

Decoding the role of polyamine metabolism in lung adenocarcinoma prognosis: A triangulated approach combining transcriptome, single-cell and Mendelian randomization analyses

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Abstract. The progression of lung adenocarcinoma (LUAD) is influenced by polyamine metabolism, which modulates anti-tumor immunity, although the underlying mechanisms remain unclear. The present study investigates the role of polyamine metabolism-related genes (PMRGs) in LUAD using transcriptomic data, single-cell RNA sequencing (scRNA-seq) and Mendelian randomization. Differentially expressed PMRGs were identified through differential expression analysis and weighted gene co-expression network analysis. Prognostic genes were selected via Cox regression and least absolute shrinkage and selection operator regression to construct a risk model. Immune infiltration, machine learning and scRNA-seq were employed to explore molecular mechanisms whilst reverse transcription-quantitative PCR (RT-qPCR) validated gene expression in LUAD tissues. A nomogram incorporating risk scores assisted in predicting LUAD prognosis (area under the curve >0.6). Distinct immune cell profiles, particularly involving B cells and CD4⁺ T cells, were observed between high- and low-risk groups. Drug sensitivity analysis identified 15 drugs with differential responses. Epithelial cells emerged as a key cluster, with dynamic changes in calcium voltage-gated channel auxiliary subunit $\alpha 2\delta 2$ (CACNA2D2) expression during pseudotime. RT-qPCR confirmed the downregulation of prognostic genes in LUAD. A polyamine metabolism-related prognostic signature (CACNA2D2, adenoreceptor β -1, immunoglobulin superfamily member 10 and carbonic anhydrase 4) associated with the tumor micro-environment was established, offering potential for enhanced prognosis prediction in LUAD.

Introduction

Lung cancer is a leading cause of cancer-related mortality worldwide and ranks second in incidence among all cancer types (1,2). In the United States alone, ~234,580 new cases and 125,070 mortalities are projected for 2024, representing the highest mortality burden of any malignancy (2). It is primarily classified into small cell lung cancer and non-small cell lung cancer (NSCLC) (3). NSCLC accounts for nearly 85% of all diagnoses, with lung adenocarcinoma (LUAD) emerging as the most prevalent histological subtype, constituting ~40% of all lung cancer cases (1). Epidermal growth factor receptor (EGFR) mutation is a key driver gene mutation in NSCLC, with research in this area being central to clinical targeted therapy (4). In patients with EGFR-mutated NSCLC, the clinical use of tyrosine kinase inhibitors (TKIs) has markedly improved treatment efficacy and driven key progress in NSCLC management (5,6). While the adverse effects of TKIs are milder compared with traditional chemotherapy (7), numerous patients, including those with LUAD, continue to face challenges such as poor treatment response, adverse reactions and unfavorable survival outcomes (8). Currently, biomarkers, such as lactate dehydrogenase (LDH), are employed in the diagnosis and evaluation of lung cancer. Elevated LDH activity, due to tumor cells' reliance on anaerobic metabolism for energy, has been linked to the onset and progression of lung cancer and is frequently used as a diagnostic adjunct in clinical practice (9). However, the specificity and prognostic value of existing biomarkers remain insufficient, highlighting the need to identify more effective biomarkers to enhance prognostic evaluation and enable personalized treatment. Tumor metabolic reprogramming is a hallmark of cancer, with metabolic abnormalities serving a critical role in tumor initiation, proliferation, invasion and other processes (10). Research into tumor metabolic pathways has thus become a vital avenue for discovering novel biomarkers and therapeutic targets (11).

Polyamines are aliphatic cations present in all mammalian cells and are essential for optimal growth in nearly every cell type (12). The three primary polyamines produced in mammalian cells are putrescine, spermidine and spermine (13). In normal cells, polyamine levels are tightly regulated by biosynthetic and catabolic enzymes (14). Abnormal regulation

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of polyamine metabolism and uptake in cancer cells leads to notably higher levels of these compounds compared with normal cells. Such dysregulation is associated with the initiation and progression of various types of cancers, including breast, colon and lung cancers (15,16). The elevated polyamine levels contribute to disease progression by promoting cell proliferation, malignant transformation and other oncogenic processes, ultimately leading to worse prognoses (17). Due to these clinical observations and the essential role of polyamines in tumor growth, the polyamine pathway represents a promising therapeutic target for cancer treatment. However, there is limited research on the role of polyamine metabolism in LUAD using bioinformatics approaches (18,19). Therefore, the specific molecular mechanisms of polyamine metabolism-related genes (PMRGs) in LUAD require further investigation.

Mendelian randomization (MR) is grounded in Mendelian genetic laws, where genotypes (genetic variations) are randomly assigned to offspring (20). This random assignment allows genetic variation to be used in studying causal relationships, helping eliminate confounding factors and enhancing the reliability of causal inference (21). By leveraging genetic variations such as single nucleotide polymorphisms (SNPs), which are associated with exposure factors, MR serves as a tool to assess causal relationships between exposures and outcomes. Through these assumptions and principles, MR provides a more accurate and reliable framework for causal inference, serving a critical role in genetics, epidemiology and clinical research (20).

In contrast to traditional transcriptomics, single-cell RNA sequencing (scRNA-seq) enables gene expression analysis at the individual cellular level, revealing intercellular variations and heterogeneities that conventional transcriptomic methods cannot capture. With higher resolution, scRNA-seq can more precisely detect gene expression differences between cells (22). This technology is widely used in cancer research to explore the complexities of the tumor microenvironment, cancer cell interactions and tumor heterogeneity (23). In lung cancer, scRNA-seq can uncover gene expression patterns across various cell types within the tumor and its microenvironment, providing insights into molecular mechanisms, potential therapeutic targets and drug resistance mechanisms (24). For example, a study analyzed clinical biopsy samples from patients with metastatic lung cancer using scRNA-seq, mapping >20,000 cancer and tumor microenvironment cells and offering valuable insights for lung cancer diagnosis and treatment (25).

Against this backdrop, the present study hypothesizes that polyamine metabolic dysregulation serves a central role in LUAD initiation and progression by modulating specific gene networks and influencing patient prognosis by regulating cellular functions within the tumor microenvironment. To validate this hypothesis, the present study developed a polyamine metabolism-based prognostic model and investigated the molecular mechanisms driving LUAD progression using integrated bioinformatics approaches. Specifically, weighted gene co-expression network analysis (WGCNA) was performed to identify key module genes associated with polyamine metabolic phenotypes. Additionally, MR analysis was performed to identify driver genes through causal inference, analyze the expression patterns and functions of prognostic genes at single-cell resolution using scRNA-seq and combine

these findings with experimental validation. This approach aims to provide a comprehensive understanding of the role of polyamine metabolism in LUAD, offering novel insights for clinical management and prognostic evaluation of patients with LUAD. In contrast to conventional transcriptomic analyses based primarily on observational correlations, the present study adopts a triangulated framework by integrating transcriptome data, scRNA-seq and MR. The application of MR enables the inference of causal relationships between PMRGs and LUAD risk, thereby providing a more robust biological foundation for the identified prognostic signature that transcends mere statistical association.

Materials and methods

Data source. The transcriptome dataset, along with relevant clinical data, was obtained from 59 control tissue samples and 513 LUAD tumor tissue samples in The Cancer Genome Atlas (TCGA)-LUAD dataset from TCGA database (<https://cancergenome.nih.gov/>) (26). Additionally, the GSE30219 (GPL570) dataset included 85 LUAD tumor tissue samples and 14 control tissue samples retrieved from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30219>) (27). The GSE31210 dataset (GPL570) comprised 226 LUAD tumor tissue samples, while the GSE131907 (GPL16791) single-cell dataset included 11 LUAD tumor tissue samples and 11 control tissue samples, also sourced from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131907>) (27). A total of 59 PMRGs were retrieved from the Molecular Signatures Database (MSigDB) (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>). Genome-wide association summary statistics for differentially expressed (DE)-PMRGs and 65,864 patients with LUAD were collected from the Genome Wide Association Studies (GWAS) database (<https://www.ebi.ac.uk/gwas/>). The GWAS data for LUAD (ieu-a-984) included 11,245 patients with LUAD and 54,619 controls with 10,345,176 SNPs, which were used as the summary association statistics for the outcome.

