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MECHANISM AND MOLECULAR BASIS OF HUANGLIAN DECOCTION IN REGULATING THE IMMUNE MICROENVIRONMENT OF COLORECTAL ADENOCARCINOMA

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ABSTRACT

This study combined network pharmacology, TCGA multi-omics, immune infiltration analysis, and single-cell transcriptome data to systematically elucidate the mechanism by which Huanglian Decoction regulates the immune microenvironment of colorectal adenocarcinoma, and constructed a target gene-based prognostic risk model. A total of 59 core targets (such as MMP1 and MMP3) were screened. Results showed that the high-risk group was enriched with M2-type macrophages and Tregs, forming an immunosuppressive phenotype and the low-risk group was dominated by CD8⁺ T cells and M1-type macrophages, showing immune activation. Single-cell analysis (GSE277905) further revealed that core targets were specifically enriched in fibroblasts, while B cells were at the hub in cell communication. This model demonstrated stable prognostic stratification ability both within TCGA and external GEO datasets (both $P < 0.05$). These suggest that Huanglian Decoction may synergistically reverse the immunosuppressive microenvironment through multiple targets, and its target model can provide new evidence for precise stratification and individualized treatment of colorectal adenocarcinoma.

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INTRODUCTION

Colorectal adenocarcinoma is a malignant tumor of glandular epithelial cells in the colon or rectal mucosa, accounting for over 90% of colorectal cancer. Currently, clinical treatment of this disease still faces significant challenges-traditional therapies represented by surgery, radiotherapy, and chemotherapy suffer from significant trauma, strong toxic side effects, and easy recurrence and metastasis^[1]. Huanglian Decoction, derived from Sheng Ji Zong Lu (General Medical Collection of Royal Benevolence), is composed of Coptidis Rhizoma (Huanglian), Zingiberis Rhizoma (Ganjiang), Artemisiae Argyi Folium (Aiye), and Mume Fructus (Wumei). It possesses the effects of clearing heat and drying dampness, warming the middle-jiao to dispel cold, and astringing to stop diarrhea^[2]. This formula has been studied and applied in gastritis, pancreatic cancer, and digestive tract diseases such as duodenum. Modern research suggests it has anti-inflammatory, anti-tumor, and immunomodulatory potential^[3-5], but its systematic regulatory mechanisms of the tumor immune microenvironment remain to be elucidated. This study integrates network pharmacology, multiomics, and single-cell analysis techniques to construct a multidimensional regulatory network of "Huanglian Decoction-Colorectal

Adenocarcinoma-Immune Microenvironment", systematically elucidating the multi-target coordinated regulatory mechanism of Huanglian Decoction, and establishing a COAD prognostic model based on target genes to evaluate its stability and clinical predictive value.

MATERIALS AND METHODS

COAD differential gene integration: After downloading RNA-seq data from the TCGA-COAD project, we used DESeq2 or edgeR to compare tumors with normal samples to screen for differentially expressed genes^[6], then we used clusterProfiler for GO/KEGG enrichment analysis and visualized results using dotplot or cnetplot^[7].

Network pharmacological analysis: We retrieved the chemical components of Coptidis Rhizoma, Zingiberis Rhizoma, Artemisiae Argyi Folium, and Mume Fructus from the TCMSP database with screening conditions: OB $\geq 30\%$, DL ≥ 0.18 . Then we obtained COAD-related genes and scores from GeneCards, OMIM, and TTD databases to screen for highly relevant target genes and constructed a PPI network map.

Construction and validation of prognostic models: We downloaded RNA-seq expression data and related clinical data of COAD patients from the TCGA database, which were used to construct a risk scoring model. The reliability and stability of the model were verified through ROC curve analysis^[8], survival analysis, independent prognostic analysis, and nomogram^[9].

