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Characterization and Application of a Copper and Lipid Metabolism-Related Gene Signature in a Prognostic Risk Model for Gastric Cancer

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Abstract

Background

Metabolic reprogramming contributes to gastric cancer progression and immune regulation. Copper and lipid metabolism are interconnected, but their combined prognostic value in gastric cancer remains unclear. This study aimed to develop and validate a copper- and lipid-metabolism-related gene signature and explore its biological relevance.

Methods

Transcriptomic and clinical data were obtained from TCGA-STAD and GSE66229. Differentially expressed copper- and lipid-metabolism-related genes were identified, followed by consensus clustering, Cox regression and LASSO Cox analysis to construct a prognostic model. The signature was externally validated, and its associations with clinical characteristics, immune infiltration, tumor mutational burden, immunotherapy-related indicators, drug sensitivity and single-cell transcriptomic features were evaluated. In vitro assays examined the function of GAD1.

Results

A total of 217 copper- and lipid-metabolism-related differentially expressed genes were identified. Consensus clustering classified gastric cancer into six metabolic subtypes with distinct survival outcomes and tumor microenvironmental features. A 15-gene prognostic signature was established and showed stable predictive performance in the TCGA and GSE66229 cohorts. High-risk patients had poorer overall survival and were characterized by stromal activation, extracellular matrix remodeling and an immunosuppressive microenvironment. Low-risk tumors showed higher tumor mutational burden and enrichment of DNA repair and metabolic homeostasis pathways. Single-cell analysis indicated that GAD1-positive cells had enhanced copper-lipid metabolic activity and elevated epithelial-mesenchymal transition, hypoxia and fatty acid metabolism scores. GAD1 knockdown suppressed gastric cancer cell proliferation, colony formation and migration.

Conclusion

This study established a copper-lipid metabolism-related prognostic signature for gastric cancer, providing a potential framework for risk stratification and biomarker development.

Keywords: Gastric cancer, Copper metabolism, Lipid metabolism, Prognostic signature, GAD1

1 Introduction

Gastric cancer (GC) remains one of the leading causes of cancer-related mortality worldwide ^[1,2]. Although substantial advances have been made in surgical techniques, chemotherapy, targeted therapy, and immunotherapy, the prognosis of patients with advanced GC remains unsatisfactory, largely owing to high recurrence rates, metastatic potential, and therapeutic resistance ^[3–8]. Therefore, the identification of reliable prognostic biomarkers and a deeper understanding of the molecular mechanisms underlying GC progression are essential for improving clinical outcomes ^[9].

Metabolic reprogramming has emerged as a hallmark of cancer and plays a pivotal role in tumor initiation, progression, and therapeutic response ^[10,11]. Tumor cells frequently rewire glucose, lipid, amino acid, and mitochondrial metabolism to meet the increased bioenergetic, biosynthetic, and redox demands required for rapid proliferation ^[10–13]. Increasing evidence indicates that metabolic alterations not only support tumor growth but also reshape the tumor microenvironment and modulate immune cell function ^[14–16]. In GC, metabolic plasticity enables tumor cells to adapt to hypoxia, oxidative stress, and nutrient deprivation, thereby promoting tumor survival and disease progression ^[12–16].

Among the diverse metabolic pathways involved in cancer progression, copper metabolism and lipid metabolism have received increasing attention in recent years ^[17]. Copper is an essential trace element involved in mitochondrial respiration, oxidative stress regulation, and multiple enzymatic reactions ^[18–20]. Dysregulated copper homeostasis has been reported in several malignancies and is closely associated with tumor proliferation, angiogenesis, metastasis, and altered therapeutic vulnerability ^[18,19,21–23]. Meanwhile, lipid metabolism provides structural components for membrane synthesis, energy substrates for tumor growth, and signaling molecules that regulate tumor progression and immune responses ^[17,24–27]. Recent studies have suggested that copper and lipid metabolism are closely interconnected in key biological processes, including oxidative stress regulation, mitochondrial function maintenance, and energy metabolic reprogramming, and may jointly contribute to tumor initiation and progression ^[18,23].

However, most existing studies have focused on the modeling and analysis of individual metabolic pathways. For instance, in the context of copper metabolism, a prognostic model based on copper metabolism-related genes has been developed in breast cancer, demonstrating that copper metabolic features can effectively predict patient survival outcomes and are closely associated with the tumor immune microenvironment. Mechanistically, these effects may involve oxidative stress and mitochondrial metabolic dysfunction induced by copper homeostasis imbalance, thereby influencing

immune cell infiltration and tumor cell proliferation ^[28]. Similarly, a copper metabolism-related gene signature established in lung adenocarcinoma has shown favorable prognostic stratification ability. Functional enrichment analysis further indicated that this model was significantly associated with key biological processes, including mitochondrial energy metabolism, redox homeostasis, and cell cycle regulation, highlighting the important role of copper metabolism in regulating tumor metabolic adaptation ^[29]. Nevertheless, these analytical frameworks remain largely restricted to a single metabolic dimension, which limits their ability to fully capture the complexity of tumor metabolic regulatory networks.

Lipid metabolic reprogramming has also been widely incorporated into prognostic modeling. For example, in colorectal cancer, prognostic models based on lipid metabolism-related genes, such as CYP19A1, have been shown not only to effectively predict patient prognosis but also to be closely associated with immunotherapeutic response. Dysregulated lipid metabolism may alter the redox state of tumor cells and remodel the tumor immune microenvironment by modulating the balance between fatty acid synthesis and oxidation, lipid peroxidation levels, and membrane lipid composition, thereby influencing sensitivity to immunotherapy ^[30]. However, these studies have similarly focused primarily on lipid metabolism as an isolated pathway and have not fully considered the synergistic or antagonistic interactions among distinct metabolic networks.

Taken together, although models based on copper metabolism- or lipid metabolism-related genes have demonstrated prognostic value and may reflect tumor biological characteristics related to oxidative stress regulation and energy metabolic reprogramming, a single metabolic pathway is insufficient to fully characterize the overall complexity of tumor metabolic reprogramming. Therefore, an integrative analysis of copper metabolism- and lipid metabolism-related genes may more comprehensively reflect tumor biological features at the level of multidimensional metabolic networks. Such an approach may facilitate the identification of more robust prognostic biomarkers, improve predictive performance, and enhance the clinical applicability of prognostic models.

In this study, we systematically analyzed genes associated with copper and lipid metabolism in GC and constructed a prognostic risk model to evaluate their clinical significance. By integrating transcriptomic profiling, immune infiltration analysis, drug sensitivity prediction, and single-cell RNA sequencing analysis, we comprehensively characterized copper-lipid metabolic reprogramming in GC and its differences across distinct risk groups. These findings may provide a theoretical basis for prognostic risk stratification and the identification of potential biomarkers in GC.

2 Materials and Methods

2.1 Data Collection

Transcriptomic and clinicopathological data for gastric cancer were obtained from The Cancer Genome Atlas stomach adenocarcinoma cohort (TCGA-STAD) and the Gene Expression Omnibus

(GEO) database. A total of 348 TCGA-STAD gastric cancer samples with available transcriptomic profiles were used for differential expression analysis, consensus clustering, molecular subtype characterization, and tumor microenvironment-related analyses. After excluding samples lacking overall survival information or complete clinicopathological data, 315 TCGA-STAD patients were retained for survival-related analyses, prognostic model construction, risk stratification, and clinicopathological association analyses. In addition, 298 gastric cancer samples from the GSE66229 cohort were included as an external validation cohort ^[31–33]. Copper metabolism-related genes (CRGs) and lipid metabolism-related genes (LRGs) were retrieved from the GeneCards database ^[34]. After duplicate genes were removed, 881 CRGs and 2,374 LRGs were obtained. Batch effects across different datasets were corrected using the "sva" R package ^[35].

2.2 Identification of Copper- and Lipid-Metabolism-Related Differentially Expressed Genes

Differential expression analysis between gastric cancer tissues and normal tissues was performed using the "limma" R package ^[36]. Genes with $|\log_2 \text{fold change}| > 1$ and false discovery rate (FDR) < 0.05 were considered significantly differentially expressed genes (DEGs). The DEGs were then intersected with CRGs and LRGs to identify candidate copper- and lipid-metabolism-related genes (CLRGs) in gastric cancer.

2.3 Functional Enrichment Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using the "clusterProfiler" R package ^[37]. Biological processes and pathways with adjusted P values < 0.05 were considered statistically significant.

2.4 Consensus Clustering Analysis

Unsupervised consensus clustering analysis was performed using the "NMF" R package ^[38] to classify the 348 TCGA-STAD samples into distinct metabolic subtypes based on the expression profiles of candidate CLRGs. The optimal number of clusters was determined according to clustering stability and consensus matrix patterns. t-distributed stochastic neighbor embedding (t-SNE) analysis was applied to visualize subtype distribution. Kaplan-Meier survival analysis and log-rank tests were used to compare overall survival among subtypes.

2.5 Gene Set Variation Analysis

Gene set variation analysis (GSVA) was performed using the "GSVA" R package ^[39] to evaluate pathway enrichment differences among metabolic subtypes. KEGG pathway gene sets were used as the reference database.

2.6 Construction of the Prognostic Risk Model

Univariate Cox regression analysis was conducted in the 315 TCGA-STAD patients with complete survival information and clinicopathological data to identify CLRGs significantly associated with overall survival (OS). LASSO Cox regression analysis was then performed using the “glmnet” R package ^[40] to identify the optimal prognostic gene signature. The risk score was calculated using the following formula:

$$\text{Risk score} = \sum (\text{coefficient} \times \text{gene expression}).$$

Patients were divided into high- and low-risk groups based on the median risk score in the TCGA cohort. Kaplan-Meier survival curves were generated to compare survival differences between the two risk groups.

2.7 Validation of the Prognostic Signature

The predictive accuracy of the model was evaluated using time-dependent receiver operating characteristic (ROC) curves generated by the "timeROC" R package ^[41]. The area under the curve (AUC) was calculated for 1-, 2-, and 3-year OS. The model was further validated in the external GSE66229 cohort using the same risk-score formula and risk-threshold strategy.

2.8 Construction of a Prognostic Nomogram

A prognostic nomogram was constructed using the "rms" R package by integrating the CLRG-derived risk score and available clinical parameters. Calibration curves were used to evaluate agreement between predicted and observed survival probabilities. The concordance index (C-index) and ROC curves were used to assess predictive performance.

2.9 Tumor Immune Microenvironment Analysis

Immune-related functional scores were calculated using single-sample gene set enrichment analysis (ssGSEA) based on predefined immune function gene sets. The CIBERSORT algorithm was used to estimate the relative abundance of immune cell populations ^[42]. The ESTIMATE algorithm was applied to calculate stromal score, immune score, ESTIMATE score, and tumor purity ^[43]. Differences in immune cell infiltration, immune-related functional scores, and immune checkpoint expression between the high- and low-risk groups were analyzed.