Differential expression analysis and module gene identification. Principal component analysis (PCA) clustering analysis was performed on TCGA-LUAD dataset to investigate clustering patterns between LUAD and normal samples using the plotPCA function from the ‘DEseq2’ (28) R package (V1.38.0; Posit Software, PBC). The primary aim of the present study was to identify differentially expressed genes (DEGs) in LUAD and correlate them with PMRG functional modules. The ‘DEseq2’ R package was utilized to identify DEGs in TCGA-LUAD tumor tissues compared with control tissues (adjusted $P < 0.05$ and $\log_2$ fold change (FC) > 2) (28). The ‘ggplot2’ package (V3.4.4) (29) and the ‘pheatmap’ package (V1.0.12) (30) were used to generate volcano plots and heatmaps, respectively. To control the false discovery rate (FDR) arising from multiple comparisons, P-values were adjusted using the Benjamini-Hochberg method. DEGs were identified based on the criteria of adjusted $P < 0.05$ and $\log_2$ FC > 2 .

WGCNA. To explore the core gene modules associated with PMRG functions, the single sample gene set enrichment

analysis (ssGSEA) method (31) was applied to determine the enrichment fraction of PMRGs (PMRG scores) within individual samples. The PMRG scores were treated as traits in WGCNA performed using the 'WGCNA' package in R (V1.71) (32) to identify module genes highly associated with PMRG scores. Initially, hierarchical clustering was applied to the samples to identify and remove any outliers. A soft threshold (β) with connectivity close to 0 was chosen by setting $R^2 > 0.85$. A scale-free network was constructed based on the selected soft threshold, which divided all genes into multiple modules visually distinguished by different colors (minModuleSize=200; deepSplit=3; mergeCutHeight=0.25). Spearman correlations between these modules and PMRG scores were computed and the genes within the modules that exhibited the highest positive and negative correlations were selected as the module genes associated with PMRG scores.

Identification of DE-PMRGs and functional annotation analysis. The core genes (DE-PMRGs) that were differentially expressed and associated with the function of PMRGs were identified through intersection analysis, forming the basis for subsequent causal verification and model construction. DEGs and PMRG module genes were intersected using the 'VennDiagram' R package (V1.7.3) (33) to obtain DE-PMRGs. Enrichment analysis of DE-PMRGs was performed using the 'clusterProfiler' R package (V4.7.1.003) (34), including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, with a significance level of $P < 0.05$ considered statistically significant. To investigate protein-protein interactions (PPIs) within the DE-PMRG group, the STRING online network tool (<https://string-db.org/>) was used to examine protein associations, with a significance threshold of $P < 0.05$ (interaction score > 0.7). A network diagram was generated and visualized using Cytoscape software (V3.9.1) (35).

MR analysis. To verify the causal relationship between DE-PMRGs and LUAD and eliminate confounding factors, two-sample MR and multivariate MR analyses were conducted using the 'extract_instruments' function from the 'TwoSampleMR' R package (V0.5.7) (36). In this analysis, DE-PMRGs were the exposure of interest, LUAD was the outcome and SNPs served as instrumental variables (IVs). The MR method relied on the following assumptions: i) IVs are strongly associated with DE-PMRG risk; ii) IVs influence LUAD risk only through their effect on DE-PMRG risk; and iii) IVs are independent of confounders.

GWAS data for expression quantitative trait loci of DE-PMRGs were used as the summary association statistics for the exposure. The exposure factors were assessed, and IVs were screened using the 'TwoSampleMR' R package (V0.5.7) (36) function 'extract_instruments' to identify IVs with significant associations with the exposure factors ($P = 5 \times 10^{-8}$). IVs for linkage disequilibrium were removed (clump=TRUE; $r^2 = 0.001$; kb=100). The 'extract_outcome_data' function was used to retrieve the outcome data and eliminate IVs significantly associated with the outcome. Simultaneously, IVs with an F-value < 10 were excluded ($F = \beta^2_{\text{exposure}} / \text{se}^2_{\text{exposure}}$).

A total of five MR methods, including MR Egger (37), weighted median (38), inverse variance weighted (IVW) (39),

simple mode (40) and weighted mode (41), were employed to perform robust causal analyses. A significant causal effect of DE-PMRGs on LUAD was determined using $P < 0.05$ from the IVW method among the five MR approaches. The 'mr_heterogeneity' function within the 'TwoSampleMR' R package (42) was used to perform a heterogeneity test (Cochran's Q test; $P > 0.05$). The 'mr_pleiotropy' function (43) was used to evaluate horizontal pleiotropy ($P > 0.05$). Finally, the 'directionality_test' function (44) was used for Steiger analysis, with correct_causal_direction=TRUE and $P < 0.05$ as the criteria for establishing the causal relationship.

Construction and validation of risk model. To identify genes associated with the prognosis of LUAD, univariate Cox regression analysis was performed on the MR acquisition genes using the 'survival' package (V3.8-3), based on LUAD samples with survival data from TCGA-LUAD dataset. This aimed to initially screen genes related to LUAD prognosis (criteria, $\text{HR} \neq 1$ and adjusted $P < 0.05$). The proportional hazards (PH) assumption test was performed for the genes obtained from univariate Cox regression ($P > 0.05$). In the univariate Cox regression analysis, FDR correction was applied to the P-values to account for multiple testing. Genes with an adjusted $P < 0.05$ and $\text{HR} = 1$ were considered candidate prognostic markers for further least absolute shrinkage and selection operator (Lasso) regression analysis. Subsequently, the 'glmnet' package (V4.1.4) was used to conduct Lasso analysis on the candidate prognostic genes. At the optimal λ value, the Lasso model minimized the error rate and successfully identified prognostic genes with non-zero regression coefficients, analyzed through 10-fold cross-validation. The risk model was constructed by multiplying the coefficients derived from Lasso regression by the gene expressions: $\text{Risk score} = \sum [\text{coef}(\text{gene}_i) \times \text{expr}(\text{gene}_i)]$ for $i = 1$ to n . Based on their risk scores, individuals were divided into high-risk and low-risk groups, using the optimal cutoff value of -0.5605245 . Survival differences between these groups were analyzed using Kaplan-Meier curves via the 'survminer' package (V0.4.9) (<https://CRAN.R-project.org/package=survminer>). Additionally, receiver operating characteristic (ROC) curves were generated using the 'survivalROC' package (V1.0.3) (<https://CRAN.R-project.org/package=survivalROC>). The validity of the risk model was further assessed by replicating the analysis in the GSE30219 dataset. The differential expression of prognostic genes between LUAD tumor and control tissues was examined using Wilcoxon tests ($P < 0.05$) on TCGA-LUAD, GSE30219 and GSE31210 datasets, visualized with box plots generated using the 'ggplot2' package (V3.4.4) (29).

Developing an independent prognostic risk score and clinical characteristic analysis. To enhance the clinical applicability of the model, a nomogram was constructed by integrating clinical features and risk scores. Univariate and multivariate Cox regression analyses were performed to assess the potential of risk score and various clinical characteristics [including sex, age, stage, tumor (T), lymph node (N) and metastasis (M) staging] as independent predictors in patients with LUAD (criteria, $\text{HR} \neq 1$ and $P < 0.05$). The 'rms' R package (V6.5-0) (45) was used to generate the nomogram, predicting 3-, 5- and 7-year

overall survival (OS) based on clinical characteristics and risk score. Calibration curves ['rms' R package V6.5-0 (45)] and decision curves ('ggDCA' R package V1.6.0) (46) were generated to assess the performance of the nomogram.

To explore the relationship between clinical characteristics and risk scores, the Wilcoxon test was used to examine differences in risk scores across various subgroups based on clinical features within TCGA-LUAD dataset ($P < 0.05$).