The immune microenvironment is associated with immune escape: We dissected the immune cell infiltration landscape of the high- and low-risk groups in COAD using the CIBERSORT algorithm, and calculated the proportions of 22 immune cell subtypes for each sample based on the RNA-seq data^[10]. We then identified differentially infiltrated immune cells between the two risk groups using the limma package in R, and visualized their distributions using boxplots. We further performed Gene Set Enrichment Analysis (GSEA) to compare the enrichment of immune-related pathways between the high- and low-risk groups. Finally, we computed the TIDE (Tumor Immune Dysfunction and Exclusion) score for each sample using the official TIDE web platform or R scripts, and evaluated the difference between the two groups using the Wilcoxon rank-sum test^[11].

Single-cell resolution localization and cellular communication: We obtained COAD single-cell RNA-seq data from NCBI GEO, TISCH, or Human Cell Atlas databases, and used the Seurat package to perform quality control, standardization, batch correction, PCA, and clustering analysis, with visualization combined with UMAP/t-SNE. Then CellPhoneDB was used to analyze intercellular ligand-receptor interaction networks, and ggplot2 and pheatmap were employed for plotting. Using the core genes screened by risk models, their average expression levels and positive cell proportions in each cell type were calculated, and bubble plots were plotted using the Seurat package DotPlot() function.

Differences in Genomic Mutation Characteristics: We obtained MAF mutation files and clinical information from the TCGA-COAD database, and divided samples into high-risk and low-risk groups based on risk models. Next, we used R-package maftools to annotate and statistically analyze mutation data, mapping mutation classification, mutation types, SNV conversion/transversion, mutation burden, and high-frequency mutation genes, and statistically stated the six SNV types and their mutation classification ratios. Then we further compared mutation characteristics and MSI score differences between high- and low-risk groups, and used Wilcoxon rank-sum test to assess differences between groups^[11]. The results are presented in box plots.

RESULTS

Identification of targets and pathways related to Huanglian Decoction in COAD: KEGG pathway enrichment analysis showed that differentiated genes were significantly enriched in key pathways such as the cell cycle and PI3K-Akt signaling pathway (Figure 1A), which play core regulatory roles in tumor cell proliferation, invasion, and immune evasion. GO bioprocess enrichment analysis further showed significant enrichment of biological processes such as cell migration, immune response, and chemotaxis (Figure 1B), indicating important biological significance in the occurrence and progression of colon adenocarcinoma. For target screening, the

active ingredient target genes, COAD-related genes, and differentially expressed genes for COAD were combined to obtain a total of 7,035 candidate genes. After further crossing, we identified 59 core target genes (Figure 1C). Network centrality analysis showed that genes such as VEGFA, IL6R, IL1B, CXCL8, MMP9, and PTGS2 had high node connectivity (Figure 1D) and were identified as potential core hub genes.

Construction and validation of COAD prognostic prediction models: The risk scoring model showed good prognosis prediction ability at 1, 3, and 5 years (Figure 2A), with the highest 5-year AUC (0.713), indicating superior long-term predictive performance. In the high-risk group, gene expression such as MMP3, VEGFA, MMP1, CCNB1, SERPINE1, and GSTM1 was upregulated (Figure 2B), associated with tumor invasion, immune evasion, and proliferation. As risk scores increased, patient survival shortened, and death events in the high-risk group became more concentrated, with prognosis significantly worse than in the low-risk group (Figure 2C-D). Nomogram analysis showed that risk score and tumor stage contributed the most to survival prediction, which were key factors for COAD prognosis (Figure 2E).

Analysis of immune cell infiltration and immune escape in high/low COAD risk groups: Immunoinfiltration analysis showed that the high-risk group was enriched with M0/M2 macrophages, Tregs, and dormant mast cells, while the low-risk group was dominated by CD8⁺ T cells, CD4⁺ memory T cells, and M1 macrophages (Figure 3A), suggesting an immunosuppressive phenotype in the high-risk group. GSEA results showed that ECM-receptor interaction and Focal adhesion were significantly positively enriched in the high-risk group (NES>0, p.adjust significantly, Figure 3B), suggesting more active matrix remodeling and migration signals, which may promote immune escape and metastasis. Meanwhile, TIDE scores in the high-risk group increased significantly (p<0.01, Figure 3C), further supporting stronger immune escape ability.

