

Original Article

Development of a colon adenocarcinoma prognostic model based on cuproptosis-related genes and analysis of immune characteristics

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Abstract: Cuproptosis is a novel form of cell death that plays a crucial role in tumor proliferation, invasion, and metastasis, and is closely associated with the prognosis of various cancers. However, its specific role and underlying mechanisms in colon adenocarcinoma (COAD) remain unclear. This study aims to identify cuproptosis-related genes (CRGs) associated with the prognosis of COAD and construct a prognostic risk model based on the expression characteristics of these genes. A total of 541 COAD samples were collected from public databases and subjected to multi-omics analysis. Based on the expression levels of CRGs, consensus clustering was employed to classify COAD patients into two subtypes, followed by the identification of differentially expressed genes (DEGs). Subsequently, weighted gene co-expression network analysis (WGCNA) was used to integrate relevant genes, and a second round of clustering analysis was performed to refine COAD patients into three subtypes, enhancing both biological and clinical relevance. LASSO and Cox regression analyses were utilized to construct a prognostic risk model for COAD. The prognostic performance of the risk model was validated using receiver operating characteristic (ROC) curves. SEC61G, SCP2, LENG8, MTUS1 and MEGF6 were identified as core genes significantly associated with patient prognosis. Comprehensive analyses using CIBERSORT, ESTIMATE, MSIsensor, and GDSC revealed distinct patterns between high-risk and low-risk subgroups of COAD. Significant differences were observed in immune cell infiltration, tumor microenvironment (TME) characteristics, tumor mutation burden (TMB), stemness features, microsatellite instability (MSI), and drug sensitivity (all $P < 0.05$). Notably, pharmacogenomic analysis indicated that high-risk patients exhibited increased sensitivity to targeted drugs such as AMG.706 (a multi-kinase inhibitor) and PHA.665752 (a c-MET inhibitor), highlighting the potential clinical application value of this model in guiding personalized treatment strategies for COAD.

Keywords: COAD, prognostic model, drug sensitivity, immune infiltration, nomogram

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide, with high morbidity and mortality. Relevant epidemiological data predict that global CRC cases will reach 3.2 million by 2040, accompanied by 1.6 million cancer-related deaths [1]. Colon adenocarcinoma (COAD) is the main subtype of CRC. Notably, an increasing number of young patients have been diagnosed with COAD in recent years [2], which has gradually become a tough challenge for global public health man-

agement. While substantial progress has been made in treatment modalities and early diagnostic techniques in the past few years, the heterogeneity and complexity of COAD lead to variations in clinical presentation and treatment response. Current treatments often fail to work well in practice due to these variations. To address this problem, we should explore the internal mechanisms and developmental rules of colon adenocarcinoma in depth. Classifying patients into different groups and searching for new treatment targets are equally important.

Copper (Cu) acts as a cofactor for numerous enzymes in the human body and is indispensable for metabolism and normal cellular activities [3]. Once copper builds up beyond the body's regulatory limits, it turns toxic, disrupting cell function and eventually triggering cell death [4]. In a 2022 study, Tsvetkov et al. identified a copper-dependent cell death pathway, known as cuproptosis, which differs from traditional apoptosis and is unaffected by other cell death pathways [5]. Copper death is closely related to mitochondrial respiration. Its mechanism involves the binding of copper to lipid compounds in the tricarboxylic acid (TCA) cycle, leading to protein aggregation and toxic stress, ultimately resulting in cell death [6, 7]. Recent years have seen growing interest in cuproptosis within cancer research, leading to a series of significant findings. In the pan-cancer study by Qin et al. [8], it was observed that cuproptosis-related genes (CRGs) are significantly expressed across various cancers and are associated with the tumor microenvironment (TME), where they play a crucial role by regulating the infiltration, activity, and function of immune cells, thereby influencing tumor immune evasion. In Yan et al.'s research [9], knocking out the CRG LIPT1 gene in HepG2 and Hep3B cells was found to suppress the activity of cancer cells in LIHC. In the study by Lubecka et al. [10], the combination of clofarabine with a phytochemical significantly reactivated the copper-induced cell death-related tumor suppressor gene CDKN2A, ultimately leading to the inhibition of cancer cell growth. Findings from these works may disclose novel therapeutic targets and support personalized treatment plans for cancer patients, which also broadens the research prospects of tumor treatment.

Advances in bioinformatics now allow researchers to obtain multi-dimensional biological data, covering genes, transcription, proteins and metabolic processes. Recent research has shown that CRGs possess significant diagnostic or prognostic value across different cancer types, such as lung cancer (LC) [11], breast cancer (BC) [12], gastric cancer (GC) [13], and hepatocellular carcinoma (HCC) [14]. Many previously reported prognostic models based on CRGs, such as the one used in the research of Luo's team [15], use just one screening step to filter candidate genes. A large number of prior works just identify differentially expressed

genes (DEGs) through comparison between tumor and normal tissues. These classic research strategies exclude tumor molecular subtypes and gene co-expression networks from analysis. For this reason, their results lack clear biological characteristics and dependable predictive value. To solve the above problems, we carried out a multi-stage gene screening process in our study. First, patient samples were divided into different molecular subgroups using CRG expression data, and we then obtained DEGs peculiar to each subgroup. Second, we adopted weighted gene co-expression network analysis (WGCNA) to mine hub genes related to these subtypes. Last, we selected the common genes from two gene sets and constructed the prognostic model accordingly.

Based on the above research background, the present study seeks to systematically evaluate the value of CRGs in predicting COAD outcomes and investigate the role of the TME in key pathological processes such as tumor resistance and immune evasion through comprehensive multi-omics analysis. This will facilitate the discovery of new therapeutic targets for COAD and provide more in-depth scientific support for cancer immunotherapy and personalized treatment strategies. Simultaneously, by developing a prognostic risk model based on CRGs, it will be possible to more accurately predict patient outcomes, provide clinicians with more effective treatment guidance, and ultimately deliver optimized therapies for COAD.

Materials and methods

Analysis of CRG expression and genomic variations in COAD

Transcriptomic data of COAD were retrieved from the TCGA and GEO databases. All raw data were sorted and normalized using Perl. Through literature review, we identified and selected 18 CRGs. Detailed information on these CRGs is provided in [Supplementary Table 1](#) [5, 16]. The expression levels of CRGs in COAD expression dataset were extracted using the “limma” package. To compare gene expression between normal and tumor samples, the data were reshaped with the “reshape2” and “ggpubr” packages. The “maftools” package was utilized to visualize mutation patterns, encompassing frequency, type, count, location,

and nucleotide alterations. In addition, copy number information for COAD was sourced from the UCSC Xena database (<https://xena.ucsc.edu/>), and the CRGs' copy numbers were extracted using Perl. The "RCircos" package in R was then used to analyze the copy number variations (CNVs) and their chromosomal distribution.

Survival analysis and subtyping of CRGs in COAD

In this study, we applied the "limma" and "sva" packages in R to integrate gene expression data from both the TCGA and GEO databases and extract CRG expression levels. Survival analysis was then conducted with the "survival" and "survminer" packages, applying a Cox regression model to assess the influence of CRGs on patient survival, with $P < 0.05$ as the significance threshold. We further constructed a prognostic network diagram to visualize the impact of CRGs. To achieve more precise classification of the samples, we conducted clustering analysis based on CRG expression levels using the "Consensus Cluster Plus" package, selecting the best K value to define the final sample subtypes.

Differential analysis between CRG subtypes

Based on the aforementioned subtyping results and survival data, statistical analysis of survival differences and survival curve plotting were conducted using the R packages "survival" and "survminer" to observe the prognostic differences among patients with different subtypes ($P < 0.05$). We further employed the "pheatmap" package to visualize CRG expression differences across subtypes, integrating gene expression with clinical data. Additionally, we conducted gene set variation analysis (GSVA) and single-sample gene set enrichment analysis (ssGSEA) and created pathway heatmaps and box plots of immune cell differences to illustrate the variations in pathways and immune cells across the different subtypes. We evaluated the effectiveness of subtyping through principal component analysis (PCA).

Screening of subtype-related core genes

First, gene expression data and subtype classification results were combined, and the "limma" and "Venn Diagram" packages were applied to identify DEGs between the two sub-

types by setting a significance threshold. Then, an in-depth analysis of COAD cuproptosis subtypes was performed using WGCNA. We first read and preprocessed the normalized expression data to exclude low-variance genes. Next, we performed sample clustering to detect and remove outlier samples, followed by reclustering of the remaining samples to generate a dendrogram. Subsequently, we selected an appropriate soft-thresholding power to conduct the WGCNA, followed by the calculation of the topological overlap matrix (TOM) for clustering. Modules were identified using dynamic tree cutting and merged based on their similarities, and the correlation between module eigengenes (MEs) and clinical features was computed to identify key modules. Finally, intersecting the results from the DEG identification and WGCNA analyses allowed us to pinpoint genes that may have a significant impact on the cuproptosis subtypes of COAD.

Functional enrichment of core genes

We employed the "clusterProfiler" package and the "org.Hs.eg.db" package for GO and KEGG pathway enrichment analysis on the intersecting genes. The filter was applied with a threshold of $P < 0.05$ for significance.

