin levels.** A: RNA levels of *MUC1*, *MUC13*, *MUC16*, *MUC4*, *MUC5AC* and *MUC5B* in cholangiocarcinoma (CCA) samples from cohort 2; B: Survival curve based on RNA levels of mucins showing the relationship between the risk factor and survival of CCA patients from cohort 2; C-F: Gene set enrichment analysis plots showing the enrichment of differentially expressed genes in high-risk patients in cell proliferation pathway, metastasis pathway, tamoxifen resistance pathway, and cisplatin resistance pathway. The *P* values and normalized enrichment score are indicated on the plots; G: Survival curve based on protein expression levels of mucins showing the relationship between the risk factor and survival of CCA patients from cohort 2; H-J: Correlation analyses of vascular invasion, regional lymph node metastasis, and tumor node metastasis stage with CCA risk factor; K: Survival curve based on RNA expression levels of mucins showing the relationship between the risk factor and survival of CCA patients from cohort 10.

## DISCUSSION

Mucins, a group of secretory proteins, play important roles in both physiological and pathological conditions. Elevated mucin levels have been observed in various tumor types, including CCA[10]. However, previous investigations into mucin functions in CCA have relied primarily on techniques such as IHC or enzyme-linked immunosorbent assay,

limiting the discovery of mucin functions to specific molecules. In contrast, in our study, we innovatively employed scRNA-seq for mucin investigation, which offers significant advantages in the following aspects: (1) Comprehensive analysis of the entire mucin family. In human, 21 mucins have been identified, with *MUC1*, *MUC4*, *MUC5AC*, and *MUC16* (CA125) being the most studied mucins in CCA. However, the functions of the remaining mucins in CCA are largely unknown. Our study considered the clinical significance and expression abundance of all mucins in CCA, leading to the identification of potential prognostic mucins, namely *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*; (2) Metabolic profiling of mucins. Metabolic reprogramming is a hallmark of cancer, and has drawn increased amounts of attention in CCA[45]. However, the metabolic states of mucin-positive cells in CCA have rarely been investigated. Our study is the first to report the metabolic characteristics of critical mucins in CCA. *FOSL1* and *HMGA1* were identified as critical transcription factors regulating active nucleotide metabolism in *MUC1*-high cells. Additionally, *GSTA1*, *GSTA2*, *ALDH1A1*, *ALDH2*, and *UCP2* were found to be overexpressed in *MUC5B*-high cells, regulating cellular oxidation processes to resist chemotherapies. These findings shed light on the mechanisms of CCA progression and treatment resistance in an unprecedented way; and (3) Interplay between mucin-positive cells and the TME. Despite numerous studies on *MUC1*-activated signaling pathways in tumor cells[10], the cellular interaction network between mucin-positive cells and TME components has seldom been illustrated. Using scRNA-seq data, we identified interactions between mucin-positive cells and myeloid cell sub-clusters. *MUC13*-high cells were found to induce macrophage infiltration into the TME, leading to subsequent M2-polarization through *PROS1*/*AXL* signaling. Additionally, *NF-κB* signaling pathway activation was observed in *MUC16*-high cells following interaction with neutrophils. These interactions elucidate the immunosuppressive status in CCA TME and offer potential targets for CCA immunotherapy. Although the number of patients used for functional mechanism investigations is limited, the CCA risk evaluation model based on mucin expression levels are applicable in large CCA cohorts, indicating the reliability of our conclusions.

Given its wide overexpression pattern and oncogenic characteristics, *MUC1* is anticipated to be a potential antitumor treatment target. However, the outcomes of clinical trials investigating *MUC1* have proven unsatisfactory[11,12]. Previous reports attributed this discrepancy to the scarcity of major histocompatibility complex class I epitopes within the *MUC1* protein, resulting in insufficient immune reactions[46]. Nevertheless, our data offer new possible explanations. Although *MUC1* is one of the most widely expressed mucins, there are numerous mucins, including *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*, which share oncogenic and metabolic characteristics with *MUC1*. Therefore, targeting *MUC1* alone may be insufficient for effective tumor therapy. Our study developed a mucin-based CCA risk evaluation system that links risk factors to multiple CCA pathological indicators, such as tumor cell proliferation, metastasis, and resistance to antitumor drugs.

In fact, the risk factor formula, which includes *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*, implies the involvement and necessity of all six mucins in CCA development. Consequently, targeting all mucins together may yield more effective therapeutic outcomes than focusing solely on *MUC1*. Notably, although the functional mechanisms of mucin-positive cells may not be directly facilitated by mucins, mucin-directed immune therapy or chemotherapy still shows strong clinical application potential. Recently, *MUC1*-directed chimeric antigen receptor (CAR) T cell therapies have shown promising killing effects both *in-vitro* and *in-vivo*[47,48]. Our data reveal attractive targets for immunotherapy, suggesting that anti-*MUC4*, anti-*MUC5AC*, anti-*MUC5B*, anti-*MUC13* or anti-*MUC16* CAR T cells may also show promising treatment effects in the future.