Analysis of chromosomal and subcellular localisation of prognostic genes. To examine the molecular localization of prognostic genes and provide insights into their functional mechanisms, the distribution of prognostic genes on chromosomes was analyzed using the 'Circos' R package (V1.2.2) (47). Subcellular localization was also assessed to identify the specific cellular regions where the prognostic genes are located. mRNA sequences (FASTA format files) of prognostic genes were obtained from the National Center for Biotechnology Information's GENE database (<https://www.ncbi.nlm.nih.gov/gene/?term=>) and analyzed for subcellular localization using the mRNALocator online database (<http://bio-bigdata.cn/>). The results were visualized by plotting histograms using the 'ggplot2' R package (V3.4.4) (29).

GeneMANIA and friends analysis. To expand the functional association network of prognostic genes and identify potential synergistic genes, GeneMANIA (<http://www.genemania.org/>) was employed to analyze genes related to the functions of prognostic genes. GeneMANIA was used to predict genes associated with the functional roles of prognostic genes and their corresponding functions.

The functional similarity of prognostic genes was assessed using GO term-based Friends analysis with the 'GOSemSim' R package (V2.24.0) (48). Similarity scores were averaged, ranked from high to low and the results were visualized.

Construction of regulatory networks. To explore the upstream regulatory mechanisms of prognostic genes, a multi-level regulatory network was constructed. The miRDB (<http://mirdb.org>) and TargetScan (<https://targetscan.org>) databases, accessed through the 'multiMiR' R package (V1.20.0) (49), were used to predict upstream microRNAs (miRNAs/miR) associated with the prognostic genes. The intersection of miRNAs identified from both databases was determined using the 'VennDiagram' R package (V1.2.3) (33), resulting in a target miRNA set. The Starbase database (<https://rnasysu.com/encori/>) was then used to predict upstream long non-coding RNAs (lncRNAs) for the target miRNAs. Additionally, the hTFtarget database (<http://bioinfo.life.hust.edu.cn/hTFtarget>) was utilized to predict transcription factors (TFs) regulating the prognostic genes. The lncRNA-miRNA-prognostic gene and TF-prognostic gene-miRNA regulatory networks were subsequently constructed using Cytoscape software.

Gene set enrichment analysis (GSEA) of prognostic models. To examine the functional differences between high- and low-risk groups and uncover the potential biological mechanisms underlying the model, GSEA was performed for GO and KEGG pathways in TCGA-LUAD dataset, comparing samples with high and low risk scores. Pathways were considered enriched

if they met the criteria of normalized enrichment score > 1 and $P < 0.05$, with the top five most significantly enriched pathways ranked by their P-values.

Immune microenvironment analysis. To explore the association between risk models and the tumor immune microenvironment and provide a basis for immunotherapy, the present study utilized the ssGSEA algorithm in the training set and performed the Wilcoxon test to compare differences between high- and low-risk groups ($P < 0.05$). Additionally, a correlation analysis was conducted in TCGA-LUAD dataset using Spearman's rank correlation coefficient to examine the relationship between prognostic genes and differential immune cell populations, with a significance threshold of $P > 0.3$ and $P < 0.05$.

Drug sensitivity analyses. To identify individualized therapeutic drugs based on risk models and enhance their clinical applicability, the present study screened for candidate drugs in the Genomics of Drug Sensitivity in Cancer 2 database (<https://www.cancerrxgene.org/>), prioritizing those associated with risk scores. Half-maximal inhibitory concentration (IC_{50}) values for various drugs were calculated for both risk groups using the 'oncoPredict' tool (V1.2.0) (50). Spearman's analysis, conducted using the R package 'psych' (V2.3.6) (<https://CRAN.R-project.org/package=psych>), was used to assess the correlation between drug IC_{50} values and risk scores. The Wilcoxon test was then employed to compare IC_{50} expression for common chemotherapeutic agents between high- and low-risk groups, with statistical significance set at $P < 0.05$.

scRNA-seq analysis. To analyze the cellular expression characteristics and key cell populations of prognostic genes at the single-cell level, the 'seurat' R package (V5.0.1) was used to process single-cell data from GSE131907, which were converted into Seurat objects (51). Quality control was applied to select high-quality cells, characterized by a gene count between 300 and 10,000 per cell and a mitochondrial proportion $< 10\%$. The LogNormalize method (52) was applied to normalize feature expression measurements of each cell against total expression. These normalized values were scaled by a factor of 10,000 and logarithmically transformed. The FindVariableFeatures function was used to identify highly variable genes (HVGs). PCA was performed to reduce the dimensionality of these genes, and relevant principal components (PCs) were selected for cluster analysis using the Uniform Manifold Approximation and Projection (UMAP) method (53). Significant marker genes within clusters were identified using the Find All Markers function, which facilitated cell type identification based on marker expression (Table SI). These marker genes were compared with those reported in the literature (27) to identify distinct cell clusters. To pinpoint key cell clusters, differential expression of prognostic genes between annotated LUAD and control tissue cells was compared using the Wilcoxon test ($P < 0.05$), with significant genes defining the key clusters.

Enrichment analysis of key cell clusters. To elucidate the biological pathways through which key cell clusters function,

functional enrichment analysis was performed on key cell clusters from LUAD samples using the R package 'ReactomeGSA' (V4.3.0) (54) to explore signaling pathways associated with LUAD progression.

Cellular communication and pseudotime analyses. Cell communication among annotated cells was analyzed in CellPhoneDB (<http://www.cellchat.org/>), using the CellChatDB.human database as a reference. The 'CellChat' package (V1.6.1) (55) was utilized to investigate cell-to-cell interactions ($P < 0.05$). The single-cell data, pre-processed and annotated via Seurat, served as the input. Based on the built-in human ligand-receptor database of CellChat, significantly overexpressed ligands or receptors were first identified across different cell populations. Communication probabilities between cell types were then calculated, and significant interactions were determined through 1,000 permutation tests. Subsequent analyses involved signaling pathway mapping, identification of communication patterns and network centrality evaluation to pinpoint key interaction axes. The core results were visualized for further interpretation. To uncover the developmental trajectories and interactions of key cell clusters, pseudotime analysis was conducted using the Monocle algorithm (V2.30.0) (56). This approach aimed to investigate the differentiation direction and explore the effect of prognostic gene expression on the differentiation degree of key cell clusters.

Prognostic gene expression analysis and reverse transcription-quantitative PCR (RT-qPCR). To validate the expression patterns of prognostic genes, clinical sample experiments were conducted to enhance the reliability of the research. Differential expression between LUAD and control samples was analyzed using the Wilcoxon test ($P < 0.05$) in TCGA and GSE30219 datasets. To further validate these findings, 10 clinical samples (5 LUAD and 5 control samples) were prospectively recruited between December 2022 and June 2023 from patients undergoing radical surgery at Hunan Cancer Hospital (Changsha, China). All participants provided written informed consent prior to sample collection. The study protocol was approved by the Ethics Committee of Hunan Cancer Hospital (approval no. SBQLL-2022-127). All clinical procedures were performed in strict accordance with the approved guidelines and the Declaration of Helsinki. The inclusion criteria for patients with LUAD in the present study were: i) Pathologically confirmed LUAD; ii) patients who underwent radical surgery; and iii) no prior history of radiotherapy or chemotherapy before the operation. The exclusion criteria included: i) Presence of other primary malignancies; ii) secondary lung cancer; and iii) samples with poor RNA integrity or microbial contamination. Regarding the control group, the control samples were obtained from adjacent healthy lung tissues (located ≥ 5 cm away from the tumor margin) from the same patients with LUAD, rather than from separate healthy individuals.

RT-qPCR was used to assess gene expression. Total RNA was extracted using the Trizol kit (Ambion; Thermo Fisher Scientific, Inc.; cat. no. 15596-018CN), and cDNA was synthesized via reverse transcription using the SureScript First Strand cDNA Synthesis Kit (Wuhan Servicebio Technology Co., Ltd).