Secondary subtyping and DEG screening

Intersection genes associated with patient prognosis were identified through survival analysis. Subsequently, we conducted consensus clustering using the "Consensus Cluster Plus" package, classifying the samples based on the expression levels of these genes. Following this classification, we performed further survival analysis, differential analyses of the intersection genes and CRGs, and visualized the results.

Development and validation of prognostic model

Patients were randomly allocated into two groups. In the training set, we applied Lasso regression and cross-validation to select significant genes for building a Cox model. The model is expressed as risk score (RS) = $\sum_{i=1}^n \beta_i * \lambda_i$, where β_i represents the risk coefficient of each prognostic gene, and λ_i refers to the genes' expression level. Applying this formula to the training data, we computed the RS and categorized the samples into different risk groups.

Next, Utilizing the “survdiff” and “timeROC” functions, we performed survival difference analyses across various risk groups and assessed the model using receiver operating characteristic (ROC) curves. Ultimately, we combined the RS data with clinical characteristics to create a nomogram for predicting outcomes in COAD patients. In addition, we explored RS differences across various subtypes and compared CRG expression patterns between different risk groups.

Immune infiltration in risk subgroups

The immune cell infiltration in COAD patients was analyzed using the CIBERSORT algorithm in R. Based on the results, immune cells significantly correlated with the RS were selected ($P < 0.05$). Following this, we calculated the correlations between the genes involved in model construction and the identified immune cells, visualizing these relationships with a heatmap.

Correlation of risk score with genomic features

Using the Perl programming language, we classified patient mutation data into risk groups and generated a corresponding waterfall plot. The correlation analysis of tumor mutation burden (TMB) was conducted using the “ggpubr” and “reshape2” packages. Additionally, we examined the relationship between the stem cell index (RNAss) and the RS. Utilizing the “estimate” package, we scored the TME across various risk groups. Finally, microsatellite instability (MSI) analysis was performed using R. The significance threshold for all analyses was set at $P < 0.05$.

Drug sensitivity of risk subgroups

First, we integrated gene expression data with RS data and imported them into the R environment. Then, using the relevant functions of the “pRRophetic” package, we evaluated the sensitivity of each drug. Finally, box plots were generated to visually display the significant differences in drug sensitivity between the high-risk and low-risk groups.

qRT-PCR verification of core genes

NCM460 and HCT116 cells at logarithmic growth phase were washed with PBS, and total RNA was extracted using RNAfast2000 RNA purification kit (Shanghai Feijie Biotechnology

Co., Ltd., China). Subsequently, 1 µg of RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (NobelabBiotech Co., Ltd., China). qPCR was performed in a 20 µL reaction system (NobelabBiotech Co., Ltd., China) using SYBR Green method with GAPDH as the internal reference gene. All experiments were performed with 3 biological replicates, and each biological sample was analyzed with 3 technical replicates. Relative quantification was conducted using the $2^{-\Delta\Delta Ct}$ method, and statistical significance was determined by a two-tailed unpaired t-test. All PCR primers were designed and synthesized by Guangzhou RiboBio Co., Ltd., with sequences provided in [Supplementary Table 2](#).

Results

Sample information

This study included 41 healthy tissue samples and 476 cancerous tissue samples from the TCGA, as well as 65 malignant tissue samples from the GEO.

Altered expression and genomic features of CRGs

First, we compared and analyzed the expression differences of 18 CRGs between normal and tumor samples. The results showed that 13 CRGs exhibited significant expression differences. Specifically, NDUFB2, NDUFV2, ATP7B, PPAT, and GLS were upregulated in tumor samples, while DBT, NDUFC1, DLST, MTF1, DPYD, PLAT, NLRP3, and ACO2 were downregulated in tumor samples. Details are provided in (**Figure 1A**). These genes are mainly involved in the mitochondrial respiratory chain (e.g., NDUFB2, NDUFV2) and copper ion transport (e.g., ATP7B). Their abnormal expression may affect tumor progression by interfering with the cuproptosis pathway. CRGs exhibited somatic mutations in 17.63% of COAD samples (79/448), with NLRP3 and DPYD as the most frequently mutated genes (5% each) (**Figure 1B**). Copy number deletions were more prevalent than gains across all 18 CRGs (**Figure 1C, 1D**), suggesting potential dysregulation of copper homeostasis in tumorigenesis.

CRG subtypes with distinct prognoses

Survival analysis identified eight CRGs (e.g., ACO2, CDKN2A) that exerted significant im-

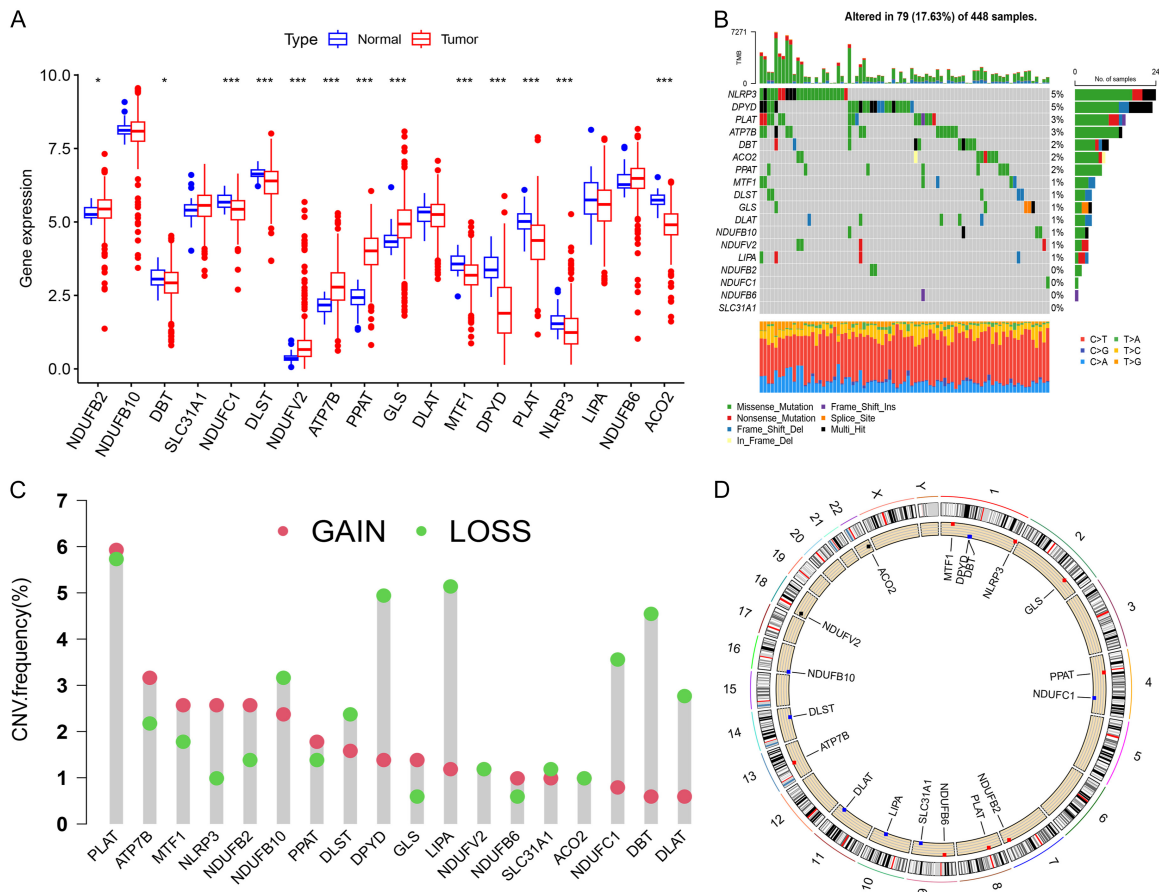


Figure 1. Differential expression and genetic alterations in CRGs in COAD. A. Expression differences of 18 CRGs between normal and tumor samples. B. Mutation frequency and types of CRGs in 448 COAD samples. C. Copy number variations (CNVs) in CRGs. D. Chromosomal distribution of CNVs in CRGs.

pacts on prognosis (Figure 2A-H), and the prognostic network revealed interactions among these genes (Figure 2I). Consensus clustering based on the expression patterns of CRGs ($k = 2$; Figure 2J) classified patients into two distinct subtypes (A/B), a result validated by PCA (Figure 2K). Survival analysis demonstrated that subtype A was associated with a better prognosis (Figure 3A), which corresponded to the enrichment of high expression of most CRGs in subtype A, whereas the opposite trend was observed in subtype B (Figure 3B). This suggests that differences in tumor cuproptosis activity can define COAD subgroups with biological disparities. GSVA showed enrichment of immune pathways in subtype A (Figure 3C), while GSEA revealed enrichment of cell cycle and extracellular matrix (ECM)-receptor interaction pathways in subtype B (Figure 3D). ssGSEA indicated that the numbers of activated CD4⁺ T cells, CD8⁺ T cells, etc., were significantly reduced

in subtype B, with an overall immune infiltration pattern distinct from that of subtype A (Figure 3E). This is consistent with the poorer prognosis of subtype B, as cell cycle dysregulation and ECM remodeling are typical features of tumor aggressiveness, and reduced immune cells impair anti-tumor immunity.