Through a comprehensive approach involving both bioinformatic analysis strategies and experimental validation, our study successfully unveils the functional mechanisms of mucin-positive cells in CCA progression, through *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*. We subsequently construct a CCA risk factor evaluation system based on the expression levels of these six mucins. The risk factor evaluation model effectively predicts CCA patient prognosis, providing a novel patient stratification method. The results obtained in our study not only enrich the current understanding of mucins in CCA progression, but also identify new targets for precision treatments of CCA.

## CONCLUSION

In conclusion, we analyzed ten clinical CCA cohorts to investigate the functions of mucins on CCA progression and prognosis. The mucin family, includes *MUC1*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC13*, and *MUC16*, regulates tumor metabolism, invasiveness, chemotherapy resistance, and cellular interaction with immune cells in microenvironments, comprehensively promoting CCA progression. We also construct a CCA risk evaluation model based on the expression levels of these mucins, applicable at both RNA and protein levels. Given the critical roles of these mucins on CCA development, our model serves as a promising tool for evaluating tumor malignancy and stratifying CCA patients.

## ARTICLE HIGHLIGHTS

### Research background

Cholangiocarcinoma (CCA) is the second most common type of liver cancer and exhibits a high mortality rate. Mucins are a family of protein that are elevated in various tumor types, including CCA. However, the comprehensive functional mechanisms and prognosis evaluation significance of mucins in CCA progression remain largely unknown.

**Research motivation**

*MUC1* has been identified as an oncogene that induce CCA progression through multiple signaling pathways. Nevertheless, how the mucin family regulate CCA is still elusive.

**Research objectives**

To investigate the functional mechanisms of mucins in CCA and to conduct a CCA risk evaluation model based on mucin expression levels.

**Research methods**

For the detection of mucin functions in CCA, single-cell RNA sequencing data from 14 CCA samples were employed, supported by comprehensive bioinformatic analyses. Validations were pursued through spatial transcriptomics and immunohistochemistry. The establishment of a CCA risk evaluation model based on mucin expression levels employed the least absolute shrinkage and selection operator regression algorithm. The risk evaluation model was constructed using RNA level of mucins, and subsequently validated by both RNA and protein levels of mucins, as well as multiple independent cohorts.

**Research results**

Elevated levels of *MUC1* and *MUC4* in CCA tumor cells were associated with activated nucleotide metabolic pathways and higher invasiveness. CCA tumor cells with heightened *MUC5AC* expression were found to induce tumor progression through the WNT signaling pathway. Robust cellular oxidation activities in *MUC5B*-high CCA tumor cells facilitated antitumoral treatment resistance. *MUC13*-high cells transformed macrophage into M2-polarization state through the PROS signaling and chemokines, including *CCL2*, *CCL4*, and *CXCR12*. Neutrophils induced the activation of nuclear factor kappa-light-chain-enhancer of activated B cells signaling in *MUC16*-high cells through the IL1B signaling, thereby promoting CCA development. Utilizing the expression levels of these mucins, a CCA prognosis evaluation model was developed and validated across multiple cohorts, which simultaneously exhibited predictive functions on the evaluation of CCA malignancy, metastasis potential, and chemotherapy sensitivity.

**Research conclusions**

Our study unveils the functional mechanisms by which mucins contribute to CCA progression, and offers a potential tool for CCA risk stratification.

**Research perspectives**

The discovery of mucin functions in CCA development and prognosis prediction indicate that mucins may be promising treatment targets for CCA.

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**FOOTNOTES**

**Co-first authors:** Chun-Yuan Yang and Li-Mei Guo.

**Author contributions:** Yang CY and Guo LM provided study concept, design, and methodology of the paper; Yang CY, Guo LM, and Luo JY performed writing, review and revision of the paper; Yang CY, Li Y, Wang GX, Tang XW, Zhang QL, and Zhang LF performed acquisition, analysis and interpretation of data, and statistical analysis; Guo LM provided technical and material support. All authors read and approved the final paper. Yang CY and Guo LM contributed equally to this work and are designated as co-first authors for two main reasons. Firstly, our study was a result of collaborative efforts, with the design and conceptualization evolving through extensive discussions between Yang CY and Guo LM. Continuous communications ensured improvement of our manuscript at both pre-submission and post-submission stages. Secondly, the overall research team encompassed authors with diverse skills from various fields. Yang CY performed bioinformatic analyses, and Guo LM was responsible for clinical validation. The co-first authorship signature respected the collaboration of different expertise, ultimately enhancing the paper's quality and reliability. In summary, we believe that designating Yang CY and Guo LM as co-first authors of our manuscript as it accurately reflects our team's collaborative spirit and equal contributions.

**Institutional review board statement:** The study was reviewed and approved by the Science and Research Office of Peking University Third Hospital.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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