Each RT-qPCR reaction contained 3 μ l cDNA, 5 μ l 2X Universal Blue SYBR Green qPCR Master Mix (Wuhan Servicebio Technology Co., Ltd) and 1 μ l each forward and reverse primer (10 μ M; Table SII). The amplification protocol began with 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 20 sec and extension at 72°C for 30 sec. The results were analyzed using the $2^{-\Delta\Delta C_q}$ method (57) with GAPDH as the internal reference gene (Table SIII). Gene expression differences between LUAD and control samples were compared using GraphPad Prism 5 (Dotmatics; $P < 0.05$).

Statistical analysis. Statistical analyses were performed using R (V4.2.2; Posit Software, PBC), with group comparisons conducted using the Wilcoxon test. Statistical significance was defined as $P < 0.05$, unless otherwise specified. For high-throughput data analyses, including differential expression and initial prognostic gene screening, multiple testing corrections (FDR/Benjamini-Hochberg method) were systematically applied to minimize false-positive results. A P-value or adjusted P-value of < 0.05 was considered to indicate a statistically significant difference.

Results

DE-PMRGs identification. PCA was performed on TCGA-LUAD dataset to characterize the distribution patterns of LUAD samples vs. normal samples. The analysis revealed a noticeable separation between the two groups in the PCA space (Fig. S1). To identify polyamine-related module genes in LUAD, differential analysis of PMRG scores was conducted between LUAD and control groups, showing significant differences ($P < 0.05$; Fig. 1A). Following WGCNA ($\beta = 9$; $R^2 = 0.85$) and module-gene screening (Fig. 1B and C), the MEblue module showed the lowest correlation with PMRG scores ($R = -0.72$; $P < 0.05$), while the MEMagenta module exhibited the highest correlation ($R = 0.70$; $P < 0.05$). These two modules identified 2,882 module genes (Fig. 1D and E). Using TCGA-LUAD data, 2,253 DEGs were found between LUAD and control samples, with 1,684 upregulated and 569 downregulated (Fig. 2A and B). By intersecting DEGs with module genes, 470 DE-PMRGs were identified (Fig. 2C).

Functional enrichment analysis and PPI network. The DE-PMRGs were involved in 1,399 GO terms, including 1,158 biological process (BP), 114 cellular component (CC) and 127 molecular function (MF) terms, as well as 45 KEGG pathways (Fig. 2D and E). Specifically, these were associated with 'ameboidal-type cell migration' and 'vascular processes in the circulatory system in BP', 'collagen-containing extracellular matrix' and 'membrane rafts' in CC, 'signaling receptor activator activity' and 'receptor-ligand activity' in MF and 'vascular smooth muscle contraction' in KEGG pathways. The PPI network analysis revealed extensive protein interactions among most DE-PMRGs, suggesting a synergistic regulation of LUAD malignant phenotypes (Fig. 2F).

Identification of 30 candidate genes. MR analysis primarily based on the results from the IVW algorithm identified 30 DE-PMRGs significantly causally associated with LUAD

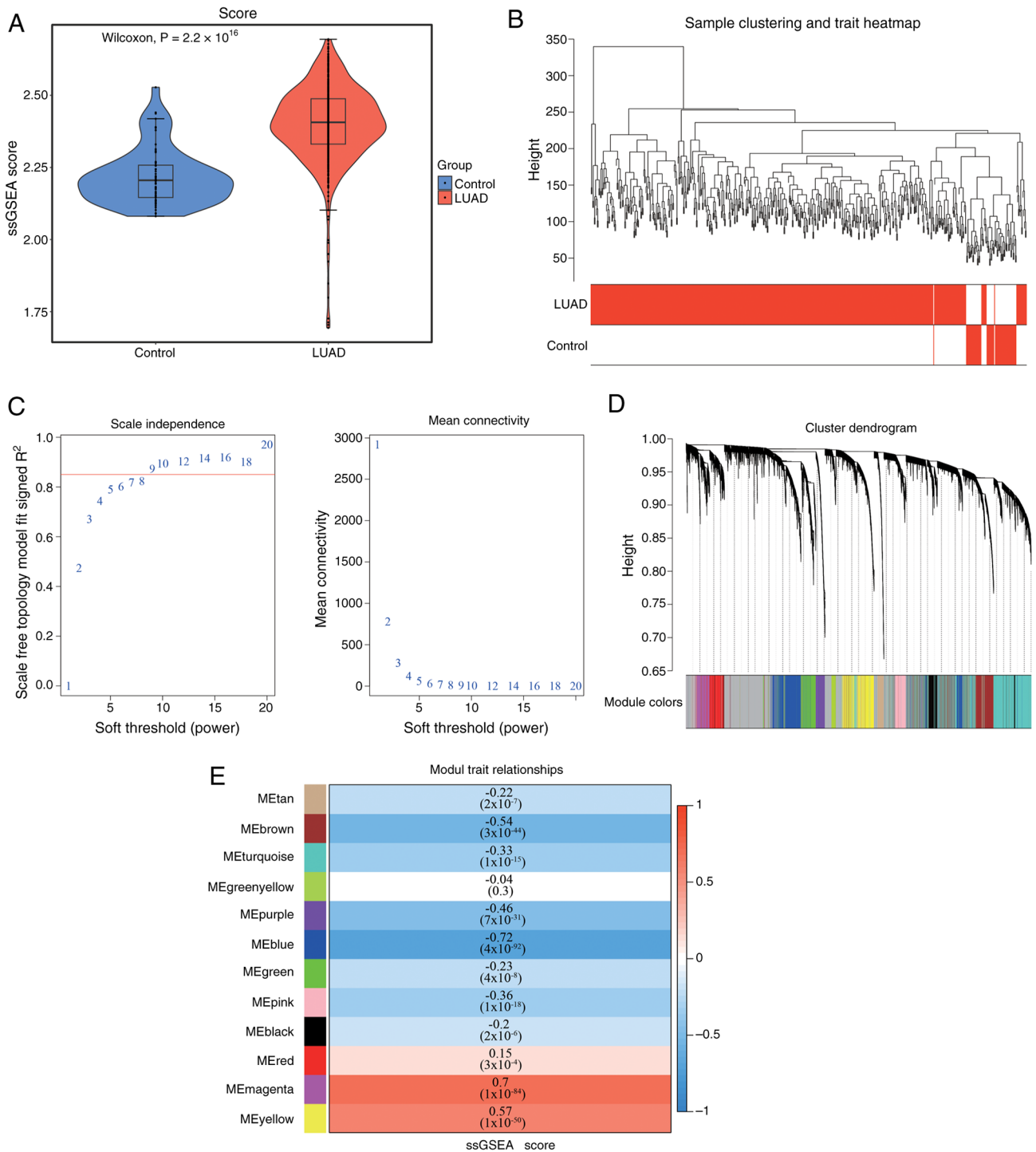


Figure 1. DE-PMRGs identification. (A) ssGSEA rating violin chart: Blue represents the control group and red represents the LUAD group. (B) Sample-level clustering and trait heatmap: Blue represents the control group and red represents the LUAD group. (C) Soft threshold selection: The horizontal axes of all panels indicate the weight parameter power value. The vertical axis of the left panel shows the scale-free fit index (R^2), while that of the right panel represents the mean adjacency function values for all genes in the corresponding gene module. (D) Identification of co-expression modules: The upper part displays the hierarchical clustering dendrogram of genes, and the lower part shows the gene modules. (E) Correlation heatmap between modules and ssGSEA. The color block on the far left corresponds to the modules, and the color bar on the far right indicates the correlation range. In the middle heatmap, deeper colors represent higher correlations, with red indicating positive correlation and blue indicating negative correlation. DE-PMRG, differentially expressed-polyamine metabolism-related genes; LUAD, lung adenocarcinoma; ssGSEA, single sample gene set enrichment analysis.