Functional profiles of subtype-related genes

We identified 861 DEGs between the cuproptosis-related subtypes (Figure 4A). WGCNA revealed nine co-expression modules, among which the yellow-green module exhibited the strongest correlation with cuproptosis subtypes ($r = 0.26$, $P = 2e-09$; Figure 4B, 4C). By intersecting this module with the identified DEGs, 408 key genes were obtained (Figure 4D). Subsequently, functional enrichment analysis was performed on these intersected genes.

Colon adenocarcinoma: cuproptosis-related model and immune analysis

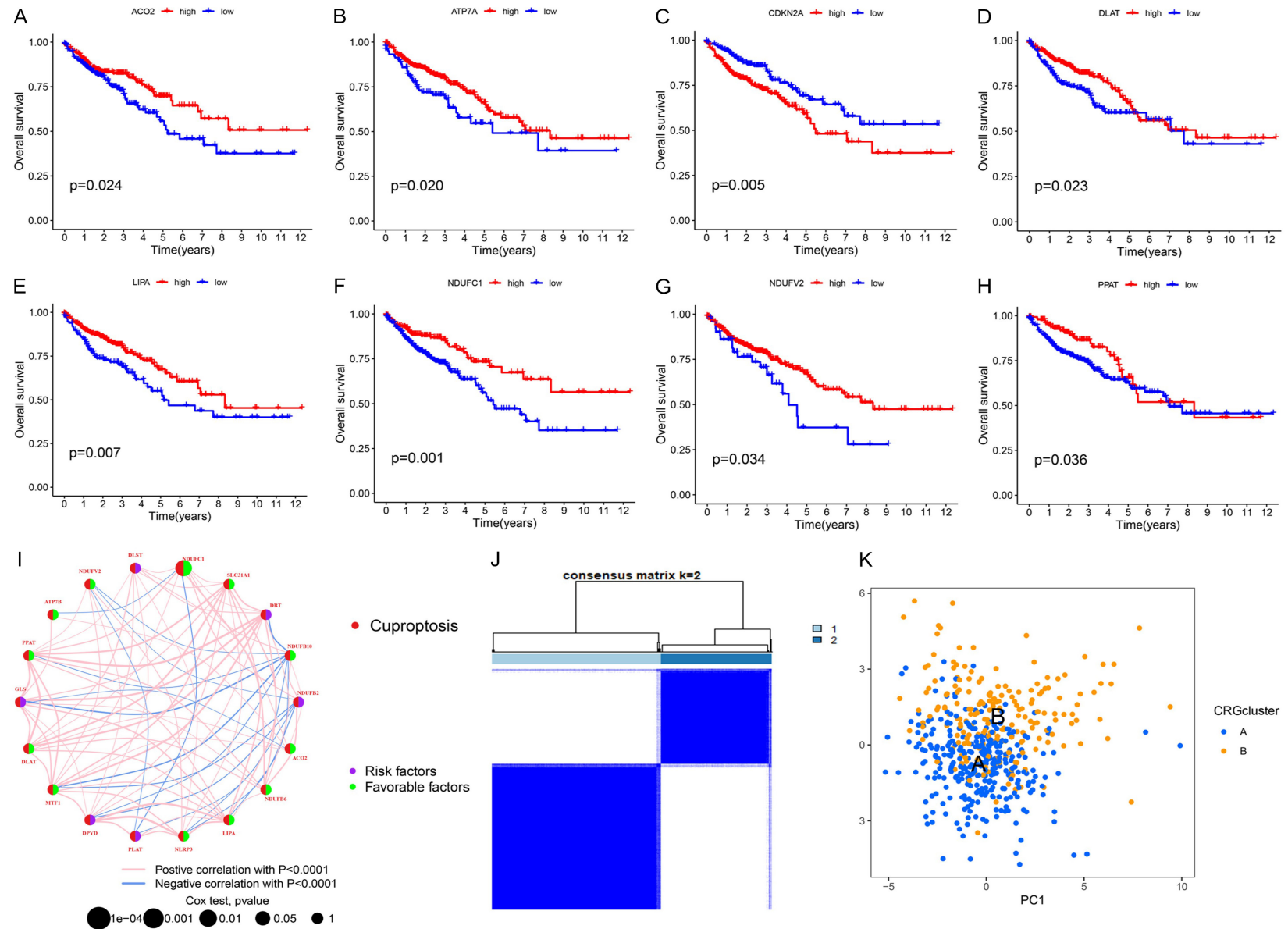


Figure 2. Prognostic significance and molecular subtypes of CRGs in patients with COAD. A-H. Survival analysis of key CRGs in COAD patients. I. Prognostic network of CRGs in relation to COAD prognosis. J. Clustering analysis with optimal $k = 2$ for COAD subtypes. K. Principal component analysis (PCA) of COAD subtypes.

Colon adenocarcinoma: cuproptosis-related model and immune analysis

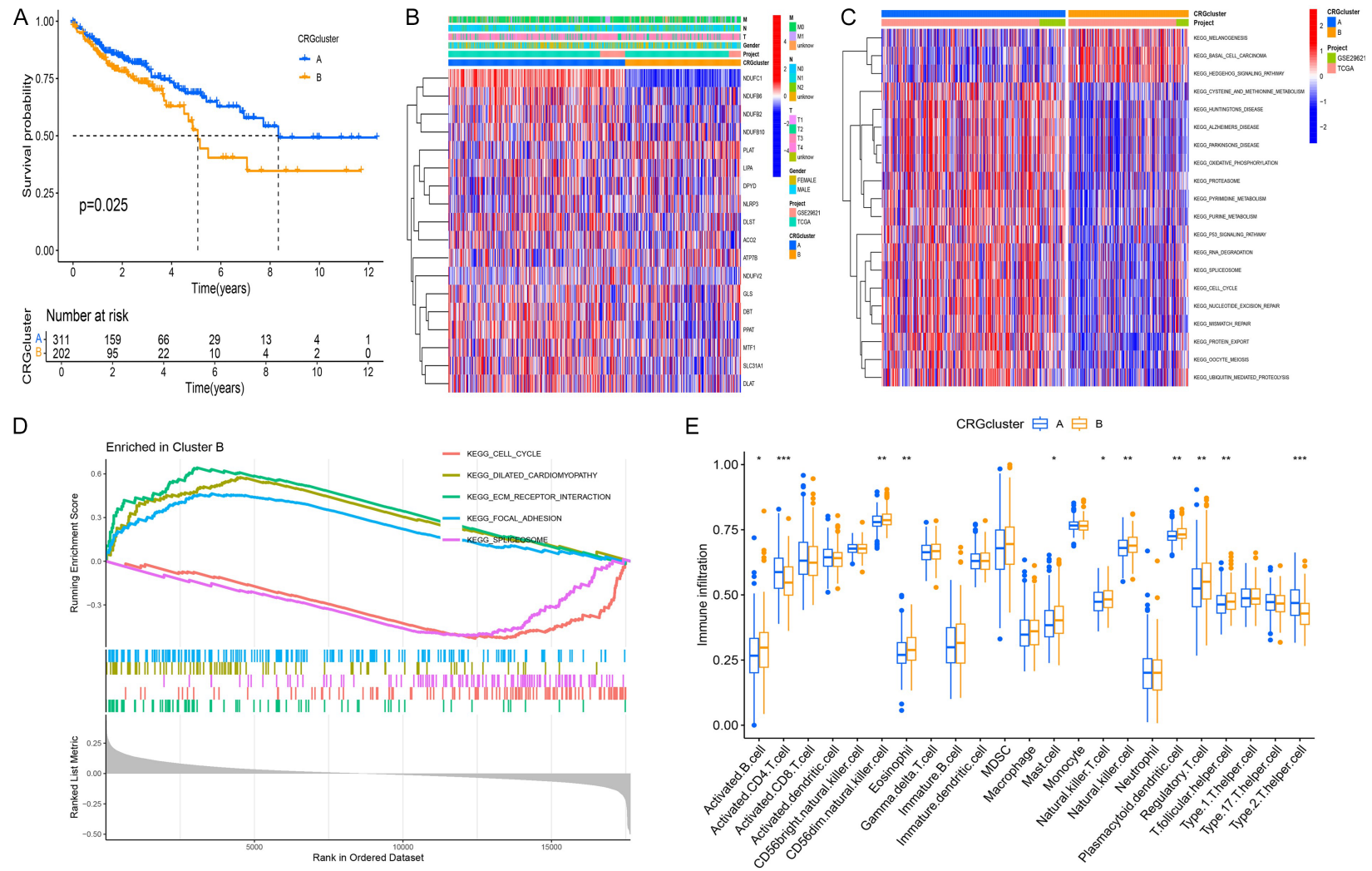


Figure 3. Subtype characteristics and functional differences of CRG clusters in COAD. A. Survival analysis of CRG subtypes. B. CRG expression analysis across subtypes. C. GSEA pathway enrichment in subtypes. D. GSEA analysis of enriched pathways in subtype B. E. ssGSEA analysis of immune cell distributions.