2.10 Differential Expression Analysis of Immune Checkpoint Genes

The expression levels of immune checkpoint-related genes were compared between the high- and low-risk groups. A panel of commonly studied immune checkpoint molecules, including PDCD1 (PD-

1), CD274 (PD-L1), CTLA4, LAG3, TIGIT, HAVCR2 (TIM-3), and PDCD1LG2 (PD-L2), was selected for analysis. Gene expression levels were extracted from the TCGA transcriptomic dataset and compared using the Wilcoxon rank-sum test. Results were visualized using boxplots generated with the "ggplot2" R package.

2.11 Correlation Analysis Between Risk Score and Immune Cell Infiltration

The relative abundance of immune cells was estimated using CIBERSORT based on the normalized gene expression matrix. Spearman correlation analysis was used to assess associations between the risk score and the infiltration levels of different immune cell types. Correlation coefficients and corresponding P values were calculated for each immune cell population. The results were visualized using correlation heatmaps generated by the "ggplot2" and "pheatmap" R packages. A two-sided P value < 0.05 was considered statistically significant.

2.12 Tumor Mutational Burden and Immunotherapy-Related Molecular Indicator Analysis

Somatic mutation data from TCGA were used to calculate tumor mutational burden (TMB). Immunotherapy-related molecular indicators, including microsatellite instability (MSI) status, immune checkpoint gene expression, and RNAss, were analyzed when corresponding data were available. Differences in TMB, MSI distribution, immune checkpoint expression, and RNAss between the high- and low-risk groups were further evaluated. [44].

2.13 Drug Sensitivity Analysis

Drug sensitivity was predicted using the "pRRophetic" R package [45] based on the Genomics of Drug Sensitivity in Cancer (GDSC) database [46]. The half-maximal inhibitory concentration (IC50) value of each candidate drug was estimated for each sample and compared between the two risk groups. Correlations between risk score and predicted IC50 values were also evaluated.

2.14 Single-Cell RNA Sequencing Analysis

Two single-cell RNA sequencing (scRNA-seq) datasets, GSE184198 and GSE163558, were obtained from the Gene Expression Omnibus (GEO) database [32]. These datasets included gastric cancer tissues and adjacent normal tissues. Data preprocessing and analysis were performed using the Seurat R package [47]. Quality control was conducted to remove low-quality cells. Cells expressing fewer than 200 genes and genes detected in fewer than three cells were excluded. In addition, cells with mitochondrial gene expression greater than 5% were filtered out to minimize the influence of damaged or apoptotic cells.

After quality control, gene expression data were normalized using the standard Seurat workflow. Highly variable genes were identified, and the top 2,000 variable genes were selected for downstream

analysis. Principal component analysis (PCA) was performed for dimensionality reduction. Based on significant principal components, cell clustering was conducted using the FindClusters function in Seurat. Clustering results were visualized using Uniform Manifold Approximation and Projection (UMAP). Cell type annotation was performed according to canonical marker genes with reference to the CellMarker 2.0 database [48].

To further investigate the potential role of key genes in gastric cancer metabolism, copper–lipid metabolic scores were calculated using the AddModuleScore function in Seurat based on the selected copper- and lipid-metabolism-related gene set. Cells with detectable normalized GAD1 expression were defined as GAD1-positive (GAD1+) cells, whereas the remaining cells were defined as GAD1-negative (GAD1–) cells. Metabolic characteristics and pathway activities were then compared between GAD1+ and GAD1– cells. Pathway activity scores, including epithelial–mesenchymal transition (EMT), hypoxia, and fatty acid metabolism, were calculated using predefined hallmark gene sets. These analyses were performed to explore the functional relevance of GAD1-associated copper- and lipid-metabolic features in gastric cancer.

2.15 Cell Culture and Lentiviral RNAi-Mediated GAD1 Knockdown

The human gastric epithelial cell line GES-1 and gastric cancer cell lines MKN7, HGC27, and AGS were used for in vitro functional validation. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and maintained at 37°C in a humidified incubator containing 5% CO₂. AGS and HGC27 cells were infected with GAD1-targeting lentiviral RNAi vectors designated 7, 8, and 9 to evaluate knockdown efficiency. Lentiviral infection was performed in the presence of infection reagent according to the manufacturer's instructions. At 48–72 h after infection, cells were harvested, and GAD1 knockdown efficiency was assessed by Western blotting.

2.16 Western Blot Analysis

To extract total cellular protein, cells in good condition were collected and lysed on ice for 1 h using a lysis buffer suitable for Western blotting and immunoprecipitation. Protein concentrations were determined using a BCA protein assay kit, and the concentration of each sample was adjusted to 3000 mg/mL by adding loading buffer. Proteins were denatured by boiling at 100°C for 10 min. Electrophoresis was initially performed at 80 V and then switched to 120 V after the dye front reached the separating gel. After electrophoresis, proteins were transferred onto PVDF membranes at a constant current of 300 mA for 120 min. The PVDF membranes were blocked at room temperature for 2 h and then incubated overnight at 4°C with anti-GAD1 and anti-GAPDH antibodies diluted in 5% non-fat milk at 1:1000. The next day, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody at room temperature for 2 h. Protein bands were then visualized using chemiluminescent detection.

2.17 Cell Viability Assay

AGS and HGC27 cells were seeded into 24-well plates at densities of 3.5×10^4 cells per well and 3.3×10^4 cells per well, respectively. Cell viability was measured on days 1, 3, and 5 after seeding. At each time point, MTT solution was added to each well at a final concentration of 0.5 mg/mL, followed by incubation for 24 h. The supernatant was then discarded, and dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance was measured at 490 nm using a spectrophotometer (BioTek, USA).

2.18 Colony Formation Assay

AGS and HGC27 cells infected with the GAD1-targeting lentiviral RNAi vector or control vector were seeded into six-well plates at a density of 1×10^3 cells per well and cultured for 10–14 days. The culture medium was replaced every 2–3 days. After visible colonies had formed, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies were then photographed and counted.

2.19 Wound-Healing Assay

Cell migration ability was evaluated using a wound-healing assay. AGS and HGC27 cells were seeded into 24-well plates and cultured until they reached complete confluence. A linear wound was generated in the cell monolayer using a pipette tip. The wound width was measured under a microscope at 0 h and 48 h after scratching to quantify the migratory ability of the cells.

2.20 Statistical Analysis

All analyses were conducted using R software (version 4.3.2). Continuous variables were compared using the Wilcoxon rank-sum test. Survival analysis was performed using Kaplan-Meier curves and log-rank tests. Unless otherwise specified, all statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

3 Results

3.1 Screening of Differentially Expressed Genes Associated with Copper and Lipid Metabolism

The overall workflow of this study is shown in Figure 1. Based on transcriptomic data from TCGA, differentially expressed genes between gastric cancer tissues and normal tissues were identified (Figure 2A). These genes were subsequently intersected with copper and lipid metabolism-related genes curated from the GeneCards database, yielding 217 candidate genes potentially involved in copper-lipid metabolic regulation in gastric cancer (Figure 2B).

Functional enrichment analyses, including KEGG pathway analysis and GO biological process analysis, revealed that these candidate genes were significantly enriched in several biological processes, including "response to xenobiotic stimulus," "steroid metabolic process," "organic hydroxy compound transport," and "lipid transport" (Figures 2C, D). These findings suggest that lipid metabolic remodeling, together with alterations in xenobiotic-response and transport-related

pathways, may be involved in the metabolic reprogramming of gastric cancer. Consistent with previous studies linking lipid metabolism to gastric cancer progression, tumor aggressiveness, and tumor microenvironmental regulation, these results further support the potential biological relevance of copper-lipid metabolism-related genes in gastric cancer ^[17,24,27].

3.2 Identification of Six Metabolic Subtypes by Unsupervised Clustering

Consensus clustering based on differentially expressed genes associated with copper and lipid metabolism classified 348 TCGA-STAD samples into six distinct metabolic subtypes (Figure 3A). This classification was further supported by t-SNE analysis, which showed clear separation among the six clusters (Figure 3B). Survival analysis was then performed among patients with available OS information and revealed significant differences in OS among these subtypes. Cluster 3 exhibited the longest OS, indicating the most favorable prognosis, whereas Cluster 4 was associated with the poorest survival outcome (Figure 3C).

Evaluation of the tumor microenvironment revealed marked heterogeneity across the six subtypes (Figures 3D-G). Cluster 4 showed the highest stromal score, immune score, and ESTIMATE score, together with the lowest tumor purity, suggesting a tumor microenvironment enriched in stromal and immune cell infiltration. Immune cell profiling further showed that, among the evaluated cell populations, NK cells had relatively low abundance, whereas fibroblasts represented the predominant stromal component. Notably, Cluster 4 displayed the highest level of fibroblast infiltration (Figure 3J). These findings suggest that Cluster 4 may represent a cancer-associated fibroblast (CAF)-dominant, stroma-driven tumor microenvironment with potential immunosuppressive and immune-exclusion features ^[14,49–52]. In contrast, immune checkpoint analysis showed that most immune checkpoint molecules were significantly upregulated in Cluster 6 (Figure 3H).

Functional enrichment analysis further revealed distinct molecular characteristics among the six subtypes (Figure 3I). Clusters associated with favorable prognosis, particularly Cluster 1 and Cluster 3, were enriched in lipid and xenobiotic metabolism-related pathways, including linoleic acid metabolism, glycerolipid metabolism, retinol metabolism, PPAR signaling, cytochrome P450-mediated drug metabolism, xenobiotic metabolism, antioxidant-related pathways, and glucuronate interconversions. These results suggest that favorable-prognosis subtypes may have enhanced metabolic homeostasis and detoxification-related activity ^[12,15].

By contrast, Cluster 4 showed marked enrichment of stromal and signaling-related pathways, including calcium signaling, vascular smooth muscle contraction, MAPK signaling, neuroactive ligand-receptor interaction, long-term potentiation and depression, and melanogenesis. Meanwhile, pathways related to proliferation and genome maintenance, such as the proteasome, p53 signaling, aminoacyl-tRNA biosynthesis, one-carbon pool by folate, pyrimidine metabolism, spliceosome, RNA degradation, cell cycle, DNA replication, and DNA repair pathways, were broadly suppressed.

Collectively, these results suggest that Cluster 4 may represent a stroma-dominant, microenvironment-driven, and potentially invasive subtype rather than a proliferation-driven phenotype, which is consistent with its unfavorable clinical prognosis.

3.3 Establishment and Validation of a CLRG-Based Prognostic Signature

Univariate Cox regression analysis identified 29 genes that were significantly associated with OS in the TCGA cohort (Figure 4A). Supplementary Figure S1A shows the expression patterns of these genes in tumor and normal tissues, indicating that most OS-related genes were markedly dysregulated in GC. In addition, the interaction network shown in Supplementary Figure S1B revealed predominantly positive correlations among these genes, suggesting their potential coordinated dysregulation in GC. Copy number variation (CNV) profiling further demonstrated frequent copy number alterations in these genes, with PON1 showing the most pronounced copy number gain and RBP4 exhibiting the highest frequency of copy number loss. The genomic distribution of CNV events is shown in Figure 4C.