($P < 0.05$; Fig. 3; Table SIV). Among these, 17 genes, including activin A receptor like type 1 and adenosine receptor β -1 (ADRB1), were protective factors for LUAD occurrence [odds ratio (OR) < 1], indicating their potential to inhibit tumorigenesis and progression via polyamine metabolism regulation. A total of

13 genes, including carbonic anhydrase 4 (CA4) and caveolin 2, were identified as risk factors (OR > 1), with their abnormal expression potentially promoting LUAD malignant transformation by disrupting polyamine metabolism. Correlation scatterplots of SNP-exposure factor effects and SNP-outcome

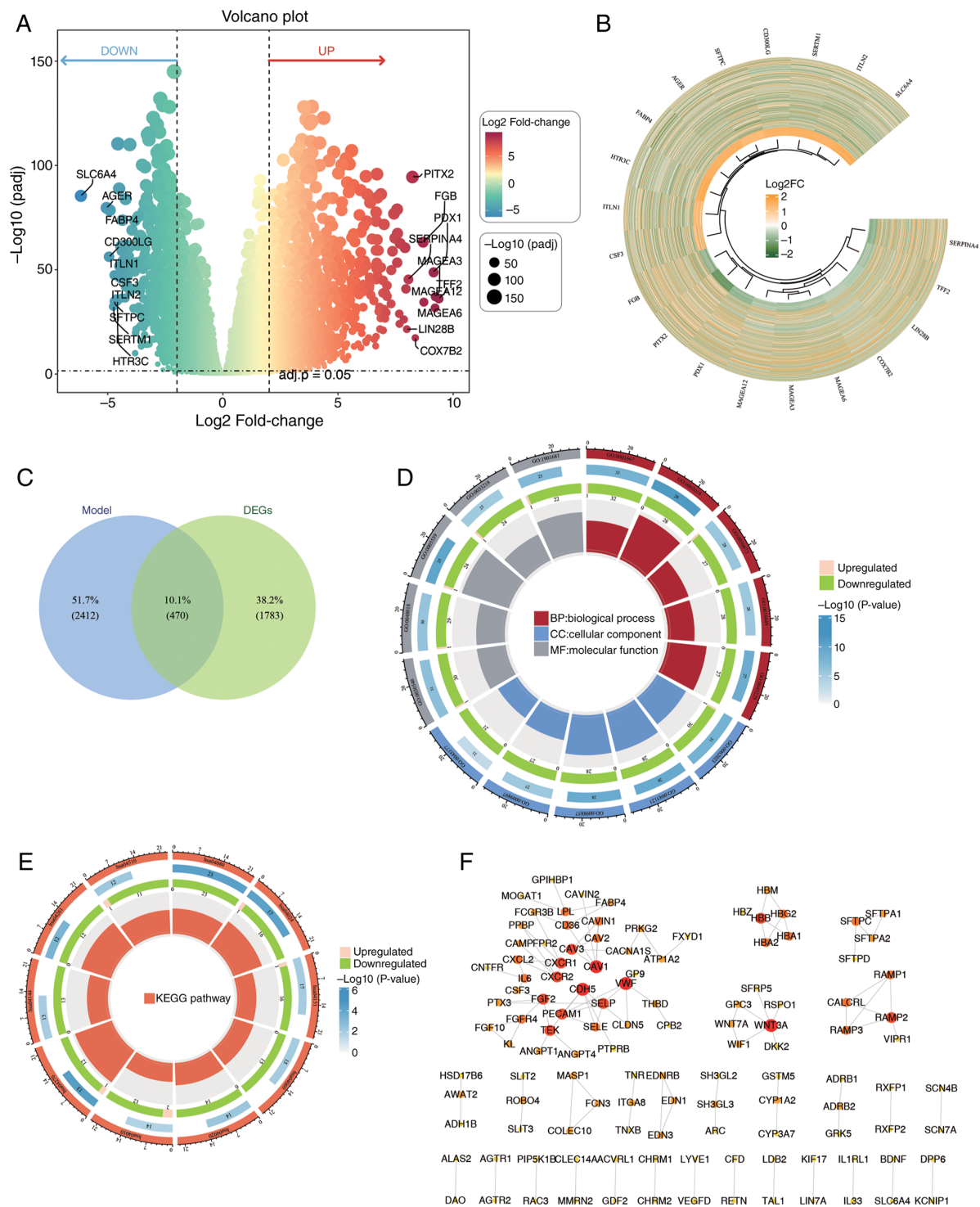


Figure 2. DEGs identification, functional enrichment analysis and PPI network between LUAD and control tissue. (A) Volcano plot highlighting the top 10 upregulated and downregulated DEGs. The vertical axis represents $-\log_{10}(\text{adj. } p)$, and the horizontal axis denotes the fold change ($\log_2 \text{FC}$); each dot corresponds to one gene, with circles representing individual samples. (B) Heatmap displaying gene expression: Top 10 upregulated and top 10 downregulated genes ranked by $\log_2 \text{FC}$. (C) Identification of 470 DE-PMRGs. (D and E) GO and KEGG enrichment analysis of DE-PMRGs: From the outermost to the innermost circle: (1) The first layer shows GO functional IDs across three categories: BP, CC and MF. (2) The second layer: Color intensity indicates significance, with the length, width and numerical labels representing the number of genes enriched in each function. (3) The third layer: The number and trend (indicated by color) of upregulated and downregulated genes in each function. (4) The innermost layer: the color of each block represents different functional categories and the size corresponds to the RichFactor of each pathway. (F) PPI network of DE-PMRGs. DEGs, differentially expressed genes; PPI, protein-protein interactions; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DE-PMRGs, differentially expressed polyamine metabolism-related genes; FC, fold change; BP, biological process; CC, cellular component; MF, molecular function; padj, adjusted P-value.

effects confirmed the high consistency between the present study results and the IVW findings (Fig. S2). The forest plots further corroborated the consistency of the IVW results (Figs. S3-S7).

MR randomness testing indicated that the 30 candidate genes adhered to Mendel's second law (Fig. S8). The heterogeneity test (Q-P-value > 0.05) indicated no heterogeneity among the samples