Colon adenocarcinoma: cuproptosis-related model and immune analysis

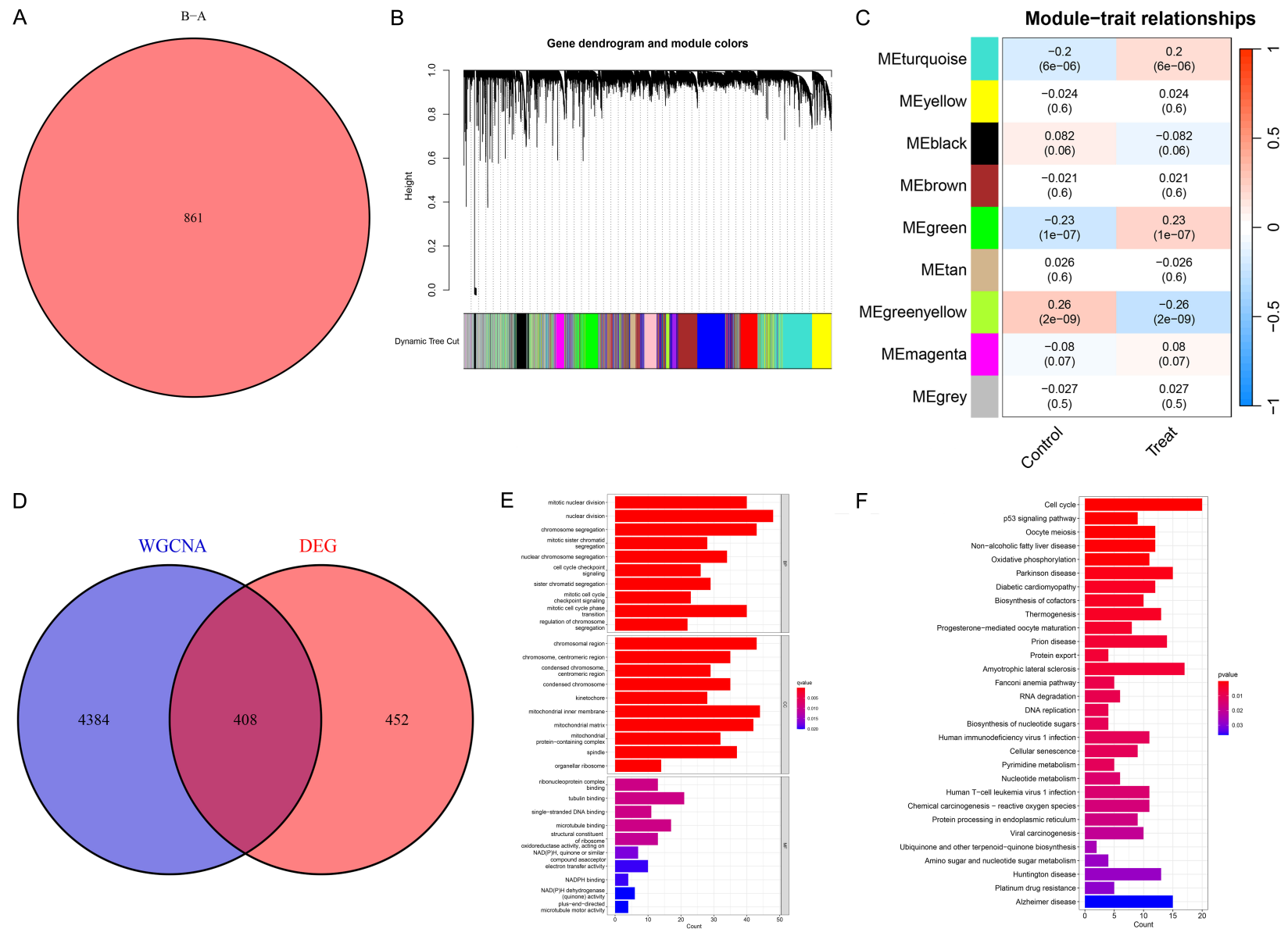


Figure 4. Identification and functional enrichment analysis of intersecting genes related to cuproptosis subtypes in COAD. A. DEGs between cuproptosis-related subtypes. B. Gene dendrogram and module colors identified by WGCNA. C. Heatmap showing correlations between gene modules and clinical features. D. Venn diagram of intersecting genes between yellow-green module and DEGs. E, F. GO and KEGG functional enrichment analysis of intersecting genes.

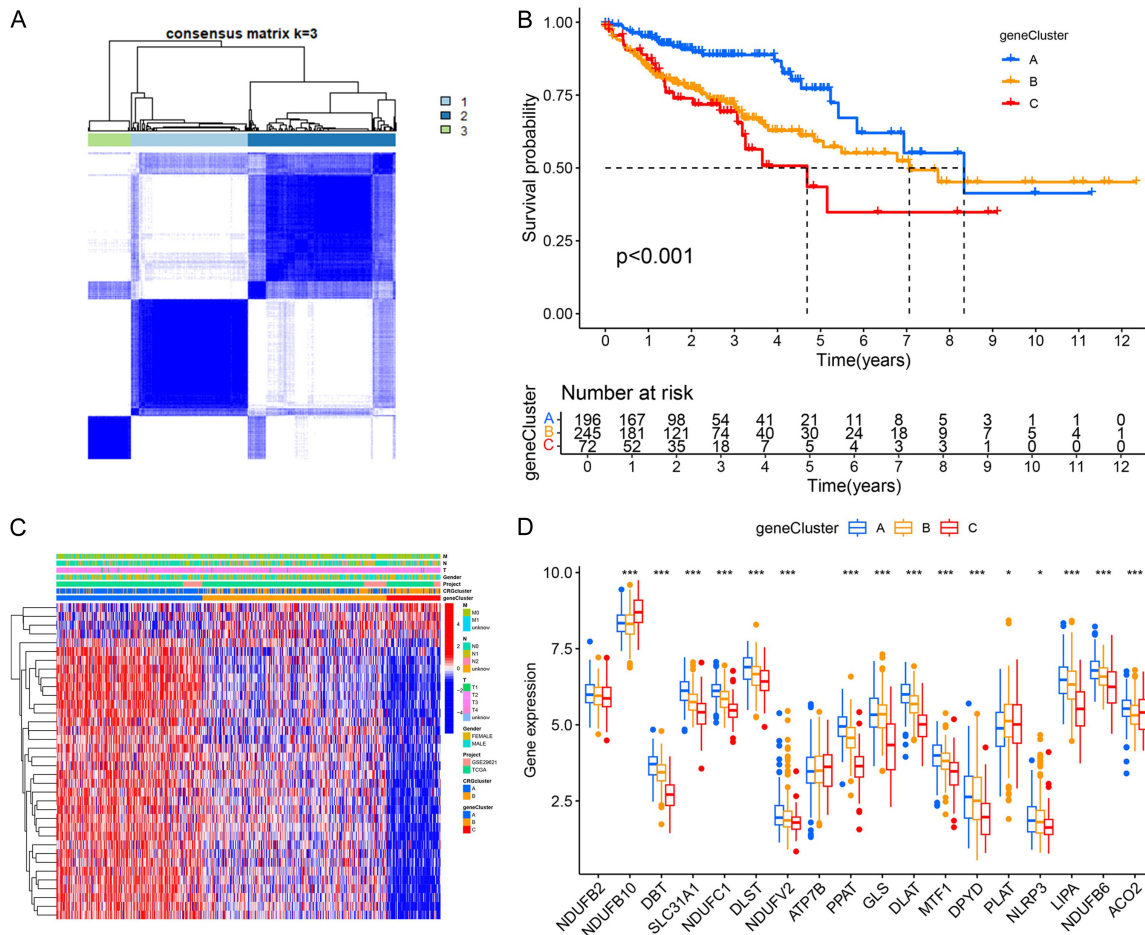


Figure 5. Subtypes identification and characterization of gene subtypes based on intersecting genes in COAD. A. Consensus clustering based on intersecting genes identifies three distinct gene clusters. B. Survival analysis of COAD subtypes A, B, and C. C. Heatmap of clinical and molecular characteristics across the three gene clusters. D. Differential expression analysis of CRGs across subtypes.

GO analysis showed that these genes were mainly enriched in biological processes related to cell cycle checkpoint signaling, with the most prominent cellular component being the mitochondrial protein complex. Key molecular functions included single-stranded DNA binding, oxidoreductase activity, and electron transfer activity (Figure 4E). In addition, KEGG pathway analysis highlighted enrichment in the cell cycle, oxidative phosphorylation, and the p53 signaling pathway (Figure 4F). Combined analysis of all results demonstrates an intimate association of cuproptosis with metabolism and anti-tumor mechanisms in COAD.

Development of a prognostic risk model

To improve the accuracy of subtype classification using cuproptosis-related DEGs, we screened 36 prognostically significant hub

genes from a total of 408 intersecting genes. These genes serve as a bridge linking CRG expression patterns to broader transcriptomic alterations. Based on consensus clustering of the expression profiles ($k = 3$), patients were classified into three subtypes: A, B, and C (Figure 5A). Survival analysis showed a step-wise decrease in survival rates from subtype A to C (Figure 5B). Notably, both the intersected genes and CRGs were significantly upregulated in subtype A but broadly suppressed in subtype C (Figure 5C, 5D). Based on the data we obtained, this revised classification can well retain the inherent biological properties of cuproptosis. More importantly, it also exposes the molecular heterogeneity within colon adenocarcinoma tissues. Using LASSO regression analysis (Figure 6A, 6B), five prognostically relevant variables were identified and used to construct a risk prediction model.