Single-cell transcriptome analysis of colorectal adenocarcinoma: After obtaining single-cell transcriptome data from colorectal adenocarcinoma from the GEO database, we retained 47,000 high-quality cells for analysis through quality control screening (Figure 4A-B). PCA showed significant separation in epithelial and immune marker gene expression from four tumor samples from three normal samples, indicating significant transcriptional differences (Figure 4C). Further unsupervised clustering identified 18 cell clusters, with the most significant differences among epithelial cells, immune cells, fibroblasts, and endothelial cells (Figure 4D). Cell communication analysis showed that dendritic cells had high connectivity with B cell networks, potentially playing key roles in tumor immune regulation and immune escape (Figure 4E).

Analysis of cell mutation profiles in high- and low-risk groups of TCGA-COAD: Somatic mutation spectrum analysis showed that the low-risk and high-risk groups had a consistent overall distribution of mutation types, but significant differences in mutation frequency and key gene abnormality characteristics (Figure 5A-B). Among them, the high-risk group exhibited a higher mutation load and more pronounced key gene abnormalities, suggesting they may have

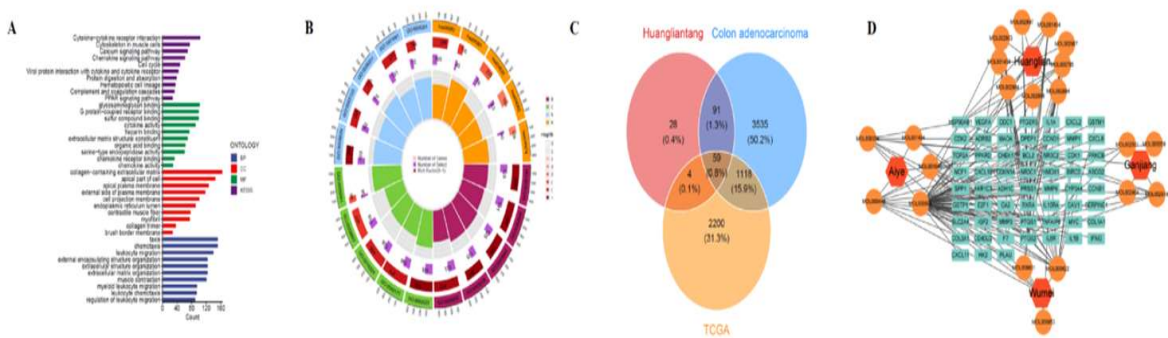


Figure 1. Analysis of disease and drug target genes. (A) KEGG and GO enrichment bar maps of COAD differentially expressed genes; **(B)** Ring maps of KEGG and GO enrichment of COAD differentially expressed genes; **(C)** Venn maps showing the intersection of target genes, COAD-related genes, and COAD differentially expressed genes in Huanglian Decoction; **(D)** PPI map