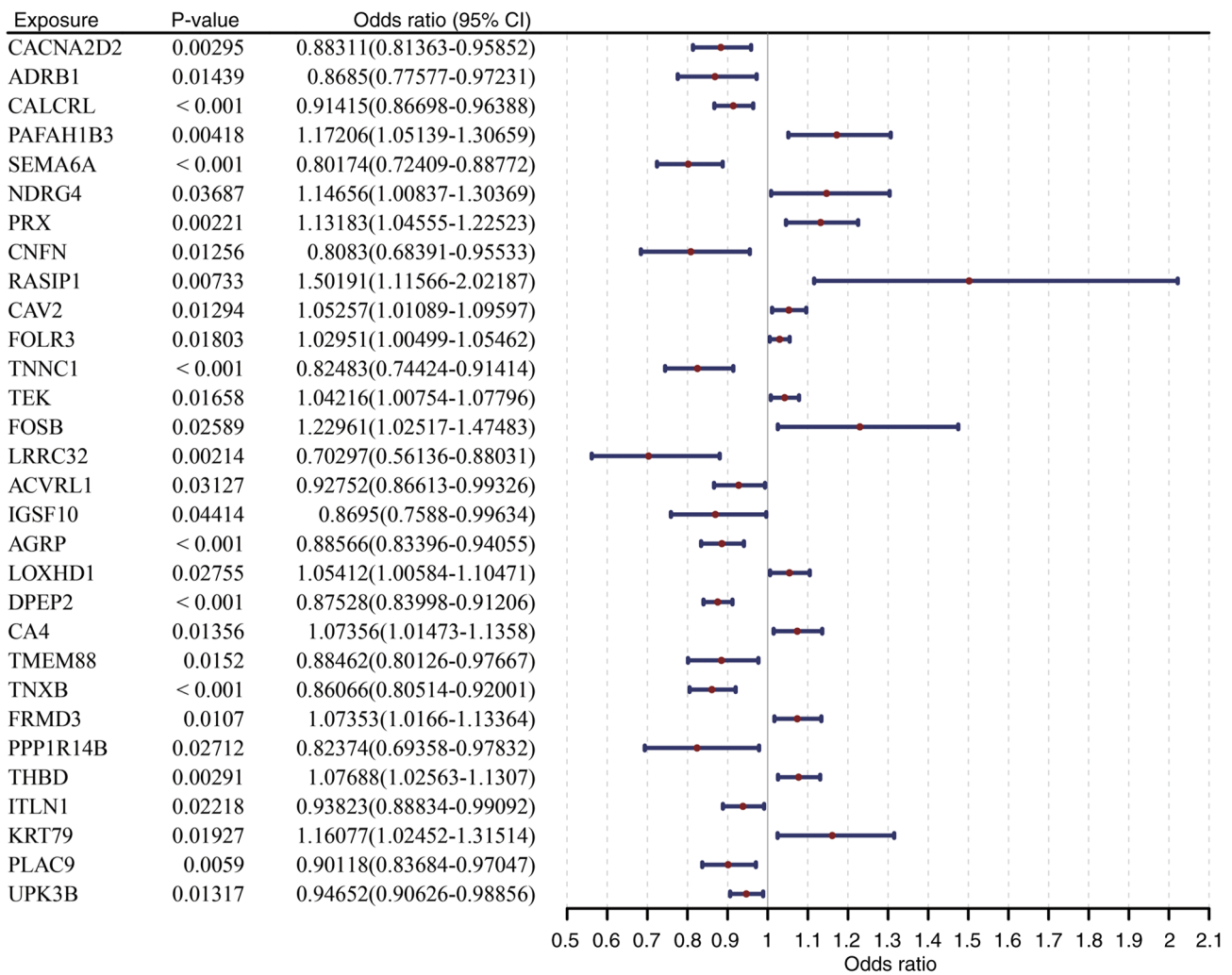


Figure 3. Mendelian screening forest map. Identification of 30 candidate genes by Mendelian randomization analysis, dominated by the results of the inverse variance weighted algorithm.

(Table SV). The horizontal pleiotropy test showed $P > 0.05$, suggesting no significant confounding effects (Table SVI). SNP elimination tests further confirmed the reliability and stability of the MR results (Figs. S9-S13). Steiger filtering analysis of the SNPs from the 30 selected candidate genes revealed that all SNPs had a TRUE direction, excluding reverse causality interference. This preliminary analysis supports the causal association between these DE-PMRGs and LUAD (Table SVII).

Development and verification of risk models. Univariate Cox regression and the PH assumption test identified four genes [calcium voltage-gated channel auxiliary subunit $\alpha 2\delta 2$ (CACNA2D2), ADRB1, immunoglobulin superfamily member 10 (IGSF10) and CA4] as prognostic markers (Fig. 4A; Table I). These four genes were selected at $\lambda = 0.01362608$ [$\log(\lambda) = -4.29577$] under the condition of the lowest evaluated error rate, indicating their retention in the model (Fig. 4B and C). A risk score model was constructed using gene expression and regression coefficients (Table II). LUAD samples from TCGA dataset were categorized into high-risk and low-risk groups based on the optimal risk score cutoff (Fig. 4D and E). Kaplan-Meier survival analysis revealed a significantly lower survival rate in the high-risk group compared with the low-risk

group ($P < 0.0001$; Fig. 4F). ROC curve analysis demonstrated that the model's area under the curve (AUC) values for predicting 3-, 5- and 7-year survival rates of patients with LUAD were all > 0.6 , suggesting modest but consistent predictive performance (Fig. 4G). The prognostic gene heatmap showed significant downregulation of IGSF10, ADRB1, CA4 and CACNA2D2 in LUAD samples (Fig. 4H).

Using the same methodology, LUAD samples in the GSE30219 and GSE31210 datasets were similarly divided into high-risk and low-risk groups (Fig. 5A and B). The survival time of patients in the high-risk group was significantly shorter than that in the low-risk group (Fig. 5C). ROC curve analysis also showed that the AUC values for predicting 3-, 5- and 7-year survival rates were > 0.6 , further supporting the stability of the model (Fig. 5D). These results demonstrate that the prognostic model constructed in the present study exhibits high accuracy and stability across both datasets, affirming its clinical value for predicting the outcomes of patients with LUAD.

Independent prognostic and LUAD nomogram construction. Univariate Cox regression identified seven factors (including risk score, age and stage) that correlated with prognosis ($P < 0.05$; Fig. 6A). The PH assumption test ($P > 0.05$) and multivariate

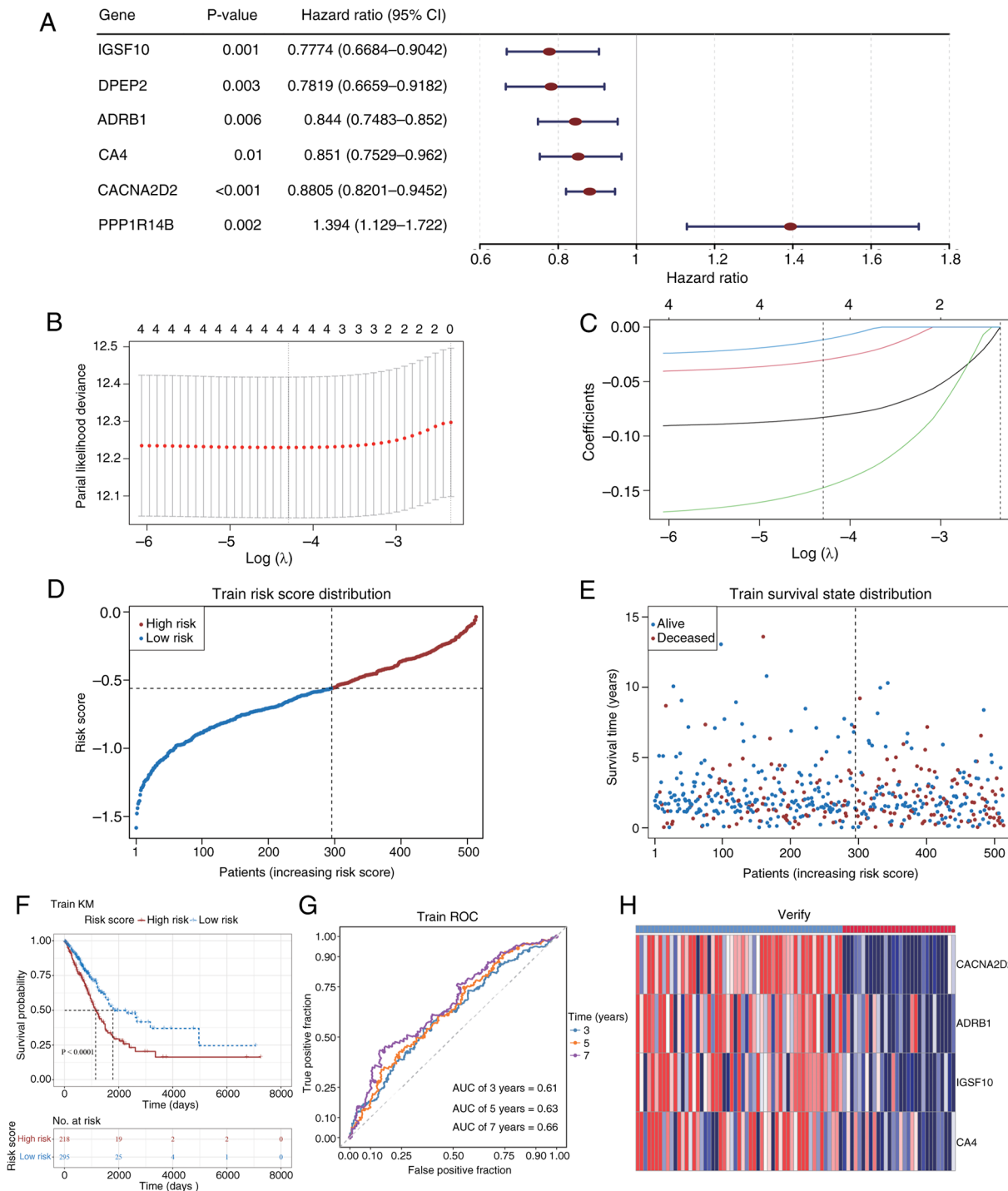


Figure 4. Development and validation of the polyamine-related risk model. (A) Forest plot of univariate Cox regression analysis for prognostic genes (n=513 patients with LUAD). (B) Lasso regression coefficient profiles of the candidate genes. (C) Cross-validation for optimal λ selection in the Lasso model. (D and E) Distribution of risk scores and survival status in the training set; red dots represent high-risk samples and blue dots represent low-risk samples. (F) Kaplan-Meier survival curves comparing high- and low-risk groups. (G) ROC curves predicting 3-, 5- and 7-year overall survival. (H) Heatmap showing the expression of four prognostic genes in the training set. AUC, area under the curve, LUAD, lung adenocarcinoma; ROC, receiver operating characteristic.