Colon adenocarcinoma: cuproptosis-related model and immune analysis

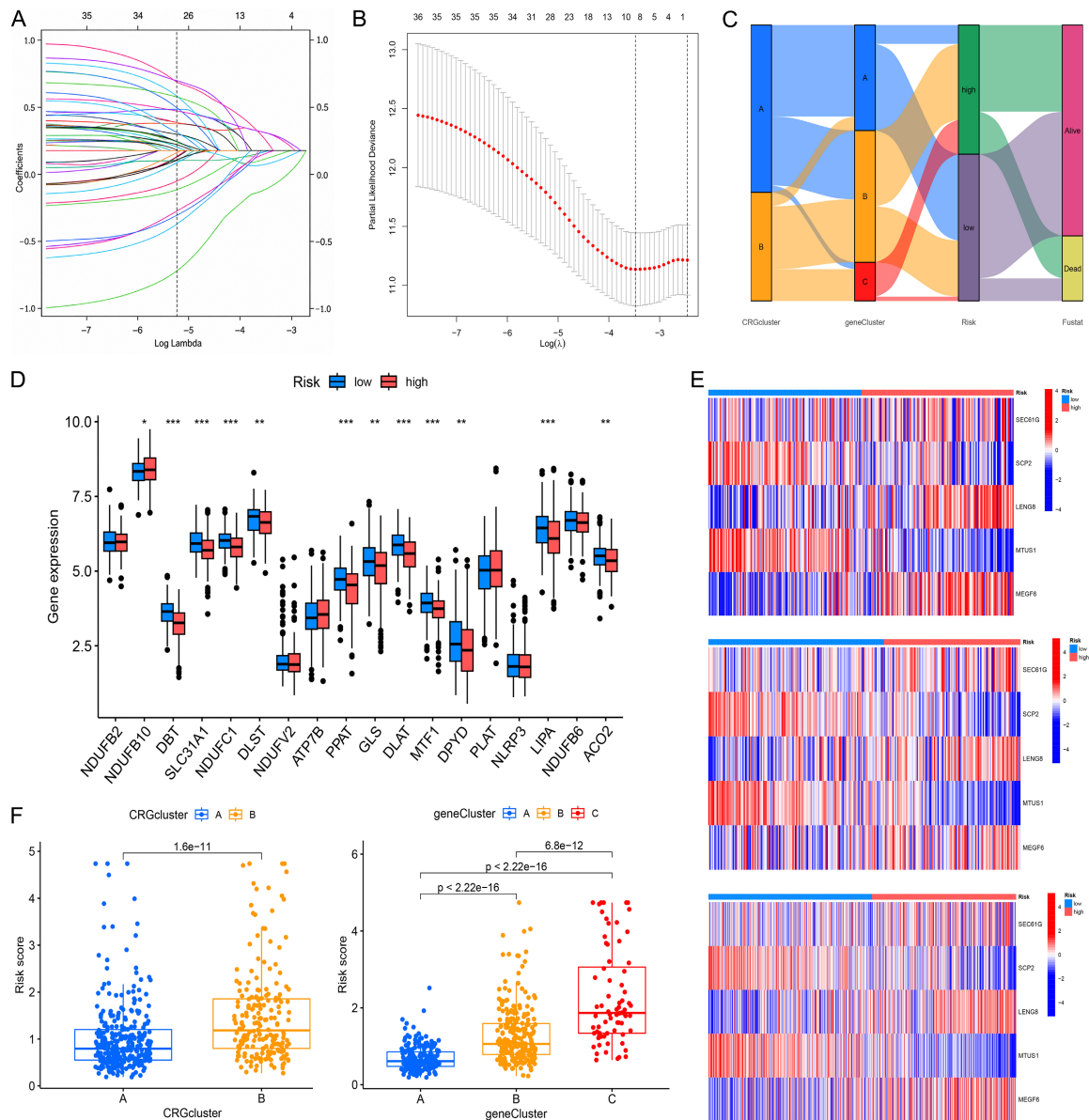


Figure 6. Construction of the prognostic model. A. LASSO coefficient profiles of intersecting genes at various values of the regularization parameter (Lambda). B. Selection of the optimal Lambda in the LASSO model using ten-fold cross-validation. C. Alluvial diagram showing the transitions among CRG clusters, gene clusters, risk groups, and survival status. D. Boxplots of selected gene expression levels in high- and low-risk groups. E. Heatmaps showing the expression profiles of selected genes across different risk groups. F. RS of samples stratified by CRG clusters (left) and gene clusters (right).

The risk score equation is: $RS = (\text{Expression of SEC61G}) \times 0.49 - (\text{Expression of SCP2}) \times 0.39 + (\text{Expression of LENG8}) \times 0.27 - (\text{Expression of MTUS1}) \times 0.50 + (\text{Expression of MEGF6}) \times 0.23$. The Sankey diagram illustrates the flow and transformation of data during the model construction process (Figure 6C). Differential analysis of CRG expression revealed that the majority of CRGs were significantly upregulated

in the low-risk group, which is consistent with the previous subtype analysis indicating that high CRG expression is associated with favorable prognosis (Figure 6D). Furthermore, differential analysis of RS showed that both CRG subtype A and gene subtype A, which exhibited the most favorable clinical outcomes, had the lowest RS values (Figure 6F), confirming RS as a robust prognostic indicator.

Colon adenocarcinoma: cuproptosis-related model and immune analysis

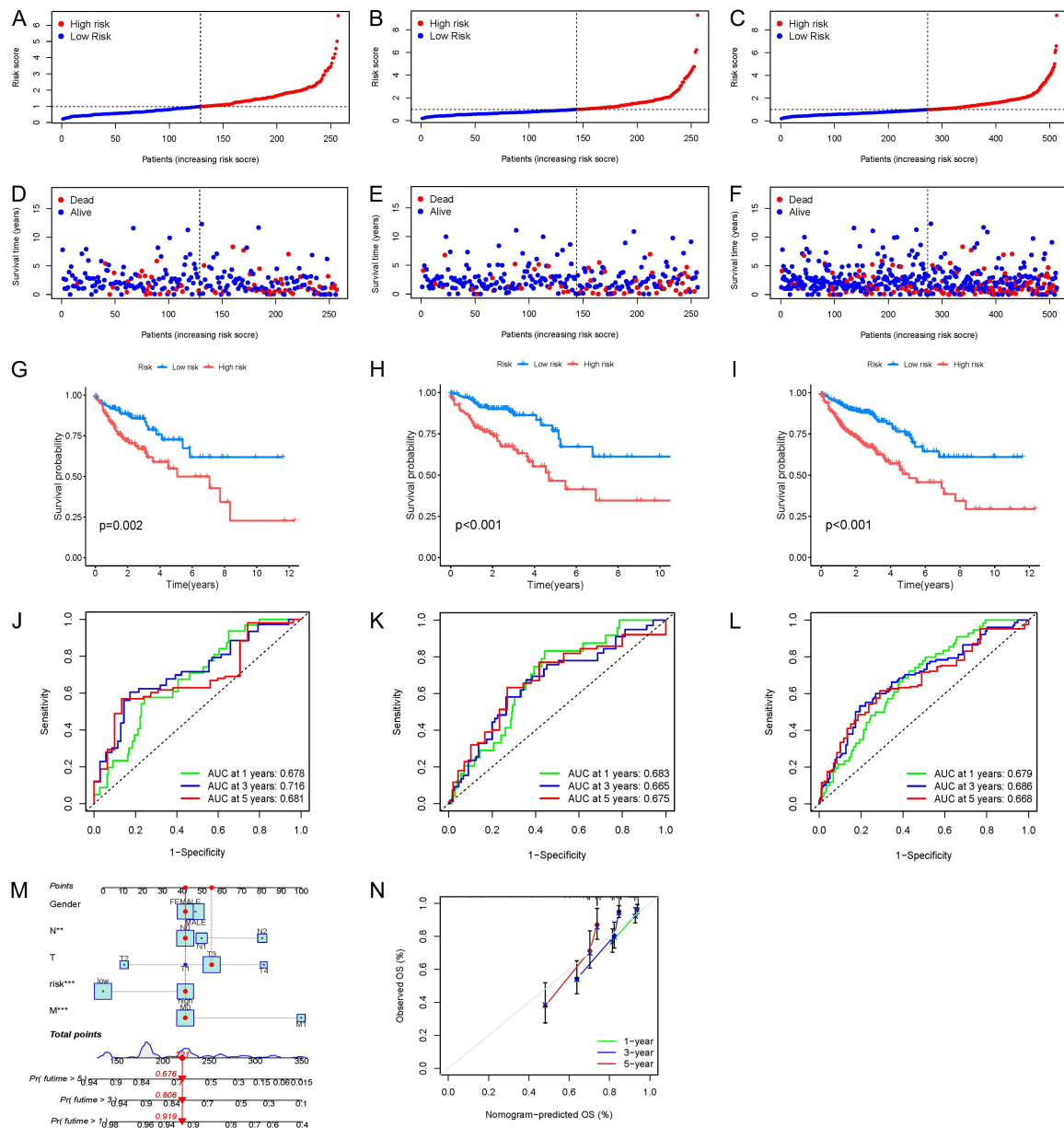


Figure 7. Validation and performance assessment of the prognostic model. A-C. Dot plot showing the distribution of RS. D-F. Survival status plot based on RS. G-I. Kaplan-Meier survival analysis stratified by risk groups. J-L. ROC curves evaluating the prognostic performance of the model. M. Nomogram for predicting 1-, 3-, and 5-year survival probabilities. N. Calibration plot assessing the predictive accuracy of the nomogram.