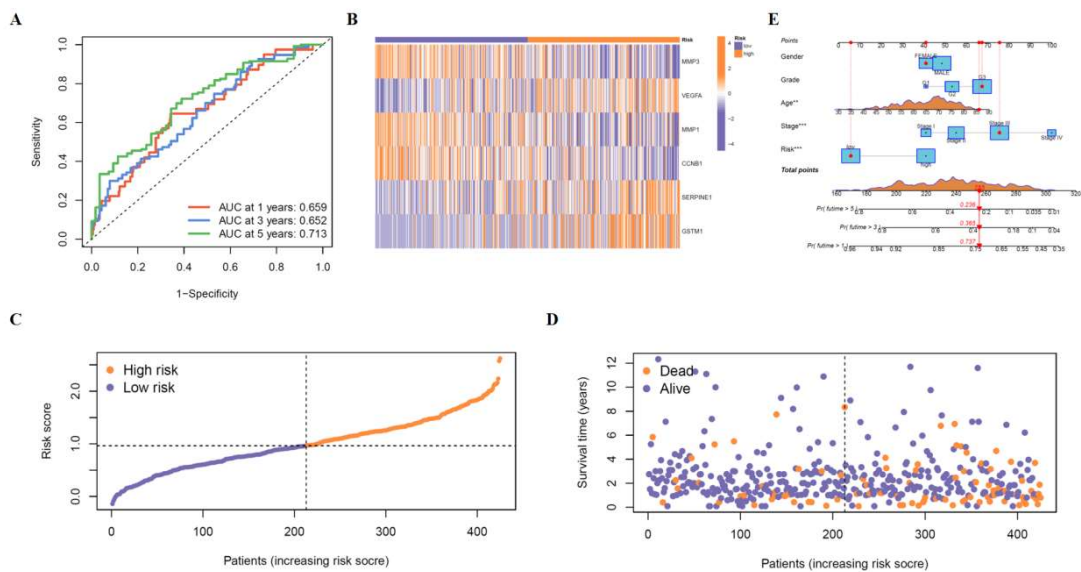


Figure 2. Validation analysis of COAD prognosis prediction models. (A) ROC curve chart; **(B)** Heatmap of gene expression differences between high- and low-risk groups; **(C)** Risk score distribution map; **(D)** Relationship chart between survival time and risk score; **(E)** Nomogram chart of COAD patient prognosis prediction based on clinical features and risk assessment

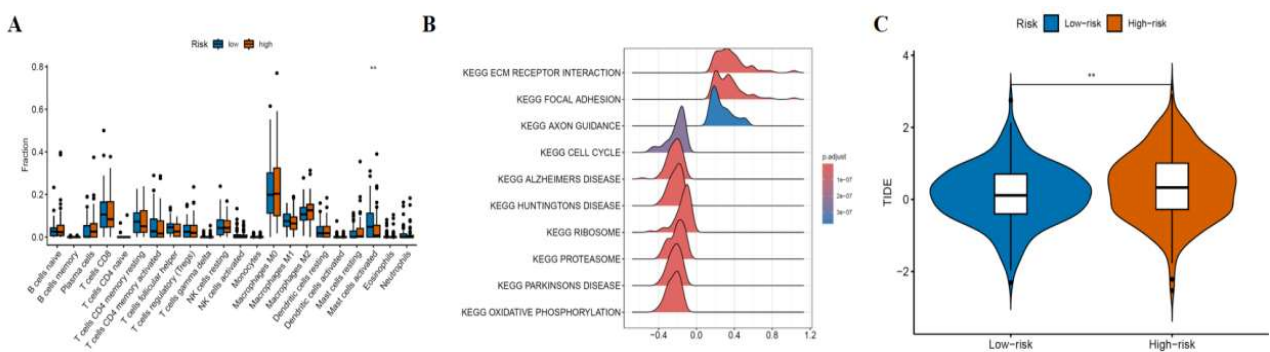


Figure 3. Analysis of immune cell infiltration and immune escape. (A) Differential analysis of immune cell infiltration; **(B)** GSEA analysis plot; **(C)** Violin plot showing the distribution of TIDE scores

more complex genomic remodeling processes and stronger immune escape abilities (Figure 5B); In contrast, the low-risk group's genome was relatively stable, possibly related to stronger DNA repair ability and immune recognition activity (Figure 5A). Analysis of the expression distribution of core genes in the COAD risk model in immune and tumor microenvironment cells. There was no statistically significant difference in microsatellite instability (MSI) levels between the high-risk and low-risk groups ($p = 0.44$).

The overall distribution of MSI indices in both groups was relatively similar and at low levels, suggesting that most COAD patients may be predominantly microsatellite stable (MSS) (Figure 6A). Further analysis of model gene expression distribution showed that six core target genes-MMP3, VEGFA, MMP1, CCNB1, SERPINE1, and GSTM1-had distinct specific expression patterns in tumor-associated immune cells and stromal cells, suggesting that they may participate in COAD progression and risk stratification by co-regulating key