To further refine the prognostic candidates, LASSO regression analysis was performed, and 15 key genes were identified for the CLRG-based prognostic signature (Figures 4D, E). A risk score model was subsequently constructed based on the regression coefficients of these genes as follows:

$$\text{Risk score} = (0.086 \times \text{MTTP}) + (0.040 \times \text{IGFBP1}) + (-0.004 \times \text{ALDH3A2}) + (-0.102 \times \text{SLC5A1}) + (-0.316 \times \text{GAD1}) + (0.812 \times \text{CYP19A1}) + (0.188 \times \text{SERPINE1}) + (0.115 \times \text{F5}) + (0.068 \times \text{ELANE}) + (0.038 \times \text{PON1}) + (0.065 \times \text{APOH}) + (0.025 \times \text{RETN}) + (0.016 \times \text{PYY}) + (0.339 \times \text{ALAS2}) + (0.410 \times \text{EGF}).$$

Patients in the TCGA cohort were stratified into high- and low-risk groups according to the median risk score. Kaplan-Meier survival analysis showed that patients in the high-risk group had significantly poorer OS than those in the low-risk group (Figure 4F). The distribution of risk scores, survival status, and gene expression patterns is presented in Figure 4G, H. Time-dependent ROC analysis further confirmed the predictive performance of the model, with AUC values of 0.725, 0.723, and 0.731 for 1-, 2-, and 3-year OS, respectively (Figure 4I).

Patients in the GSE66229 cohort were stratified using the median risk score derived from the TCGA training cohort. Consistent with the results from the training cohort, patients in the high-risk group had significantly shorter OS than those in the low-risk group ($P < 0.05$; Figure 4J). The distribution of risk scores, survival outcomes, and expression profiles in the validation cohort is shown in Figure 4K, L. Time-dependent ROC curves for the validation cohort are shown in Figure 4M. Collectively, these findings suggest that the CLRG-based signature may serve as a reliable prognostic tool for GC and may contribute to outcome risk stratification.

3.4 Evaluation and Validation of the CLRG-Based Risk Signature

Given the predictive performance of the CLRG-based signature, stratified survival analyses were conducted in the TCGA cohort according to key clinicopathological characteristics, and Kaplan-Meier curves were generated for each subgroup. As shown in Figure 5A-F, patients in the low-risk group consistently showed better OS across subgroups stratified by age (>65 vs. ≤65 years), sex (female vs. male), clinical stage (I-II vs. III-IV), T category (T1-T2 vs. T3-T4), nodal status (N0 vs. N1-N3), and metastatic status (M0 vs. M1). However, the discriminatory capacity of the signature was attenuated in the T1-T2 subgroup, possibly owing to the generally favorable prognosis and limited number of survival events among early-stage patients. Similarly, risk stratification was less pronounced in the M1 subgroup, which may be attributable to the uniformly poor prognosis of metastatic disease and the relatively small sample size.

The associations between the risk score and clinicopathological variables were further examined. As shown in Figure 5G-M, patients with advanced clinical stage, higher N category, or death status (fustat = 1) showed significantly higher risk scores ($P < 0.05$). By contrast, no statistically significant differences in risk score were observed across the remaining clinicopathological subgroups.

3.5 Development and Evaluation of a Prognostic Nomogram

A prognostic nomogram integrating the CLRG-derived risk score and clinicopathological variables was developed to estimate the 1-, 3-, and 5-year survival probabilities of patients with GC (Figure 6A, D). In this nomogram, higher total scores corresponded to lower predicted survival probabilities. Calibration curves demonstrated good agreement between the predicted survival probabilities and the observed outcomes (Figure 6B, E). In addition, the nomogram achieved AUC values of 0.712 in the training cohort and 0.766 in the validation cohort, outperforming conventional clinical indicators. These results suggest that the nomogram has robust predictive performance and favorable generalizability (Figure 6C, F).

3.6 Functional Enrichment Analysis of the High- and Low-Risk Groups

A KEGG pathway enrichment heatmap was generated to illustrate the biological differences between the high- and low-risk groups (Figure 7A). The high-risk group showed marked enrichment in pathways associated with extracellular matrix remodeling, intercellular signaling, vascular regulation, and inflammatory responses, including the renin-angiotensin system, TGF-beta signaling pathway, calcium signaling pathway, cell adhesion molecules (CAMs), ECM-receptor interaction, regulation of the actin cytoskeleton, and multiple glycosaminoglycan and glycosphingolipid biosynthesis-related pathways. Activation of these pathways suggests that high-risk tumors may have

enhanced invasive and metastatic potential, accompanied by active stromal remodeling and pro-inflammatory signaling [14,53–56].

By contrast, the low-risk group was predominantly enriched in pathways related to genetic information processing, protein synthesis, and cellular energy metabolism, including ribosome, DNA replication, mismatch repair, homologous recombination, proteasome, and tricarboxylic acid (TCA) cycle pathways. These findings suggest that tumors in the low-risk group may retain more active genome-maintenance capacity and metabolic homeostasis, which may be consistent with less aggressive biological behavior [10,11].

To further characterize the biological differences between the two risk groups, Gene Set Enrichment Analysis (GSEA) was performed using the KEGG pathway database (Figure 7B, C). Consistent with the enrichment heatmap, the high-risk group was significantly enriched in pathways related to cell adhesion, extracellular matrix organization, and intercellular signal transduction, including the calcium signaling pathway, CAMs, ECM-receptor interaction, focal adhesion, and neuroactive ligand-receptor interaction. These pathways are closely associated with tumor invasion, metastasis, and cellular communication within the tumor microenvironment, suggesting that high-risk tumors may exhibit enhanced migratory and invasive properties [54–56].

In contrast, the low-risk group showed significant enrichment in pathways involved in genetic information processing and protein degradation, such as base excision repair, DNA replication, nucleotide excision repair, proteasome, and RNA degradation. These pathways reflect active DNA repair and protein turnover processes, suggesting that tumor cells in the low-risk group may maintain relatively greater genomic stability and metabolic efficiency, corresponding to a less aggressive tumor phenotype.

3.7 Analysis of Immune Function and Immune Microenvironment

Correlation analysis between the risk score and immune cell infiltration revealed that the risk score was significantly associated with multiple immune and stromal cell populations, including monocytic lineage cells, neutrophils, endothelial cells, and fibroblasts (Figure 7D). Comparisons of immune-related functional scores between the high- and low-risk groups showed that patients in the high-risk group exhibited significantly enhanced activity in several immune functions, including chemokine receptor activity (CCR), parainflammation, and type II IFN response (Figure 7E). These findings suggest that the CLRG-based risk score may be closely associated with immune and stromal remodeling in the tumor microenvironment.

Further immune infiltration analysis showed that the high-risk group had higher proportions of monocytes, M0 macrophages, and resting dendritic cells, whereas the low-risk group exhibited increased infiltration of follicular helper T cells, activated NK cells, and M1 macrophages (Figure 7F). Given that activated NK cells and M1 macrophages are generally implicated in antitumor immune

responses, this immune cell distribution may partially explain the relatively favorable prognosis observed in the low-risk group ^[16,26].

Further evaluation of the tumor immune microenvironment demonstrated significant differences between the two risk groups (Figures 7G-J). Compared with the low-risk group, the high-risk group showed significantly higher stromal score and ESTIMATE score ($P < 0.01$), whereas tumor purity was significantly lower ($P < 0.001$). In addition, the immune score was moderately but significantly elevated in the high-risk group ($P = 0.036$). These results indicate that high-risk tumors may contain greater stromal and immune cell infiltration, accompanied by reduced tumor purity, suggesting a more complex and remodeled tumor microenvironment. This pattern is consistent with previous evidence that stromal activation, immune infiltration, and extracellular matrix remodeling are closely associated with gastric cancer progression and therapeutic resistance.

Moreover, correlation analysis between individual genes in the prognostic model and immune cell populations showed that most model genes were significantly associated with various immune cell types (Figure 7K). Differential immune checkpoint expression and prognostic comparisons between the high- and low-risk groups further indicated that patients in the high-risk group had poorer survival outcomes and an immune-altered phenotype (Figures 7L, M). Together, these findings suggest that the high-risk group is characterized by immune and stromal remodeling and may warrant further evaluation in the context of immunotherapy response.

3.8 Mutation Landscape, Tumor Mutational Burden, and Immunotherapy-Related Molecular Indicators

Somatic mutation analysis was performed using TCGA mutation data to characterize the mutation landscape associated with the prognostic signature (Figure 8A). The low-risk group exhibited higher TMB scores than the high-risk group (Figure 8B), and patients with elevated TMB had prolonged OS (Figure 8C). Combined stratification based on risk score and TMB further showed that patients in the low-risk/high-TMB subgroup had the most favorable prognosis among the four subgroups (Figure 8D).

Immunotherapy-related indicators were then assessed. The distribution of microsatellite instability (MSI) status differed between the two risk groups, and MSI-high tumors tended to have lower risk scores (Figure 8E, F). To further characterize the mutational landscape, waterfall plots were generated separately for the high- and low-risk groups (Figure 8G, H). In the high-risk group, frequent mutations were observed in genes such as TTN, TP53, MUC16, ARID1A, LRP1B, SYNE1, FLG, FAT4, and CSMD3 (Figure 8G). The low-risk group also displayed a broad mutation spectrum, but with a distinct distribution of mutation frequencies and altered genes (Figure 8H). In addition, the risk score was negatively correlated with RNAss (Figure 8I), suggesting a potential association between the prognostic signature and stemness-related features in gastric cancer.

Overall, these findings indicate that the low-risk group may exhibit molecular features commonly associated with favorable immunotherapy-related biomarkers, whereas the high-risk group may represent a more stromal- and immune-remodeled phenotype.

3.9 Drug Sensitivity Analysis

Drug sensitivity analysis was performed using 198 antitumor agents from the GDSC database. Predicted IC50 values were calculated for each sample and compared between the high- and low-risk groups. The predicted IC50 values of several agents, including 5-fluorouracil, afatinib, osimertinib, and paclitaxel, were higher in the high-risk group or positively correlated with the risk score, suggesting that patients with lower risk scores may be more sensitive to these agents in silico (Figure 9A-D, I-L).

Conversely, the predicted IC50 values of cediranib, staurosporine, dasatinib, and dactolisib were lower in the high-risk group or negatively correlated with the risk score, suggesting that patients with higher risk scores may have greater predicted sensitivity to these agents (Figure 9E-H, M-P). These findings indicate that the CLRG-based signature may have potential value in guiding individualized drug sensitivity prediction, although experimental and clinical validation is required.

3.10 Single-Cell Transcriptomic Analysis Reveals Metabolic Characteristics of GAD1-Positive Cells in Gastric Cancer

To investigate the cellular distribution of copper- and lipid metabolism-related genes within the gastric cancer tumor microenvironment, scRNA-seq datasets from GSE184198 and GSE163558 were integrated, yielding 48,997 cells for downstream analyses. After data integration and quality control, UMAP dimensionality reduction revealed multiple distinct cell clusters (Figure 10A). Based on highly variable genes (Figure 10B) and canonical marker genes, these clusters were annotated into major cell types, including epithelial cells, endothelial cells, fibroblasts, macrophages, monocytes, and T cells (Figure 10C). The top 15 marker genes for each cluster were visualized using a heatmap, further supporting the reliability of cell-type annotation (Figure 10D).