Excellent predictive ability of the model

Correlation analysis of risk scores revealed distinct expression patterns of the core genes, with a significant positive correlation observed between the risk score and mortality risk (Figures 6E, 7A-F). Survival analysis confirmed that patients in the high-risk group had significantly shorter overall survival ($P < 0.05$), and this finding was consistently validated in the training cohort, validation cohort, and entire

cohort (Figure 7G-I). ROC curve analysis showed that the area under the curve (AUC) values for the training cohort, validation cohort, and entire cohort were all greater than 0.65, indicating favorable prognostic predictive performance of the model (Figure 7J-L). A nomogram constructed based on the risk score and clinical variables could predict the 1-, 3- and 5-year survival rates of patients (Figure 7M), and the calibration curve verified its predictive accuracy (Figure 7N).

Immune and genomic differences in risk groups

CIBERSORT analysis indicated that M0 macrophages were positively correlated with the RS, while M1 macrophages, neutrophils, and resting CD4 memory T cells showed a negative correlation with RS (**Figure 8A-D**). The relationship between model genes and immune cells is illustrated in **Figure 8E**. TME analysis revealed significantly higher immune and stromal scores in the low-risk group (**Figure 8F**), suggesting enhanced immune cell infiltration in the TME, which may promote antitumor immunity. The waterfall plot reveals that the gene mutation frequency is higher in the higher-risk group (**Figure 8G, 8H**). TMB analysis demonstrated that the low-risk group exhibited significantly higher TMB, which was negatively correlated with RS (**Figure 8I, 8J**). Elevated TMB is generally associated with increased tumor neoantigen production, potentially enhancing immune recognition and elimination of tumor cells. We carried out MSI analysis and found more MSI-H cases in the low-risk group. These patients also had lower risk scores (**Figure 8K, 8L**). Clinically, MSI-H tumors are known to be more sensitive to immune checkpoint inhibitors. We further analyzed RNAss. The data showed an obvious negative link between RNAss and risk score (**Figure 8M**). It means that tumor stem cell activity and malignant proliferation are relatively restrained in low-risk individuals. We also evaluated responses to different drugs. The high-risk group was more sensitive to AMG.706 and PHA.665752. The low-risk group gained better effects from AKT inhibitor VIII and mitomycin C (**Figure 9A-H**). These varied responses make it feasible to carry out stratified and personalized treatment. The model we built can therefore serve as a useful reference for designing combination treatment strategies.

Differential expression of model genes verified

Quantitative PCR (qPCR) analysis revealed significant differential expression of five key genes in the COAD prognostic model between COAD cells (HCT116) and normal colon epithelial cells (NCM460). Specifically, the expression of the LENG8 gene was significantly upregulated, with an increase of 2.28 ± 0.36 - fold (**Figure 10A**). Similarly, the expression level of the MEGF6 gene increased by 2.20 ± 0.28 fold (**Figure**

10B), and the expression of the SEC61G gene was upregulated by 1.82 ± 0.16 fold (**Figure 10E**). These upregulation patterns are consistent with their presumed oncogenic roles. Conversely, the expression of the MTUS1 gene was downregulated to 0.39 ± 0.05 fold (**Figure 10C**), and the expression of the SCP2 gene was downregulated to 0.51 ± 0.07 fold (**Figure 10D**). These downregulation trends strongly support their hypothesized tumor suppressive functions.

Discussion

COAD has exhibited a steadily increasing incidence worldwide in recent years [17]. Considerable heterogeneity exists among individual patients, and tumor lesions occurring at different anatomical sites also display distinct clinical characteristics [18]. These complex biological features greatly hinder clinical treatment and routine disease management. Conventional prognostic systems, which mainly depend on basic clinicopathological parameters and tumor staging, have obvious limitations in practical application. Therefore, it is urgently necessary to establish novel prognostic models with improved accuracy and better clinical practicability. Cuproptosis, a recently discovered form of programmed cell death [19], has been shown to suppress tumor cells by regulating intracellular copper ion homeostasis. However, its association with COAD prognosis and tumor immunity remains insufficiently elucidated. By integrating multi-omics data, we constructed a multi-biomarker prognostic model based on CRGs to enhance the accuracy of survival prediction and provide a foundation for personalized treatment strategies.

Analysis of public databases revealed significant differential expression of most CRGs between normal and tumor tissues. Among these genes, the significant downregulation of NDUFC1 in COAD impairs the function of the electron transport chain (ETC), disrupts the coupling between the mitochondrial respiratory chain and the TCA cycle, and hinders the interaction between copper ions and acylated components of the TCA cycle as well as the aggregation of acylated proteins, thereby inhibiting the cuproptosis pathway [5, 20]. In contrast, the upregulation of PPAT not only meets the energy demand of COAD for rapid proliferation

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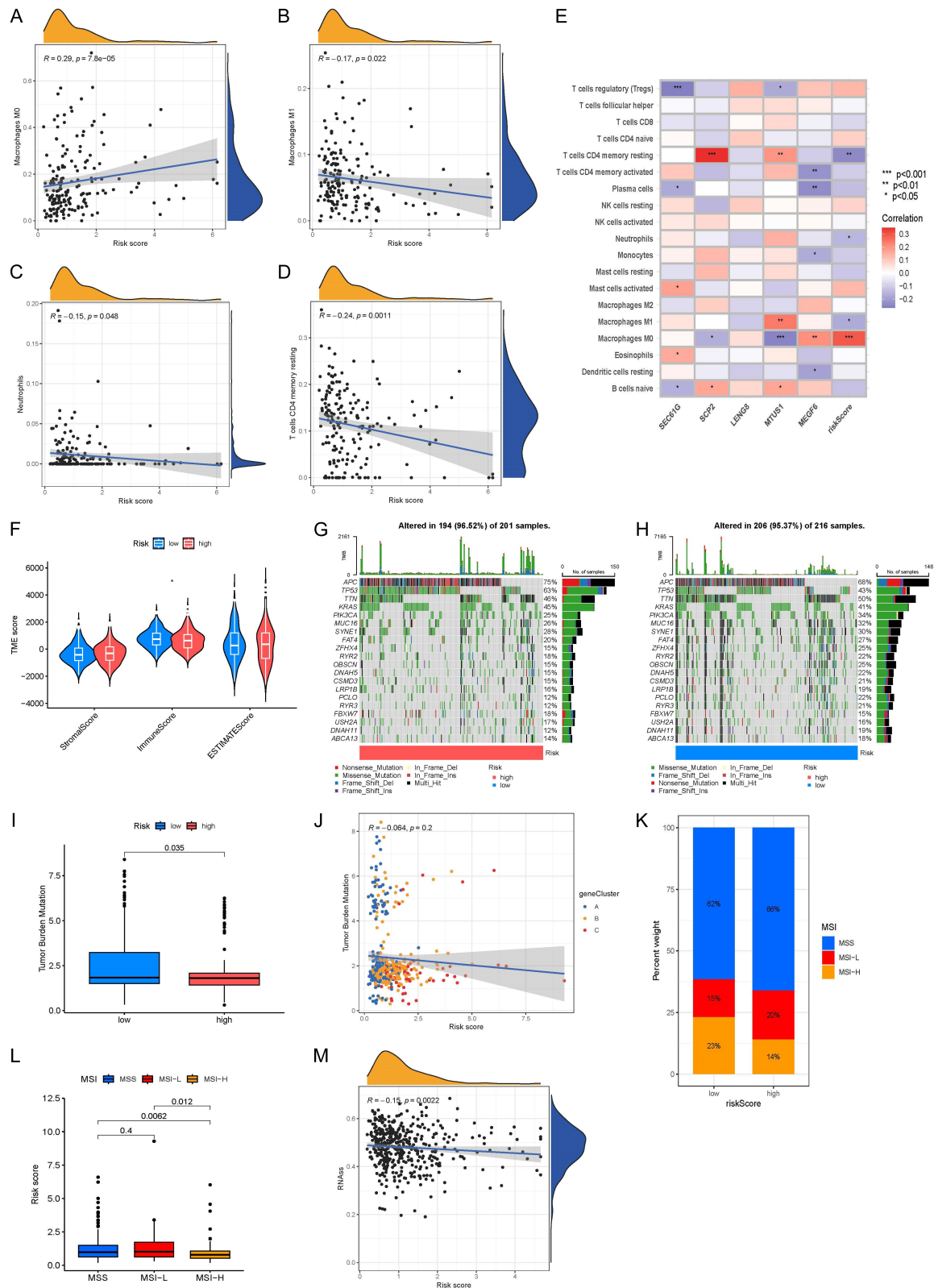


Figure 8. Multi-omics characteristics of the tumor microenvironment and genomic features stratified by risk score in COAD. A-D. Correlation between immune cell abundance and RS. E. Heatmap showing the correlation between immune cells and model genes. F. TME analysis comparing stromal and immune scores across risk groups. G, H. Waterfall plot showing gene mutation frequencies in high- and low-risk groups. I, J. TMB analysis stratified by risk groups. K. MSI analysis comparing MSS, MSI-L, and MSI-H by risk groups. L. Boxplots comparing RS among MSS, MSI-L, and MSI-H groups. M. Correlation between stem cell RNAss and RS.