analysis confirmed risk score, T stage, M stage and N stage as independent prognostic factors ($P < 0.05$; Fig. 6B, Table III). A nomogram was developed incorporating these independent prognostic factors (Fig. 6C), allowing for the prediction of survival outcomes for patients with LUAD at 3-, 5- and 7 years. The prognostic model demonstrated certain diagnostic potential in predicting the probabilities of 3-, 5- and 7-year survival (Fig. 6D). Decision curve analysis indicated that the net benefit

curve corresponding to the nomogram remained a significant distance from the extreme curves ('All' and 'None') for the 3-, 5- and 7-year assessments. Furthermore, the net benefit of the nomogram surpassed that of individual variables such as T stage, N stage and M stage across the entire threshold probability range, suggesting that the model provides superior net benefits for clinical decision-making in the individualized prognostic assessment of patients with LUAD (Fig. 6E and F).

Table I. Test of the proportional hazards assumption.

Gene	χ^2	df	P-value
CACNA2D2	0.0968983245862793	1	0.755584002022931
ADRB1	1.84307799655053	1	0.174590615317016
IGSF10	1.40028673042861	1	0.236675568527714
DPEP2	6.60470088373115	1	0.010170988792036
CA4	0.173824923446468	1	0.676734610858642
PPP1R14B	4.09566218136085	1	0.0429933856854411

df, Degrees of freedom; CACNA2D2, calcium voltage-gated channel auxiliary subunit $\alpha 2\delta 2$; ADRB1, Adenoreceptor β -1; IGSF10, immunoglobulin superfamily member 10; DPEP2, dipeptidase 2; CA4, carbonic anhydrase 4, PPP1R14B; protein phosphatase 1 regulatory inhibitor subunit 14B.

Table II. Prognostic gene regression coefficients.

Gene	Coefficient
CACNA2D2	-0.0828842
ADRB1	-0.0302789
IGSF10	-0.147514
CA4	-0.0116366

CACNA2D2, calcium voltage-gated channel auxiliary subunit $\alpha 2\delta 2$; ADRB1, Adenoreceptor β -1; IGSF10, immunoglobulin superfamily member 10; CA4, carbonic anhydrase 4.

Variations in risk assessment within subgroups defined by clinical features in TCGA-LUAD revealed significant disparities in risk scores across subgroups based on age, T stage, N stage and overall clinical stage ($P < 0.05$; Fig. 6G).

Exploration of prognostic genes. The GeneMANIA database identified 20 genes associated with the functions of prognostic genes, including solute carrier family 4 (SLC4) member 4, SLC4 member 1 and alkaline ceramidase 2. These prognostic genes were predicted to be involved in 105 functions, such as peptide hormone secretion, insulin secretion, regulation of hormone secretion and voltage-gated cation channel activity, among others (Fig. 7A). The functional similarity of prognostic genes was ranked from highest to lowest based on the average similarity scores. Results indicated that IGSF10, ADRB1, CA4 and CACNA2D2 exhibited low mean functional similarity (threshold > 0.5 ; Fig. 7B).

Chromosomal localization revealed that CACNA2D2 and IGSF10 were primarily located on chromosome 2, ADRB1 on chromosome 10 and CA4 on chromosome 17 (Fig. 7C). Subcellular localization analysis showed that ADRB1, IGSF10 and CA4 were predominantly located in the cytoplasm, whereas CACNA2D2 was primarily localized in the endoplasmic reticulum (Fig. 7D).

The miRDB and TargetScan databases predicted upstream miRNAs for the prognostic genes, identifying 97 and 111 miRNAs, respectively. A total of 26 target miRNAs were common between the two databases. The upstream

lncRNAs of these target miRNAs were predicted using the Starbase database, resulting in the identification of 73 lncRNAs. The lncRNA-miRNA-mRNA regulatory network was then constructed (Fig. 7E). Notably, lncRNAs XIST and NEAT1 co-regulated OLFML2B via hsa-miR-18a-5p. Additionally, 37 TFs (such as FOXA2, histone deacetylase 2 and TATA-box binding protein associated factor 3) were predicted to regulate IGSF10, ADRB1 and CACNA2D2 in the hTFtarget database. Both IGSF10 and ADRB1 were found to be simultaneously regulated by CEBPB in the miRNA-mRNA-TF network (Fig. 7F).

Enrichment, immune cell infiltration, and drug sensitivity analysis of high- and low-risk groups. At a significance threshold of $P < 0.05$, the high-risk group was significantly enriched for 756 GO pathways, including structural components of ribosomes, ribosomal subunits, complex-containing mitochondrial proteins and oxidative phosphorylation (Fig. 8A). In KEGG pathway analysis, 29 pathways were significantly enriched in the high-risk group, such as proteasome, ribosome and pentose phosphate pathways, while the low-risk group was enriched in 11 KEGG pathways, including 'α-linolenic acid metabolism' and 'taste transduction' (Fig. 8B).

Immune infiltration analysis revealed significant variations in 15 immune cell types between high- and low-risk groups, including CD56⁺ natural killer cells, memory B cells and T follicular helper cells (Fig. 8C). Additionally, eosinophils showed a strong positive correlation with all prognostic genes (Fig. 8D).

A total of 33 drugs were identified with significant correlations between their IC₅₀ values and risk scores ($|cor| > 0.3$; $P < 0.05$), including CCT007093, Parthenolide, CMK, RO-3306 and A-443654 (Fig. 8E). Among these, the IC₅₀ values of 15 drugs showed significant differences between the high- and low-risk groups, with drugs such as Parthenolide, CMK and RO-3306 exhibiting lower IC₅₀ values in the high-risk group ($P < 0.05$; Fig. 8F).

Significant enrichment of signalling pathways and identification of key cell clusters. After filtering, the GSE131907 dataset retained 83,883 cells and 25,498 genes (Fig. S14A). The data were standardized, and 2,000 HVGs were extracted (Fig. S14B). Clustering analysis of the top 20 PCs identified 21 distinct cell clusters (Fig. S14C and E). A total of eight cell types were annotated based on marker genes, including