We next examined the expression pattern of GAD1, a gene included in the prognostic model, at the single-cell level. GAD1 expression was generally low and detected in only a small subset of cells (Supplementary Figure S2B). Cells with detectable GAD1 expression were therefore defined as GAD1-positive (GAD1+) cells for subsequent analyses. Compared with GAD1-negative (GAD1-) cells, GAD1+ cells exhibited significantly higher Cu-Lipid scores (Figure 10E), and UMAP visualization further showed that cells with high Cu-Lipid scores were enriched in specific cell populations (Figure 10F).

Functional pathway analysis demonstrated that GAD1+ cells displayed increased activity in epithelial-mesenchymal transition (EMT), hypoxia, and fatty acid metabolism pathways (Figure 10G).

These pathways are closely linked to tumor invasiveness and metabolic plasticity [25,26,57,58]. Kaplan-Meier survival analysis based on the TCGA cohort further revealed that patients with high Cu-Lipid scores had significantly worse OS than those with low scores ($P = 0.0063$; Figure 10H), suggesting that copper- and lipid metabolism-related features may play important roles in gastric cancer progression.

3.11 Functional Validation of GAD1 in Gastric Cancer

Although GAD1 has been increasingly implicated in tumor-associated metabolic regulation, its biological function in gastric cancer remains incompletely understood. To further validate the role of GAD1 in gastric cancer, *in vitro* cell models were established for functional assays. Western blot analysis showed that GAD1 expression was markedly higher in gastric cancer cell lines than in the human gastric epithelial cell line GES-1, with relatively high expression observed in HGC27 and AGS cells (Figure 11A). Therefore, HGC27 and AGS cells were selected for subsequent GAD1 knockdown and functional validation experiments.

To silence GAD1 expression, lentiviral RNAi-mediated knockdown was performed in AGS and HGC27 cells using three independent GAD1-targeting RNAi lentiviral vectors, designated 7, 8, and 9. Western blot analysis was used to evaluate knockdown efficiency. The results showed that all three lentiviral RNAi vectors reduced GAD1 protein expression to varying degrees, with vector 9 showing the most pronounced knockdown efficiency. Therefore, vector 9 was selected for subsequent functional assays (Figure 11B). In the subsequent experiments, the GAD1-silencing group was labeled as si-GAD1, and the control group was labeled as Vector. Functional assays demonstrated that GAD1 knockdown significantly suppressed the malignant phenotypes of gastric cancer cells. MTT assays showed that, compared with the Vector group, the si-GAD1 group exhibited reduced cell viability in both AGS and HGC27 cells in a time-dependent manner (Figure 11C, D). Colony formation assays further revealed that the clonogenic capacity of gastric cancer cells was markedly impaired in the si-GAD1 group (Figure 11E). In addition, wound-healing assays showed that GAD1 silencing significantly inhibited the migratory ability of AGS and HGC27 cells (Figure 11F). Collectively, these findings indicate that GAD1 is highly expressed in gastric cancer cells, and that lentiviral RNAi-mediated GAD1 knockdown significantly suppresses gastric cancer cell proliferation, colony formation, and migration, suggesting that GAD1 may contribute to the malignant progression of gastric cancer.

4 Discussion

Metabolic alterations are increasingly recognized as important determinants of biological heterogeneity in gastric cancer, particularly through their effects on stromal remodeling, immune regulation, and therapeutic responsiveness. In the present study, we systematically characterized

copper- and lipid-metabolism-related genes in gastric cancer and established a prognostic model that was closely associated with tumor microenvironment features, immune heterogeneity, and predicted therapeutic response.

Previous studies have demonstrated molecular interactions between copper metabolism and lipid metabolism in tumors. Intracellular accumulation of copper ions can promote the generation of reactive oxygen species (ROS) through redox reactions, thereby inducing lipid peroxidation and triggering oxidative stress-related autophagy. This process may also activate the Nrf2/PPARgamma signaling pathway, thereby regulating lipid synthesis and lipid metabolic reprogramming ^[59]. In addition, copper can promote lipid droplet degradation by regulating lipid droplet autophagy, also known as lipophagy, and enhance polyunsaturated fatty acid (PUFA)-mediated cytotoxicity, thereby influencing the metabolic state and growth of tumor cells ^[60].

At the molecular mechanistic level, copper ions can be reduced by ferredoxin 1 (FDX1) and subsequently bind directly to lipoylated proteins in the mitochondrial TCA cycle, such as DLAT and DLD. This interaction induces aggregation of lipoylated proteins and loss of iron-sulfur cluster proteins, thereby causing proteotoxic stress and mitochondrial dysfunction and ultimately triggering a copper-dependent form of cell death known as cuproptosis ^[20,61]. This process suggests that copper can directly act on lipid-related post-translational modifications, such as protein lipoylation, and mitochondrial metabolic pathways, thereby establishing a critical link between energy metabolism and lipid metabolism.

Lipid metabolic status may also reciprocally regulate copper-mediated cellular effects. Previous studies have shown that lipid metabolic reprogramming, including alterations in fatty acid synthesis and oxidation, can modulate cellular sensitivity to copper-induced toxicity and cuproptosis by affecting mitochondrial redox status and energy metabolism, thereby playing an important role in tumor progression and therapeutic response ^[62].

Taken together, copper metabolism and lipid metabolism form a complex bidirectional regulatory network through multiple mechanisms, including oxidative stress, lipid droplet homeostasis, mitochondrial function, and protein lipoylation. These findings suggest that integrative analysis of pathways related to both metabolic processes may enable a more comprehensive characterization of tumor metabolic reprogramming and provide new perspectives for prognostic assessment and targeted therapy.

One of the major findings of this study is that gastric cancer patients could be classified into distinct copper-lipid metabolic subtypes with markedly different clinical outcomes and microenvironmental characteristics. In particular, the subtype with the poorest prognosis showed prominent stromal activation, elevated immune and stromal scores, reduced tumor purity, and fibroblast enrichment. This pattern is consistent with accumulating evidence that CAFs are key orchestrators of gastric cancer progression by promoting extracellular matrix remodeling, metastasis, stemness, treatment resistance,

and attenuation of antitumor immunity [49,50]. Recent studies further indicate that fibroblast populations in gastric cancer are highly heterogeneous and that specific fibroblast subsets are associated with distinct biological programs, prognosis, and immunotherapy-related phenotypes [51]. In gastric cancer peritoneal metastases, CAF-macrophage crosstalk has also been shown to shape the immune landscape and govern response to immune checkpoint blockade, further supporting the view that stromal activation is not merely a bystander event but a major determinant of disease aggressiveness and therapeutic vulnerability [52].

Our pathway analyses showed that the high-risk group was enriched in extracellular matrix organization, focal adhesion, cell adhesion, and related stromal signaling programs. These findings are biologically plausible because recent evidence suggests that the extracellular matrix itself can directly participate in immune escape in gastric cancer. For example, collagen-rich extracellular matrix has been reported to promote immune evasion through activation of IL4I1-AHR signaling, while ECM-remodeling regulators such as PLOD1 may facilitate metastasis and immune escape by reshaping the stromal niche [63,64]. Therefore, enrichment of ECM-related pathways in the high-risk group may reflect a microenvironment that supports tumor invasion, metastatic dissemination, and immune exclusion. In contrast, the low-risk group was enriched in pathways related to DNA repair and metabolic homeostasis, suggesting a comparatively more stable biological state.

Another notable observation is the close association between the prognostic signature and immune contexture. High-risk tumors displayed increased infiltration of monocytes, macrophages, fibroblasts, and endothelial cells, together with higher stromal scores and lower tumor purity, indicating a stromal-enriched and potentially immunosuppressive ecosystem. This interpretation is in line with recent work showing that the gastric cancer microenvironment is highly heterogeneous and frequently characterized by immunosuppressive cell populations and tumor-promoting stromal interactions [50,52]. By contrast, the low-risk group showed relatively higher proportions of activated NK cells and M1 macrophages, which are generally linked to more effective antitumor immune activity. Taken together, these results suggest that copper-lipid metabolic patterns may be tightly connected to immune heterogeneity and stromal-immune coupling in gastric cancer.

The single-cell analysis in this study further highlighted the potential importance of GAD1-positive cells as a metabolically distinct population. GAD1-positive cells exhibited higher copper-lipid metabolic activity and enrichment of EMT, hypoxia, and fatty acid metabolism programs. These observations are consistent with recent gastric cancer studies showing that microenvironmental hypoxia can drive fatty acid uptake and metastatic behavior. In particular, hypoxia-induced CD36 expression has been shown to promote peritoneal metastasis through enhanced fatty acid uptake in gastric cancer cells [25]. In addition, recent reviews have emphasized that cuproptosis-related biology may have particular relevance in gastric cancer because copper-dependent cell death, mitochondrial metabolism, and metabolic rewiring are closely interconnected in this disease [65]. Therefore, our

findings raise the possibility that GAD1-positive cells represent a metabolically adaptive subpopulation with enhanced invasive and stress-tolerant potential. Consistent with this possibility, our in vitro functional assays further showed that GAD1 was highly expressed in gastric cancer cell lines, and lentiviral RNAi-mediated GAD1 knockdown significantly suppressed cell viability, colony formation, and migration in AGS and HGC27 cells, as demonstrated by Western blot, MTT, colony formation, and wound-healing assays.

From a clinical perspective, the prognostic model may also have potential relevance for therapeutic stratification. The low-risk group showed higher TMB, favorable MSI-related features, and distinct immune-related characteristics, suggesting that this subgroup may harbor molecular features commonly associated with responsiveness to immune checkpoint blockade. This interpretation is consistent with gastric cancer studies showing that biomarkers such as PD-L1 expression, MSI status, Epstein–Barr virus positivity, and TMB may help inform immunotherapy-related assessment, although each marker has important limitations ^[66–71]. Conversely, the high-risk group exhibited features of stromal activation and immune remodeling, suggesting that immune cell infiltration alone may not necessarily reflect effective antitumor immunity. This is consistent with the concept that gastric cancer can display an immune-infiltrated but functionally suppressed microenvironment, particularly when stromal barriers and immunosuppressive circuits dominate.

Overall, our study suggests that copper-lipid metabolic reprogramming is not only associated with prognosis in gastric cancer but may also reflect a broader biological framework involving stromal remodeling, immune suppression, and therapeutic responsiveness. By integrating bulk transcriptomic analysis, immune landscape evaluation, drug sensitivity prediction, and single-cell transcriptomics, we provide evidence that copper-lipid metabolic features may help capture clinically meaningful heterogeneity in gastric cancer.

Several limitations should be acknowledged. First, this study was based mainly on retrospective bioinformatics analyses using public datasets, which may introduce selection bias. Second, although the model showed robust performance in an external cohort, further validation in larger multicenter and prospective cohorts is still required. Third, the mechanistic links among copper metabolism, lipid metabolism, stromal remodeling, and immune regulation remain to be experimentally clarified. Future studies combining multi-omics profiling with functional assays will be important for determining whether the pathways highlighted here are directly actionable in gastric cancer.