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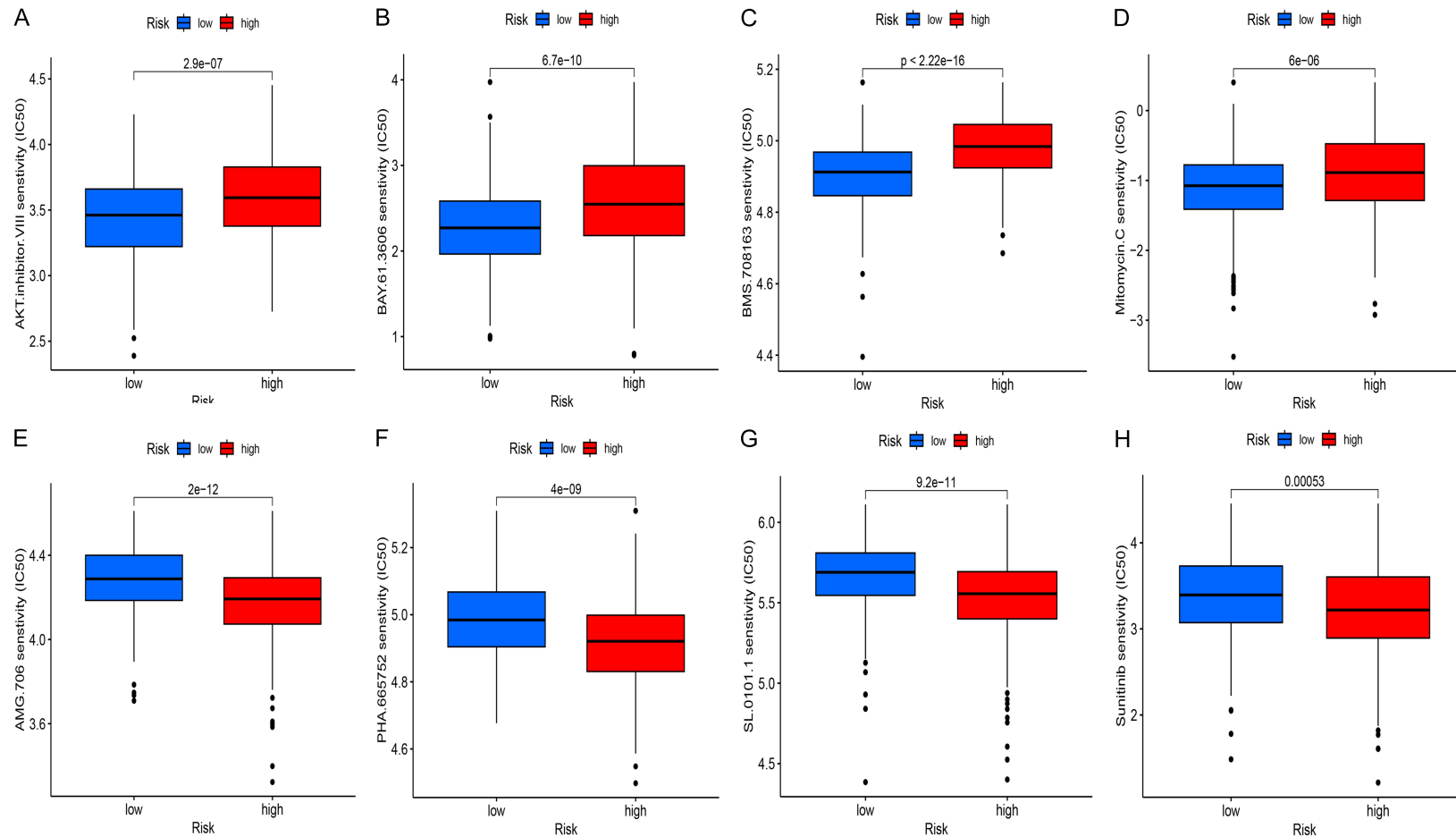


Figure 9. Drug sensitivity analysis of different agents between high-risk and low-risk groups. A-H. Boxplots showing the IC₅₀ values of different drugs (AKT inhibitor VIII, BAY 61-3606, BMS-708163, Mitomycin C, AMG-706, PHA-665752, SL-0101, Sunitinib) in high-risk and low-risk groups.

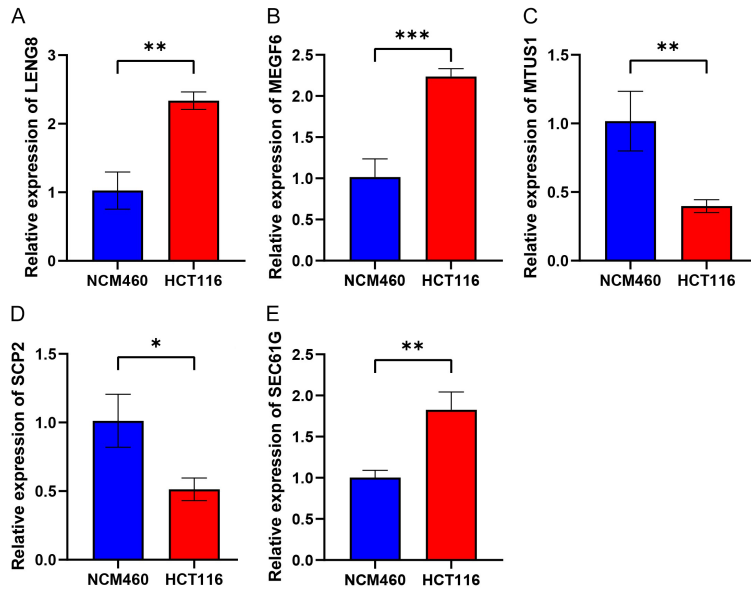


Figure 10. Validation of differential expression of the model genes. A-E. The relative expression levels of five model genes (LENG8, MEGF6, MTUS1, SCP2, and SEC61G) in the normal colon epithelial cell line NCM460 and the colon adenocarcinoma cell line HCT116 are presented.

by enhancing purine synthesis, but its metabolic intermediate adenosine can also inhibit the infiltration of M1 macrophages, which indirectly promotes tumor immune escape [21]. ACO2, a core enzyme linking the TCA cycle to lipid metabolism, is significantly downregulated in COAD samples and associated with malignant phenotypes [22]. Its functional deficiency inhibits the TCA cycle, prompting metabolic reprogramming in tumor cells to rely on glycolysis for energy supply and promoting lipid synthesis. Meanwhile, it enhances lipid desaturation by upregulating stearoyl-CoA desaturase (SCD1), thereby driving tumor progression. CRGs collectively influence the occurrence, development, and clinical progression of COAD through their respective roles in energy metabolism, immune regulation, and lipid synthesis [23-25]. Using the above genes as markers, we carried out cluster analysis to categorize all patient samples into two subgroups, namely subtype A and subtype B. Survival outcomes differed obviously between the two groups: patients in subtype A achieved a more favorable prognosis. Meanwhile, most CRGs were highly expressed in subtype A, while their expression levels declined in subtype B.

Results from gene set enrichment analysis suggested that multiple tumor-promoting pa-

thways were enriched in subtype B. Further GSVA analysis also confirmed the enrichment of three key pathways: melanogenesis, basal cell carcinoma pathway and Hedgehog signaling pathway. The melanogenesis and basal cell carcinoma pathways have been implicated in skin cancer development [26], while the Hedgehog signaling pathway promotes invasion and metastasis in various cancers by regulating cell proliferation, differentiation, and survival [27, 28]. GSEA further demonstrated that, compared to subtype A, subtype B showed greater enrichment in the cell cycle pathway, ECM-receptor interaction pathway, focal adhesion pathway, and spliceosome pathway. Dysregulation of the cell cycle path-

way leads to uncontrolled cancer cell proliferation [29]. Aberrant ECM-receptor interactions can induce ECM stiffening and promote malignant transformation [30, 31]. Abnormal expression of key focal adhesion pathway components (e.g., integrins, FAK) enhances tumor invasion and metastasis [32, 33]. Spliceosome pathway dysregulation promotes cancer cell growth through the generation of aberrant mRNA splice variants [34, 35]. The abnormal activation of these pathways explains why patients in subtype B tend to have worse clinical outcomes. It also helps us better understand the biological distinctions between the two subtypes.