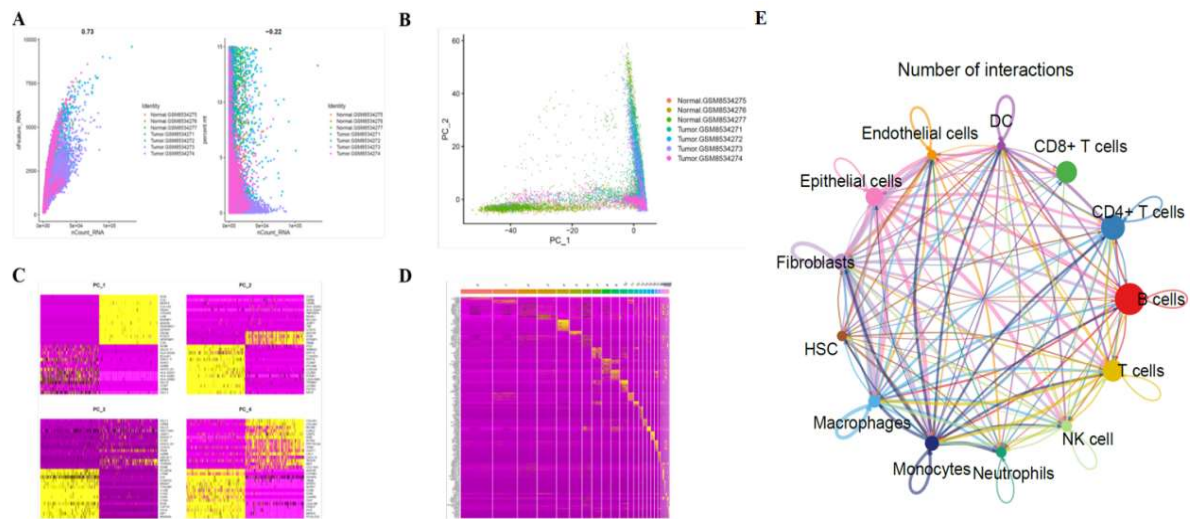


Figure 4. Single-cell analysis of CRC. (A) High-quality gene quality control map; (B) Characteristic distribution map; (C) PCA scatter plot; (D) Cluster heatmap; (E) Cell interaction network map