In summary, we established a copper-lipid metabolism-related prognostic model for gastric cancer and demonstrated its close association with tumor microenvironment features, immune heterogeneity, and predicted therapeutic response. These findings provide additional evidence that the crosstalk between copper metabolism and lipid metabolism may contribute to gastric cancer progression and may offer a useful framework for biomarker development and personalized therapeutic strategies.

Author Contributions

Xueying Sun: Software, Validation, Visualization, Writing – original draft; **Xin Li:** Visualization, Writing – original draft; **Jingwen Tian:** Methodology, Software; **Xin Wang:** Data curation, Visualization; **Yangyang Lu:** Supervision, Methodology, Writing – review & editing; **Zuohui Yan:** Conceptualization, Writing – original draft, Writing – review & editing; **Weiwei Qi:** Data curation, Formal analysis, Funding acquisition, Software.

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Conflict of interest

The authors declare no conflict of interest.

Data Availability

The datasets used and analyzed in this study were obtained from publicly available databases. The main findings and relevant materials are included in this article and its supplementary files. Further information is available from the corresponding author(s) upon reasonable request.

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Clinical trial number

Clinical trial number: not applicable.

Ethics, Consent to Participate, and Consent to Publish declarations

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable. This study used publicly available and de-identified datasets from TCGA and GEO, including TCGA-STAD, GSE66229, GSE184198, and GSE163558. No newly collected human samples, animal experiments, or clinical trial intervention was involved.

Supplementary information

Supplementary File 1 contains Supplementary Fig. S1 and Supplementary Fig. S2. Supplementary File 2 contains the full uncropped gels and blots images, together with annotated versions indicating the cropped regions used in the Western blot analyses shown in Fig. 11.

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Study Design for Copper and Lipid Metabolism in Gastric Cancer

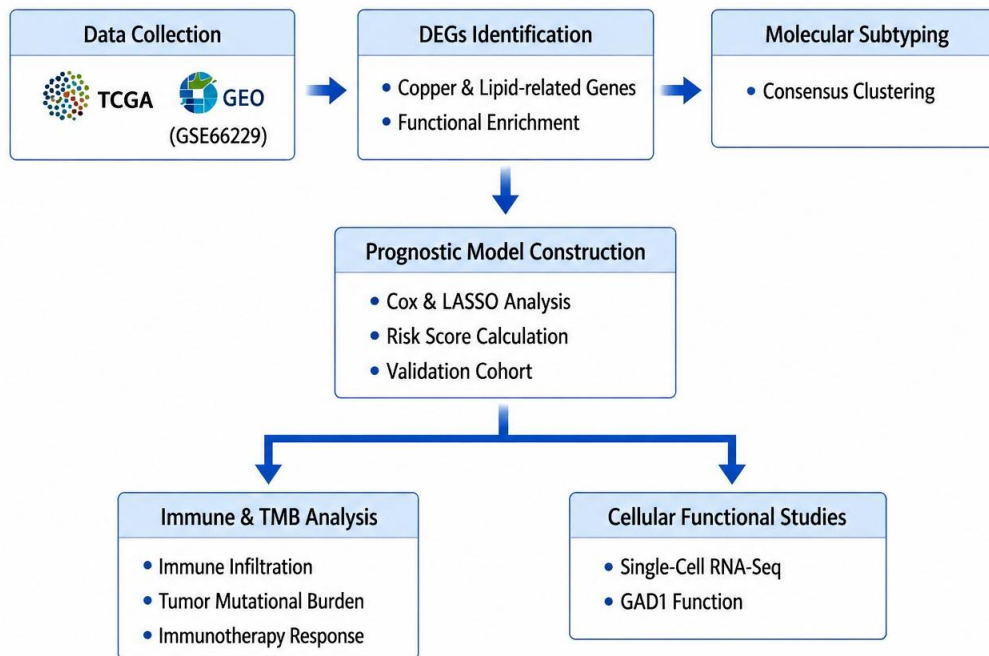


Fig. 1 Workflow of this study.

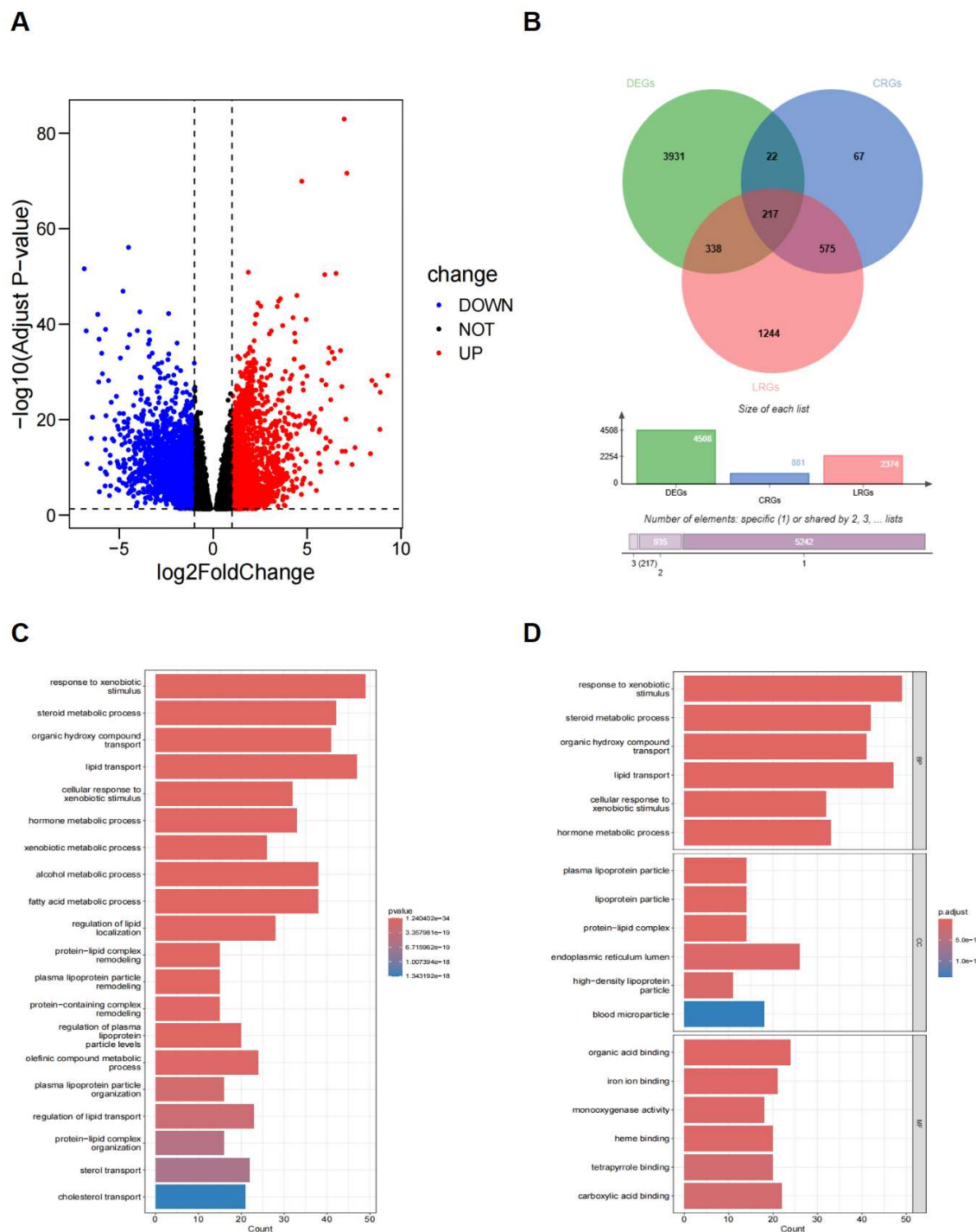


Fig. 2 Gene screening and functional enrichment analysis. (A) Volcano plot of DEGs in TCGA-STAD. (B) Venn diagram showing the intersection of differentially expressed genes, copper metabolism-related genes (CRGs), and lipid metabolism-related genes (LRGs) in gastric cancer (n = 217). (C, D) KEGG and GO enrichment analyses of the 217 differentially expressed CRGs and LRGs.

tumor purity (G) across different clusters. (H) Boxplots showing differences in immune checkpoint expression among the six clusters. (I) GSVA heatmap showing biological functions and signaling pathways across the six clusters. (J) Boxplots showing differences in immune cell infiltration among the six clusters. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