Although CRG-based subtyping revealed significant differences in prognosis and core pathway gene expression, we hypothesize that the effects of CRGs are mediated through the regulation of a broader transcriptional network. These downstream effector genes and co-regulated signaling pathways work together to drive the unique biological features of each subtype. Next, we took the prognosis-related differentially expressed genes from the two subtypes and cross-referenced them with WGCNA-derived subtype-associated genes to find overlapping candidates. We then ran consensus clustering on these shared genes and

sorted all samples into three subgroups labeled A, B and C. This step helped us classify patient samples more accurately and comprehensively. After that, we applied multivariate Cox regression and LASSO regression to build a prognostic risk model using the five overlapping genes. We also adopted survival curve analysis and ROC curve analysis to fully verify how well this model could stratify patient risks and predict survival outcomes.

We further explored the expression features of SEC61G, SCP2, LENG8, MTUS1 and MEGF6 in COAD, and explored their functions during tumor growth. According to our results, SEC61G, LENG8 and MEGF6 are high-risk genes. When these genes are highly expressed, patients tend to experience tumor deterioration and worse prognosis. In comparison, SCP2 and MTUS1 represent low-risk genes, and their elevated expression is connected with improved survival. Our conclusions match those reported in prior studies. SEC61G, as the γ subunit of the SEC61 complex, contributes to tumor regulation by mediating endoplasmic reticulum (ER) protein translocation and maintaining ER-cytoplasmic calcium homeostasis [36]. In COAD, SEC61G activates EGFR and its downstream pathways via calmodulin, promoting cell cycle progression from G1 to S phase to drive tumor growth [37]. Although literature on LENG8 is limited, studies by Song et al. [38] have confirmed the prognostic predictive value of its alternative splicing events and expression levels in clear cell renal cell carcinoma (KIRC). In the present study, the association between its high expression in COAD and poor prognosis further expands the potential application of this gene in precise tumor typing. MEGF6, a transmembrane protein containing multiple epidermal growth factor (EGF)-like domains, promotes COAD cell migration by inducing epithelial-mesenchymal transition (EMT) through activation of the transforming growth factor β (TGF β)/SMAD signaling pathway [39]. Its high expression in 5-fluorouracil-resistant cells suggests involvement in drug resistance mechanisms [39]. In hepatocellular carcinoma, lenvatinib-induced MEGF6 upregulation triggers myostatin activation, leading to sarcopenia and enhanced tumor proliferation, whereas MEGF6 knockdown simultaneously achieves muscle protection and tumor suppression, highlighting its multifaceted role [40]. SCP2, a key lipid transport protein with established tumor-suppressive functions, is upregulated by the

tumor-suppressive long non-coding RNA LINC-00261 through inhibition of the transcription factor FOXP3, thereby suppressing angiogenesis and cell cycle progression in pancreatic cancer cells [41]. This aligns with our findings in COAD, where SCP2 acts as a protective factor, validating its role in tumor metabolic regulation. MTUS1, a tumor suppressor gene located at 8p22, encodes ATIP1 and ATIP3 proteins, which induce cancer cell apoptosis by blocking the extracellular signal-regulated kinases (ERK) signaling pathway [42]. Cheng et al. [43] confirmed that low MTUS1 expression in COAD is associated with poor prognosis. In head and neck squamous cell carcinoma, MTUS1 exerts tumor-suppressive effects by inducing mitochondrial pyroptosis via reactive oxygen species (ROS) [44].

TME is gaining importance in cancer research, playing a crucial role in tumor initiation, progression, and response to treatment. To this end, we used CIBERSORT to further analyze immune cell infiltration in COAD and investigate its correlation with RS. The results indicated that M0 macrophages were positively correlated with RS, while M1 macrophages, neutrophils, and resting CD4 memory T cells were negatively correlated with RS, consistent with previous findings. High infiltration of M0 macrophages may promote their differentiation into a pro-tumorigenic M2 phenotype, thereby exacerbating tumor progression and contributing to a high-risk profile [45, 46]. Conversely, M1 macrophages can activate cytotoxic T lymphocytes (CTLs) by presenting antigens or secreting pro-inflammatory cytokines, thereby promoting T cell proliferation and interferon-gamma (IFN γ) secretion, inhibiting tumor growth [47]. Neutrophils can inhibit COAD progression by modulating the tumor-associated microbiome [48] or by eliminating antibody-labeled cancer cells via a trogoptosis mechanism [49]. Additionally, resting CD4 memory T cells, as a subset of antigen-specific CD4 T cells, are associated with improved prognosis when enriched [50]. These cells are regarded as indicators of prognosis in several types of cancer, including breast cancer [51], multiple myeloma [52], and lung adenocarcinoma [53]. In summary, the improved prognosis in the low-risk group may stem from enhanced immune cell activity driven by the upregulation of CRGs, providing new insights into their immunomodulatory roles within the TME.

The TMB analysis shows that the low-risk group has a higher TMB than the high-risk group, and there is a negative correlation between TMB and RS. This relationship may be attributed to the fact that a higher TMB is often associated with the release of more neoantigens, which triggers a stronger immune response and increases the likelihood of tumor clearance by the immune system [54]. This partially explains why the low-risk group exhibits higher TMB. Additionally, MSI indicates genomic instability typically resulting from defects in the DNA mismatch repair (MMR) system [55]. High TMB and MSI-H status are typically associated with a stronger immune response, and MSI-H tumors typically show improved responsiveness to immune checkpoint inhibitors [56], which explains why patients in the low-risk group tend to have a better response to immunotherapy.

Several limitations of the present study should be acknowledged. First, all original analytical data were retrieved from public TCGA and GEO databases. Only qPCR assays in cell lines were performed to verify the expression of core model genes. No external validation was conducted using clinical COAD tissues, the International Cancer Genome Consortium (ICGC) cohort, or multicenter clinical samples. Accordingly, potential data bias may compromise the generalizability and clinical predictive performance of the prognostic model. Second, this study failed to explore novel regulatory mechanisms and unique signaling pathways of core genes. The biological functions of key genes including *NDUFC1* and *PPAT* were not validated through in vitro and in vivo functional experiments, and the clinical applicability of the candidate drugs predicted by the model also lacks sufficient clinical evidence. In follow-up work, we will incorporate ICGC datasets and multicenter clinical samples. Tumor tissues will be used for external validation to refine the prognostic model and verify its stability. We will also conduct functional experiments to explore the biological roles of core genes and identify new pathways linked to cuproptosis and colon adenocarcinoma.

Conclusion

In this study, we successfully built a novel COAD prognostic signature based on cuproptosis-related genes. By analyzing this risk model, we screened out core molecular biomarkers and

further illustrated the intricate crosstalk among cuproptosis mechanisms, immune microenvironment changes, and tumor progression of COAD. Collectively, this signature offers a reliable clinical reference for prognostic assessment and individualized treatment decision-making for colon adenocarcinoma patients. Additionally, the study underscores the potential of targeting cuproptosis-related pathways in COAD therapy. Although these findings require further clinical validation, our research offers new insights into the pathogenesis of COAD and contributes to the improvement of therapeutic approaches.

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Disclosure of conflict of interest

None.

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Colon adenocarcinoma: cuproptosis-related model and immune analysis

Supplementary Table 1. List of cuproptosis-related genes

Gene Symbol	Full Gene Name
NDUFB2	NADH: Ubiquinone Oxidoreductase Subunit B2
NDUFB10	NADH: Ubiquinone Oxidoreductase Subunit B10
DBT	Dihydrolipoamide Branched-Chain Transacylase E2
SLC31A1	Solute Carrier Family 31 Member 1
NDUFC1	NADH: Ubiquinone Oxidoreductase Subunit C1
DLST	Dihydrolipoamide S-Succinyltransferase
NDUFV2	NADH: Ubiquinone Oxidoreductase Subunit V2
ATP7B	ATPase Copper Transporting Beta
PPAT	Phosphoribosyl Pyrophosphate Amidotransferase
GLS	Glutaminase
DLAT	Dihydrolipoamide Acetyltransferase
MTF1	Metal Regulatory Transcription Factor 1
DPYD	Dihydropyrimidine Dehydrogenase
PLAT	Plasminogen Activator, Tissue Type
NLRP3	NLR Family Pyrin Domain Containing 3
LIPA	Lipase A, Lysosomal Acid, Cholesteryl Esterase
NDUFB6	NADH: Ubiquinone Oxidoreductase Subunit B6
ACO2	Aconitase 2

Supplementary Table 2. The primer sequences for qPCR

Primer		Sequence 5'-3'
GAPDH	Forward	CCCTGACATACCAAGCACT
	Reverse	CCTTTGCGCTCCTCTCAGAA
LENG8	Forward	ACAAATCCATCAACCGTTCC
	Reverse	GCCAGTTTGTTCAGGTGGTT
MEGF6	Forward	CGGAGAACCGTCTACTACATGG
	Reverse	CAACGGCCACCGTGAAAAC
MTUS1	Forward	TGCTTCGAAACCTTCGACTT
	Reverse	TGTCAATGCCTCAAACCTTGG
SCP2	Forward	GGAGCTGAGAATTCAAGAGACT
	Reverse	AGTCACCAAAAACATAGCCAAC
SEC61G	Forward	TCTACGCTCTCAATCCGCCATC
	Reverse	TCTTTTCTGTCGGGTTTGGTGCA