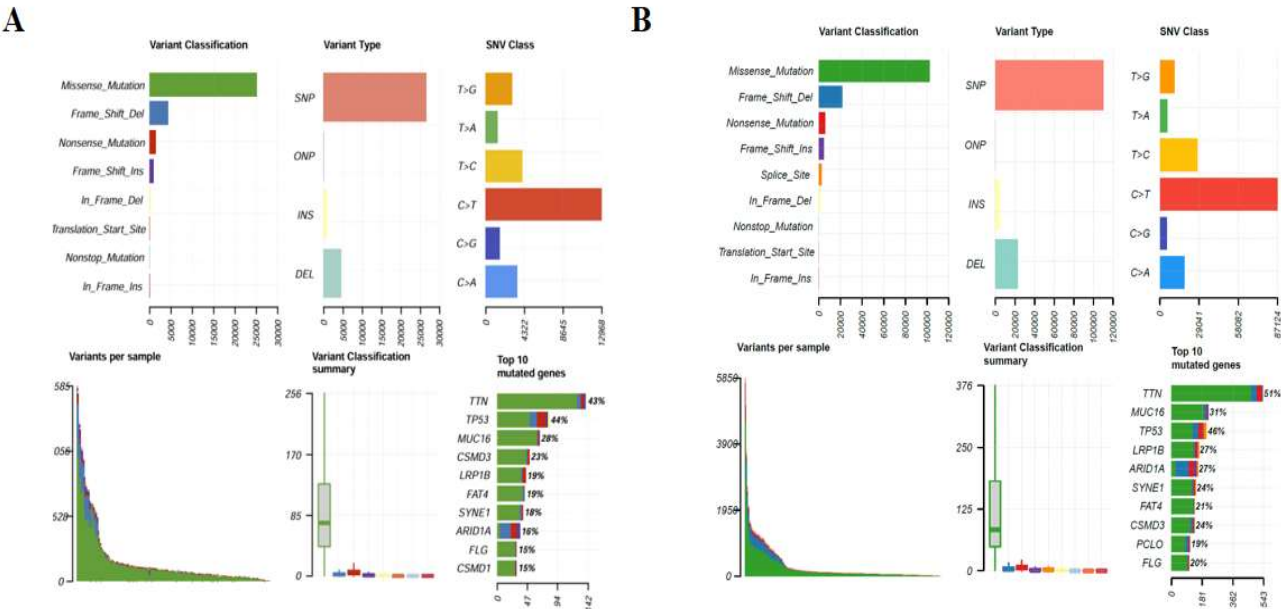


Figure 5. Analysis of cell mutation spectra in high- and low-risk TCGA-COAD groups. (A) Mutation types, mutation load, and mutation frequency maps in the low-risk group; (B) Mutation types, mutation load, and mutation frequency maps in the high-risk group.

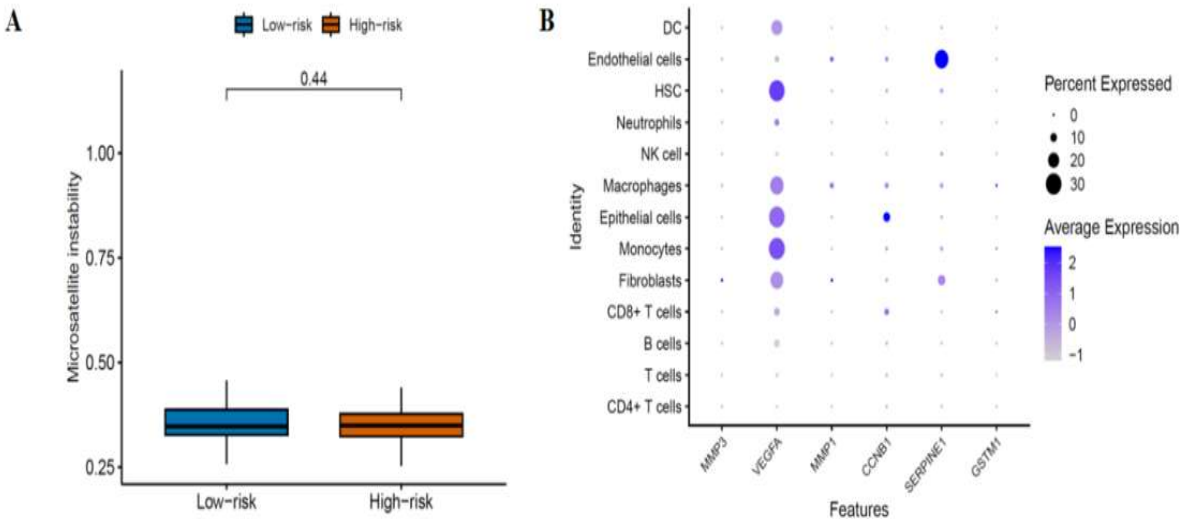


Figure 6. Clinical analysis of COAD. (A) MSI analysis box plot; (B) Model gene bubbleplot

biological processes such as cell cycle, extracellular matrix remodeling, angiogenesis, and immune response (Figure 6B).

SUMMARY

This study conducted a multidimensional meta-analysis centered on "Huanglian Decoction - Colorectal Adenocarcinoma - Immune Microenvironment," systematically revealing that Huanglian Decoction may regulate tumor progression through a multi-component, multi-target synergistic mechanism. Results showed that key components of Huanglian Decoction act on core targets such as MMP1, CCNB1, and VEGFA, and are associated with immune pathways including PD-L1 expression and Th17 differentiation, promoting increased infiltration of CD8⁺ T cells and decreased proportion of M2-type macrophages, suggesting its potential to reverse the immunosuppressive microenvironment. At the single-cell level, further suggestions indicate that B cells occupy a pivotal position in cell communication, with core targets more concentrated in fibroblasts, suggesting that matrix remodeling may be an important component. The LASSO-Cox risk model built on core targets performed stably in training and validation cohorts and was independent of TMB/MSI status, offering good prognostic stratification value and providing new evidence for precise management of MSS-type "cold tumors" patients.

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