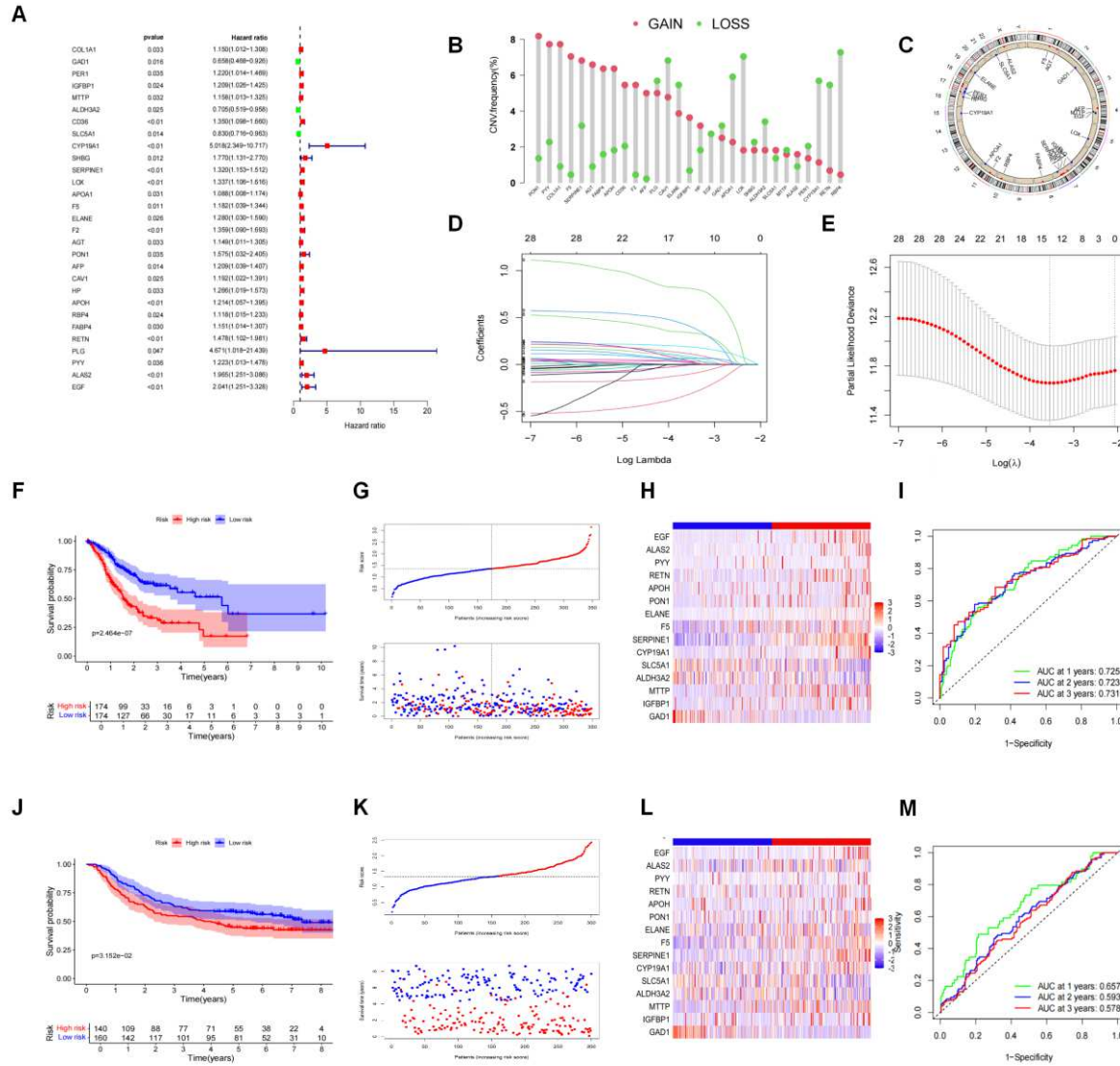


Fig. 4 Construction and validation of the CLRG-based prognostic signature. (A) Univariate Cox regression analysis identified 29 genes significantly associated with OS. (B) CNV alterations in OS-related genes. (C) Chromosomal locations of CNV alterations in OS-related genes. (D, E) LASSO regression analysis identifying 15 genes for the CLRG-based prognostic signature. (F) Kaplan-Meier curve for high- and low-risk groups in the TCGA cohort. (G) Distribution of risk score and survival status in the TCGA cohort. (H) Heatmap of the 15 signature genes in the TCGA cohort. (I) ROC curves evaluating the predictive accuracy of the signature in the TCGA cohort. (J) Kaplan-Meier

curve for high- and low-risk groups in the GEO cohort. (K) Distribution of risk score and survival status in the GEO cohort. (L) Heatmap of the 15 signature genes in the GEO cohort. (M) ROC curves evaluating the predictive accuracy of the signature in the GEO cohort.

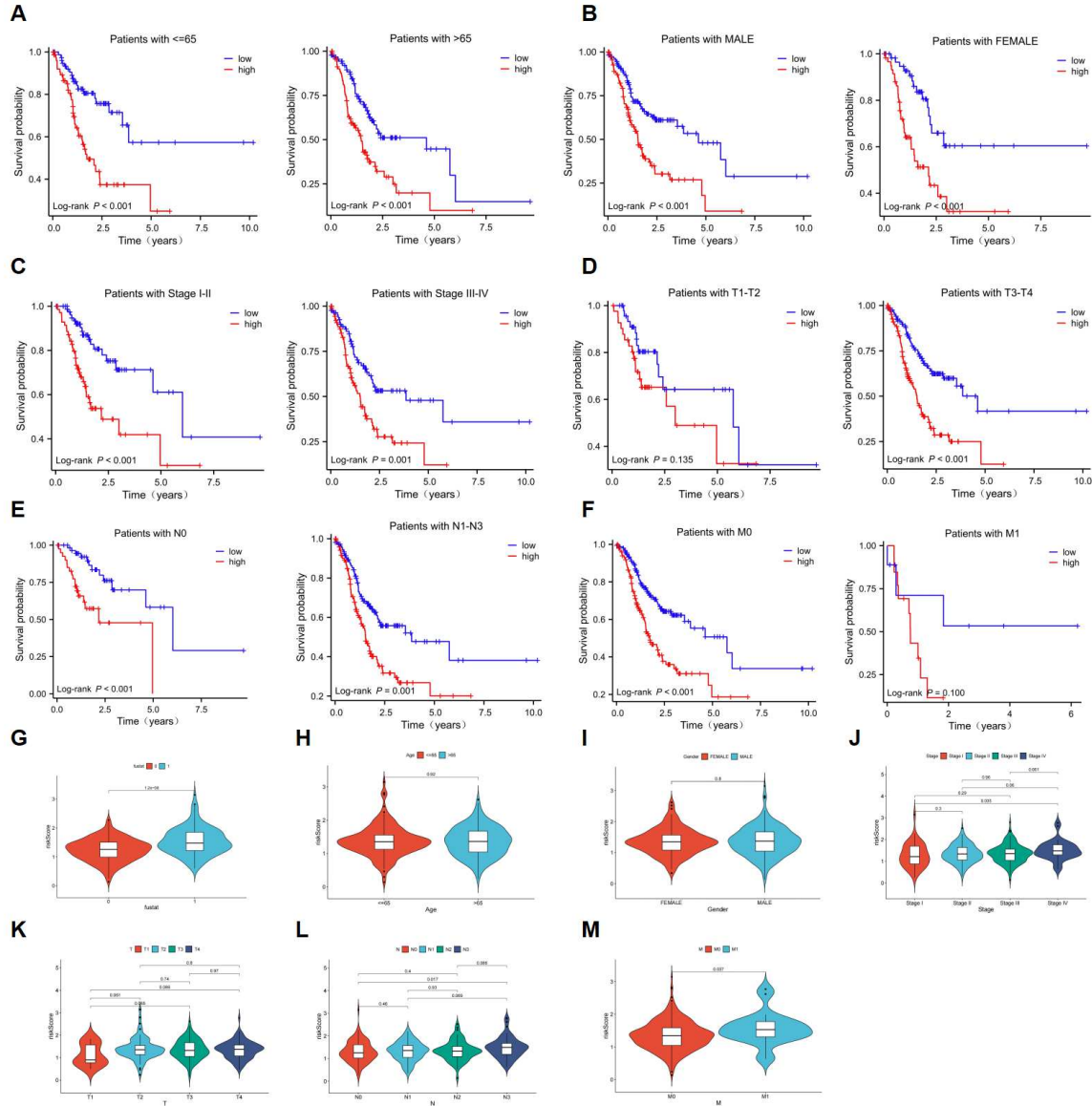


Fig. 5 Associations between the risk signature and clinicopathological parameters. (A-F) Stratified Kaplan-Meier survival analyses comparing high- and low-risk groups in the TCGA cohort according to age, sex, clinical stage, T stage, N stage, and M stage. Patients in the low-risk group generally showed longer OS than those in the high-risk group. (G-M) Systematic evaluation of risk scores across clinical parameters in GC samples, including survival status, age, sex, clinical stage, T stage, N stage, and M stage.

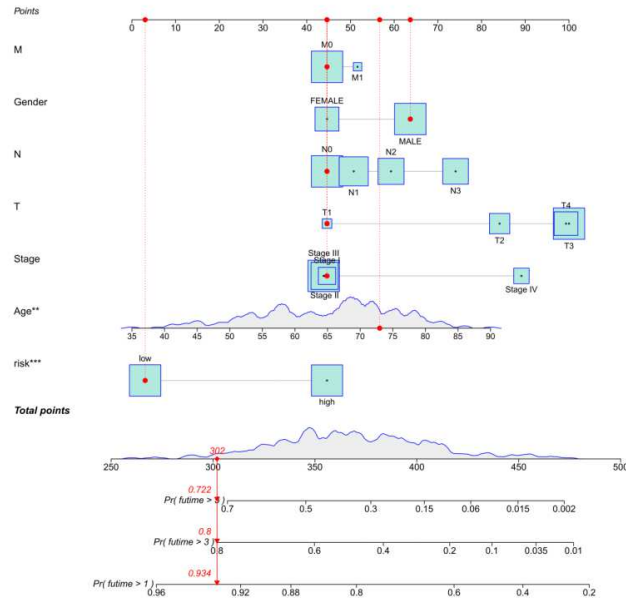
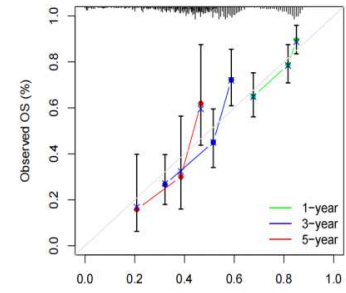
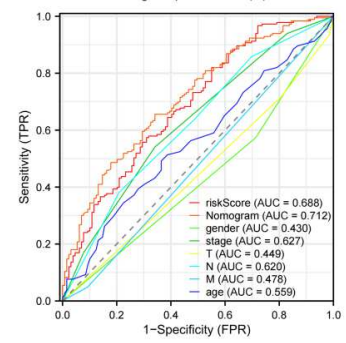
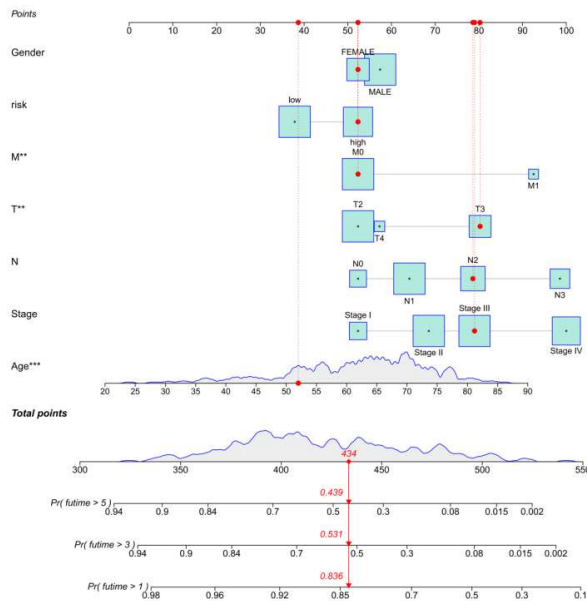
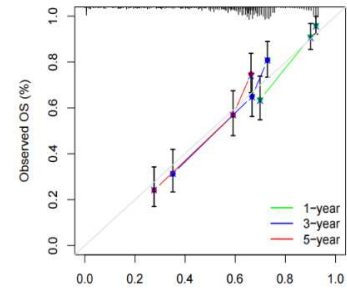
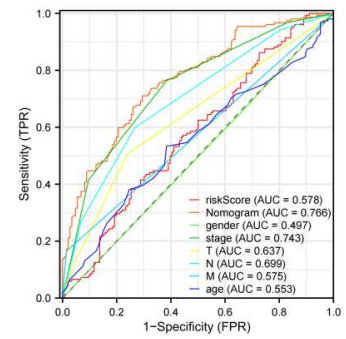
A**B****C****D****E****F**

Fig. 6 Development and evaluation of a prognostic nomogram. (A, D) Nomograms predicting the 1-, 3-, and 5-year survival probabilities of patients with GC in the TCGA cohort (A) and GEO cohort

(D). (B, E) Calibration curves for the nomograms in the TCGA cohort (B) and GEO cohort (E). (C, F) ROC curves assessing the prognostic accuracy of the nomogram and clinical features in the TCGA cohort (C) and GEO cohort (F).

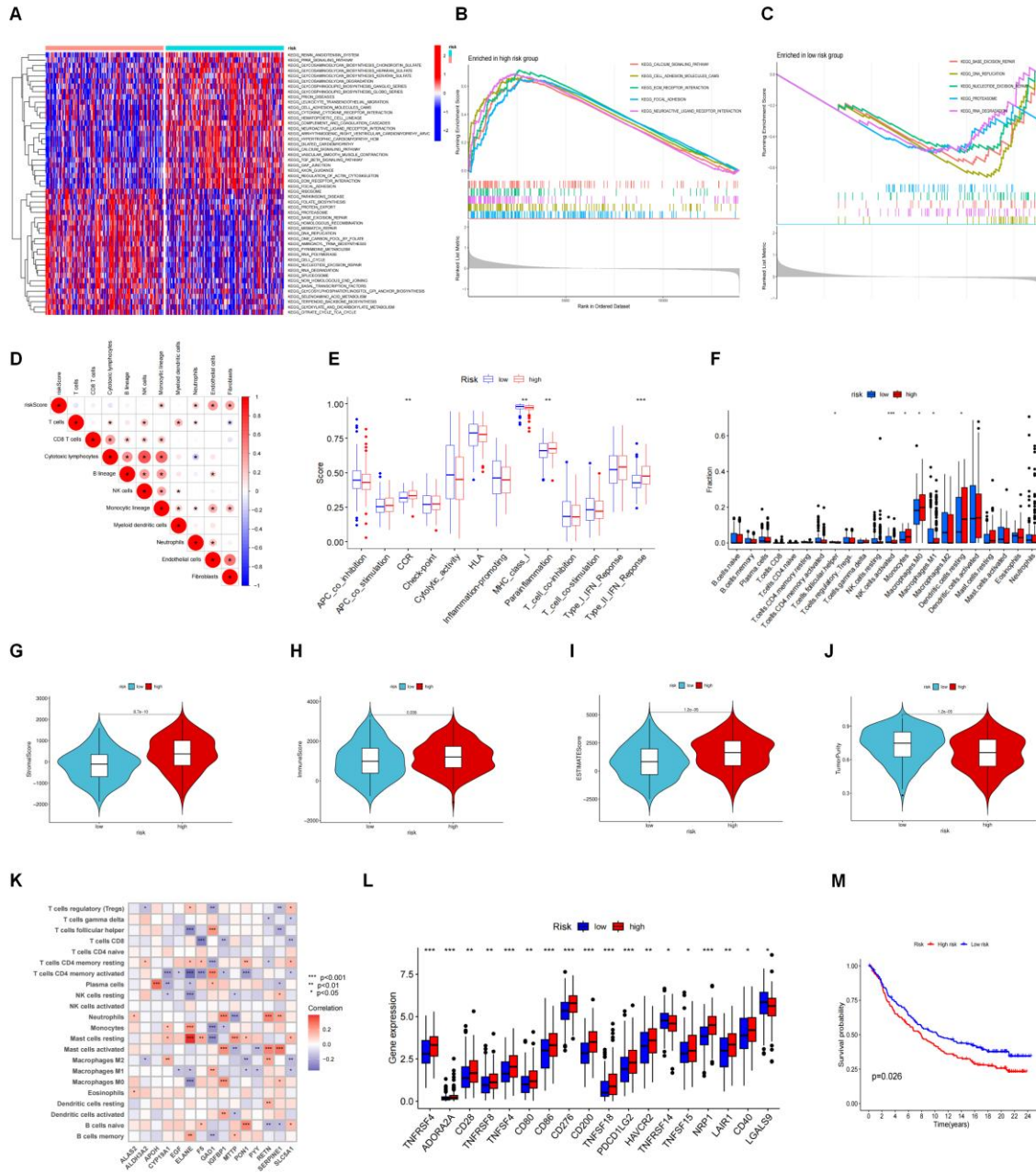


Fig. 7 Functional enrichment and immune landscape analyses. (A) KEGG pathway enrichment heatmap comparing the high- and low-risk groups. (B, C) GSEA results of KEGG pathways between the high- and low-risk groups. (D) Correlation analysis between risk score and immune cell infiltration. (E) Evaluation of immune-related functional scores between risk groups. (F) Comparison of immune cell infiltration between risk groups. (G-J) Violin plots showing stromal score (G),

immune score (H), ESTIMATE score (I), and tumor purity (J) between the high- and low-risk groups. (K) Heatmap of correlations between CLRGs and immune cells. (L) Comparison of immune checkpoint expression between risk groups. (M) Survival analysis stratified by immune checkpoint-related gene expression, showing worse prognosis in the high-risk group.

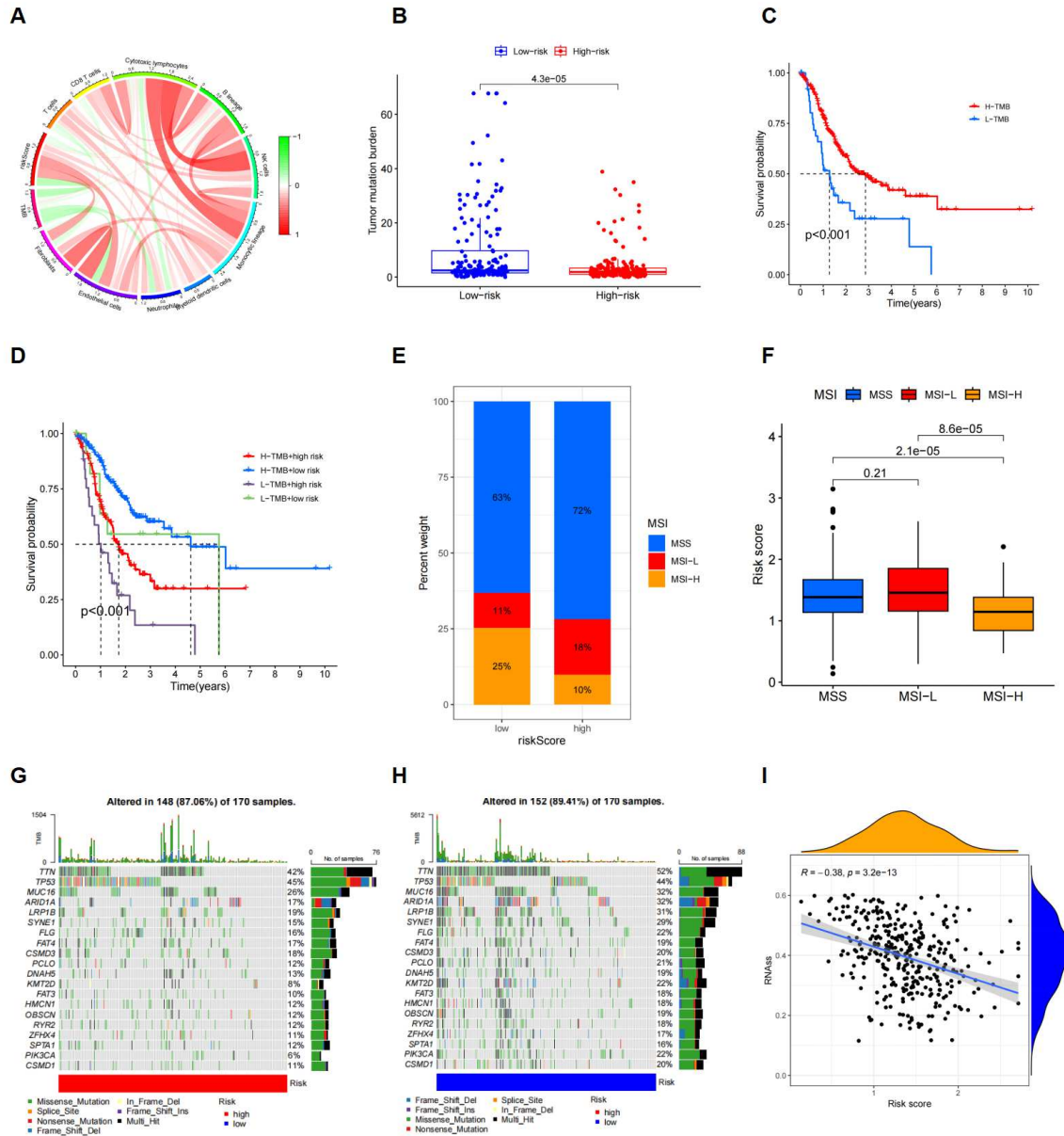


Fig. 8 Mutation landscape, tumor mutational burden, and immunotherapy-related indicators.

(A) Overview of somatic mutation characteristics in the TCGA cohort. (B) Comparison of TMB between the high- and low-risk groups. (C) Kaplan-Meier survival analysis according to TMB status. (D) Kaplan-Meier survival analysis combining risk score and TMB status. (E) Proportional distribution of MSI status between the high- and low-risk groups. (F) Comparison of risk scores

among MSI subtypes. (G, H) Mutation landscapes of the high- and low-risk groups. (I) Correlation analysis between risk score and RNAss.

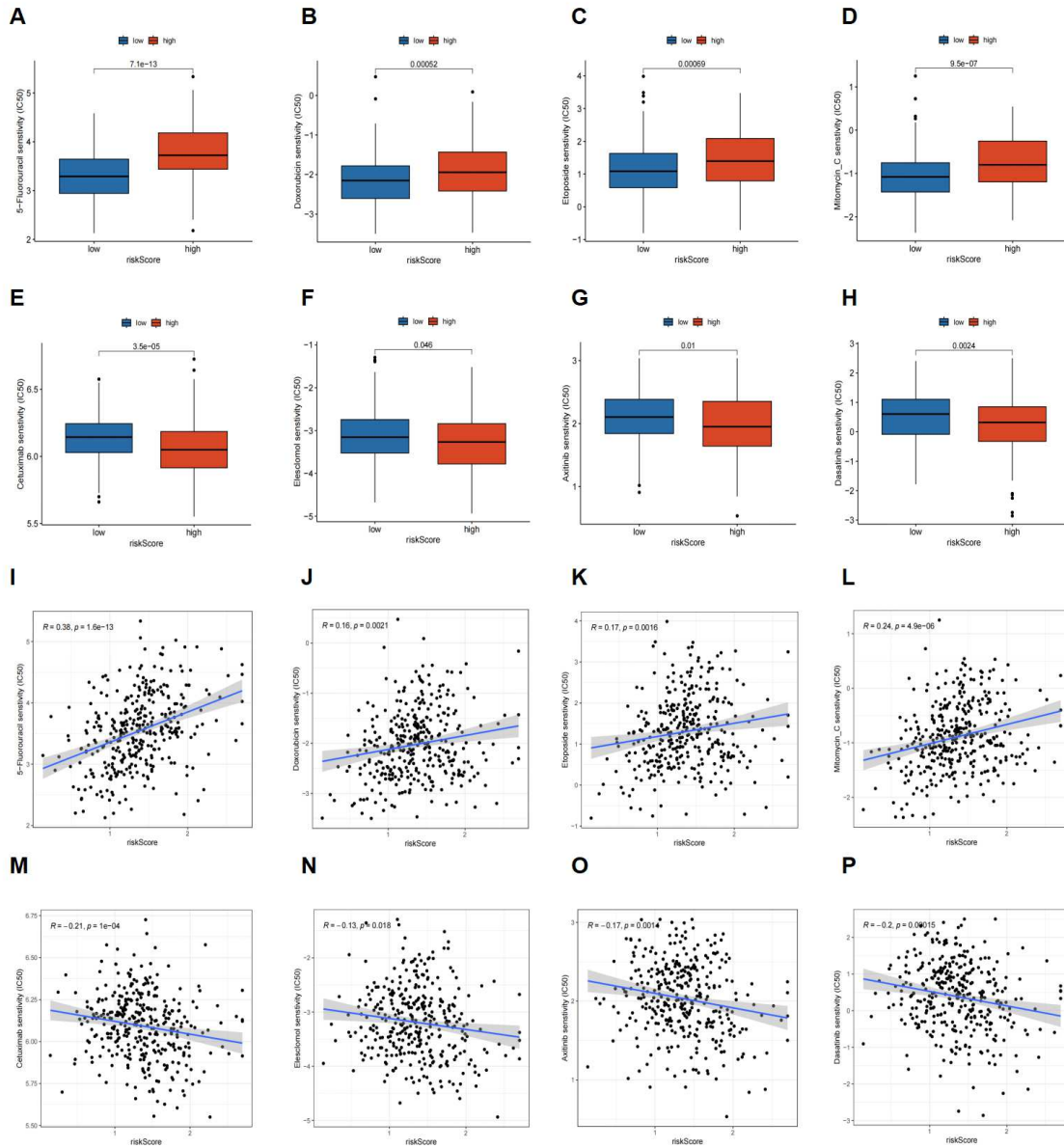


Fig. 9 Drug sensitivity analysis. (A-H) Comparison of predicted IC₅₀ values for 5-fluorouracil (A), afatinib (B), osimertinib (C), paclitaxel (D), cediranib (E), staurosporine (F), dasatinib (G), and dactolisib (H) between risk groups. (I-P) Correlations between risk score and predicted IC₅₀ values for 5-fluorouracil (I), afatinib (J), osimertinib (K), paclitaxel (L), cediranib (M), staurosporine (N), dasatinib (O), and dactolisib (P). *, **, ***, and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

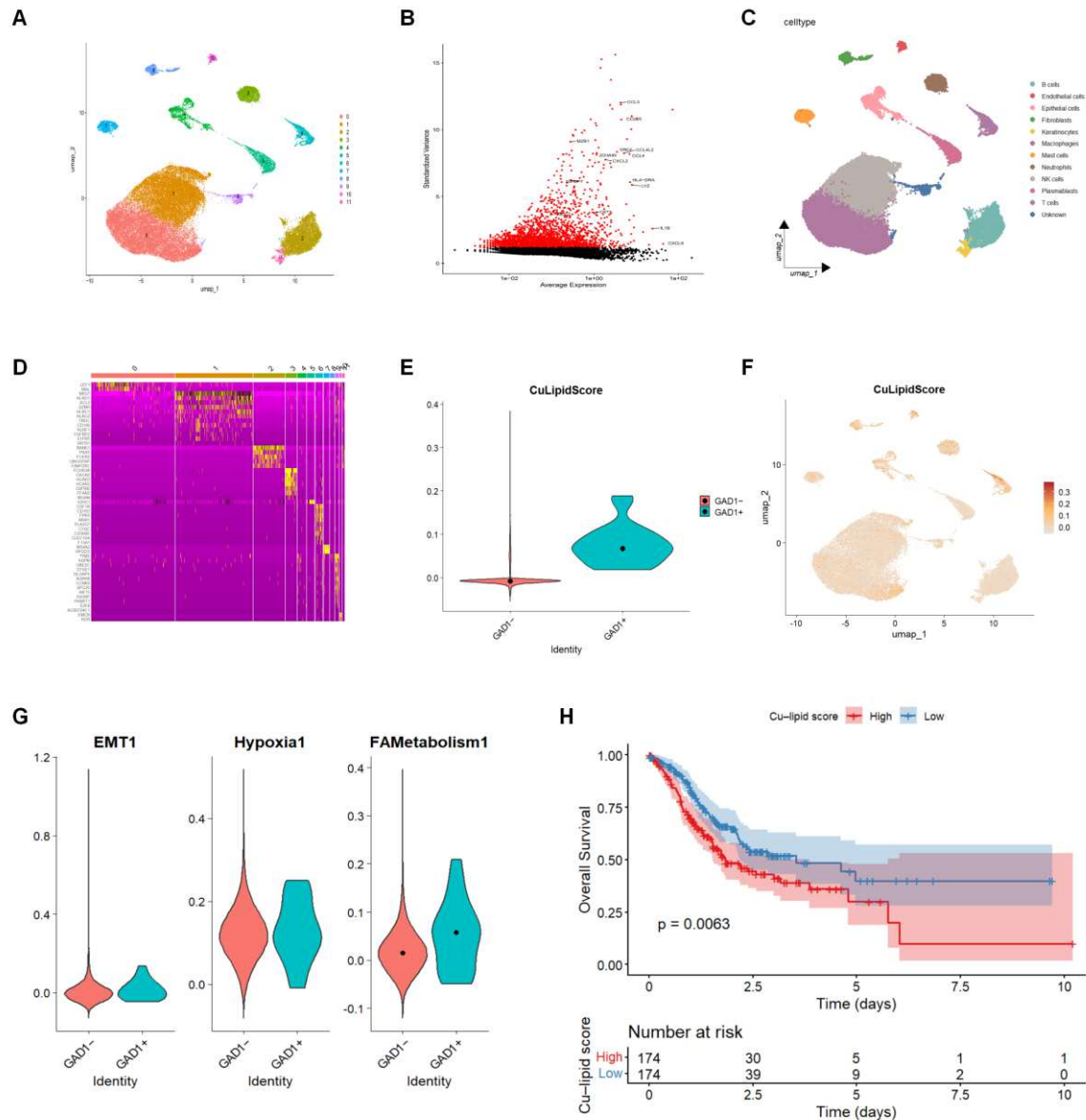


Fig. 10 Single-cell transcriptomic analysis reveals metabolic characteristics of GAD1-positive cells in gastric cancer. (A) UMAP visualization showing cell clustering. (B) Scatter plot of highly variable genes (HVGs). (C) UMAP plot showing annotated cell types based on canonical markers. (D) Heatmap of the top 15 marker genes for each cell cluster. (E) Comparison of Cu-Lipid scores between GAD1-positive and GAD1-negative cells. (F) UMAP visualization showing the distribution of Cu-Lipid scores. (G) Pathway activity comparison between GAD1-positive and GAD1-negative cells, including EMT, hypoxia, and fatty acid metabolism. (H) Kaplan-Meier survival analysis based on Cu-Lipid score.

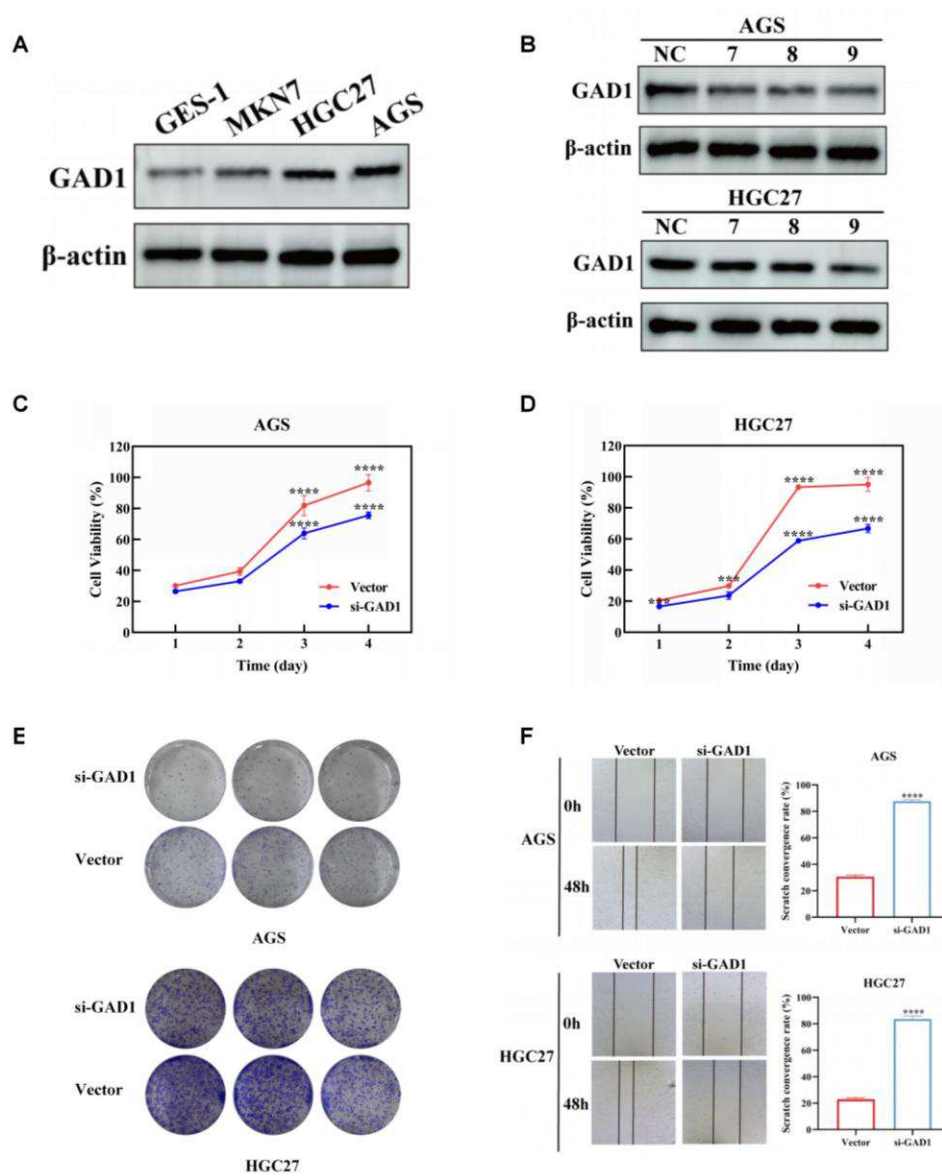


Fig. 11 Functional validation of GAD1 in gastric cancer cells. (A) Western blot analysis of GAD1 protein expression in the human gastric epithelial cell line GES-1 and gastric cancer cell lines MKN7, HGC27, and AGS. (B) Western blot analysis of GAD1 knockdown efficiency in HGC27 and AGS cells infected with three independent GAD1-targeting lentiviral RNAi vectors, designated 7, 8, and 9. Vector 9 showed the most effective GAD1 knockdown and was selected for subsequent functional assays. (C, D) MTT assays showing the effect of GAD1 knockdown on the viability of AGS and HGC27 cells. (E) Colony formation assays showing the clonogenic ability of AGS and HGC27 cells in the Vector and si-GAD1 groups. (F) Wound-healing assays showing the migratory ability of AGS and HGC27 cells after GAD1 knockdown. In this figure, si-GAD1 denotes the GAD1-silencing group

generated by lentiviral RNAi-mediated knockdown, and Vector denotes the lentiviral control group. Data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

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