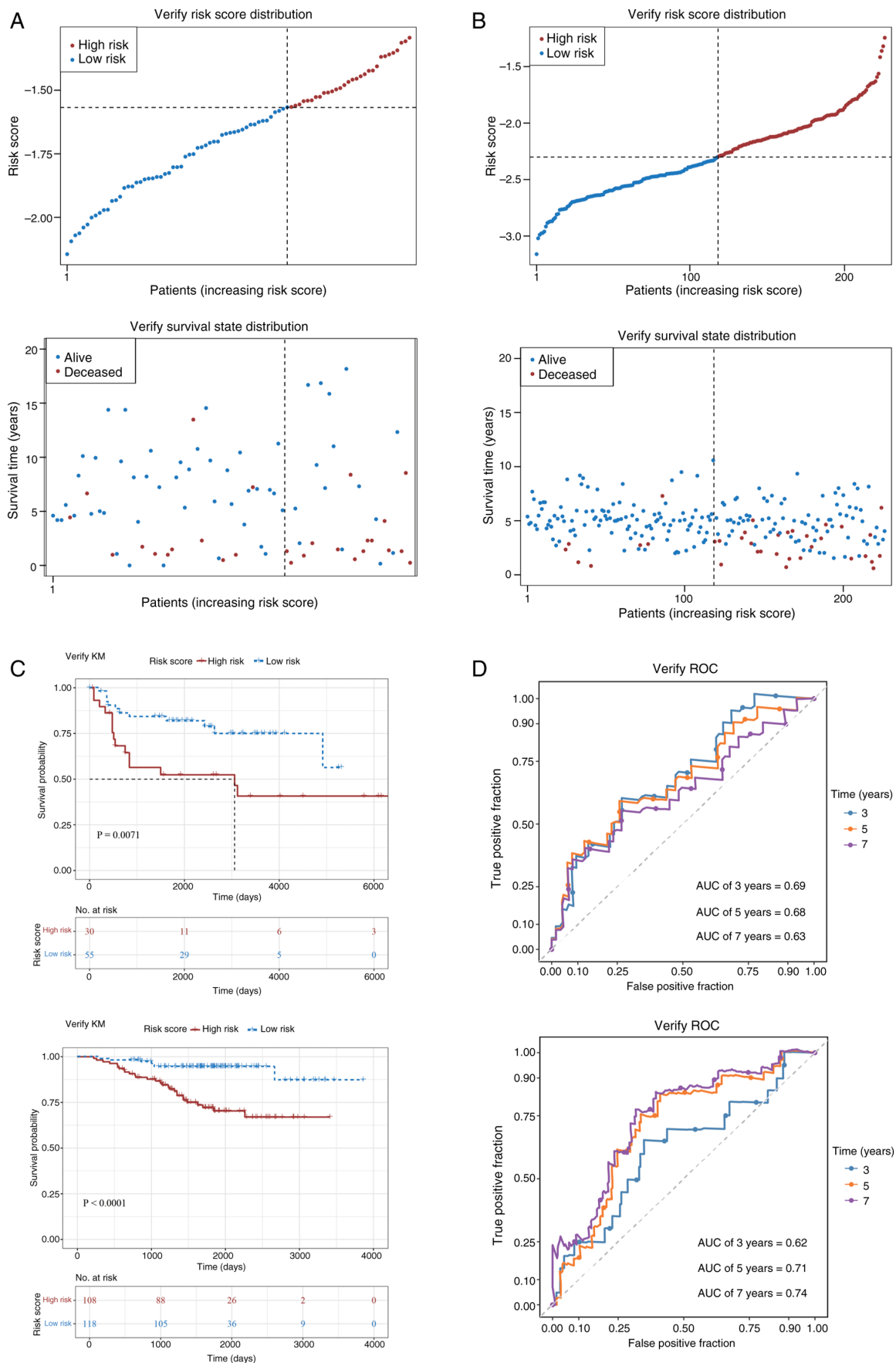


Figure 5. Validation of the prognostic risk model in independent cohorts. Risk score distribution and survival status mapping in the external validation datasets (A) GSE30219 (n=85) and (B) GSE31210 (n=226). (C) Kaplan-Meier survival curves for the high- and low-risk groups in the combined validation set, showing significant differences in overall survival. (D) ROC curve analysis evaluating the accuracy of the prognostic model in forecasting 3-, 5- and 7-year survival outcomes in the validation cohorts. Red lines and blue lines in risk plots follow the same definitions as in Fig. 4. Log-rank test P-values are provided for survival comparisons. KM, Kaplan-Meier; ROC, receiver operating characteristic; AUC, area under the curve.

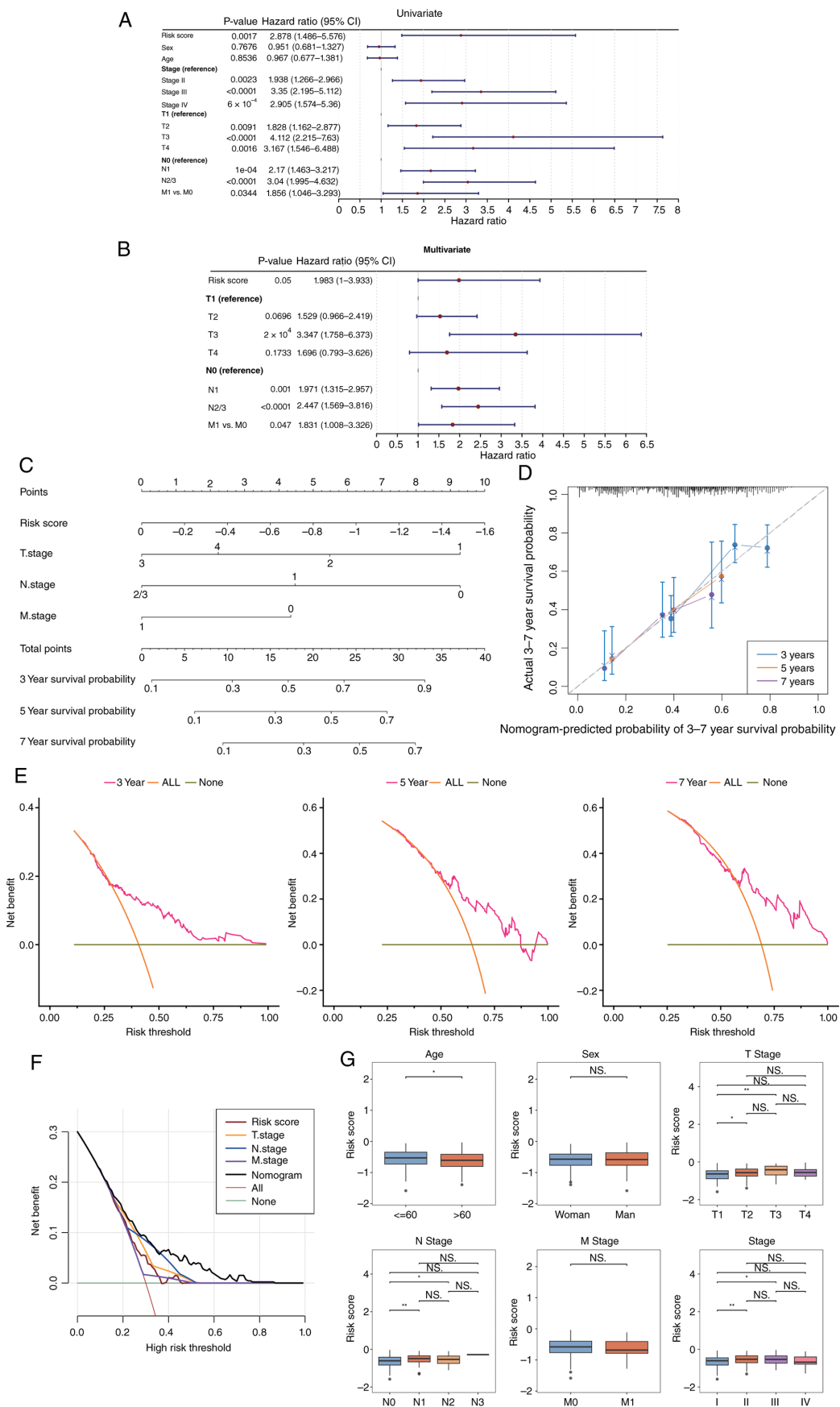


Figure 6. Independent prognostic factors and LUAD nomogram construction. (A) Univariate and (B) multivariate Cox regression analyses for screening independent prognostic factors in LUAD. (C) Nomogram model: Variables in the model are key genes, and ‘Total Points’ represents the sum of individual scores corresponding to the values of all variables. (D) Calibration curve of the nomogram model with predicted probability on the x-axis, actual probability on the y-axis. A calibration curve slope closer to 1 reflects higher model prediction accuracy. (E and F) Decision curve analysis curve with threshold probability on the x-axis, and net benefit rate (after subtracting harms from benefits) is on the y-axis. (G) Box plot showing risk score differences across clinical characteristics. *P<0.05 and **P<0.01. ns, not significant; OS, overall survival; T, tumour; N, lymph node; M, metastasis; LUAD, lung adenocarcinoma,

Table III. Test of the proportional hazards assumption.

Characteristic	χ^2	df	P-value
Risk score	0.24141431262829	1	0.623186484679978
Stage	9.02122082345755	3	0.0290100705831387
T stage	2.54548021781013	3	0.467127830054141
N stage	5.39966572425955	2	0.0672167462647642

df, Degrees of freedom; T, tumor; N, lymph node.

epithelial cells, fibroblasts, endothelial cells, T lymphocytes, natural killer cells, B lymphocytes, myeloid cells and mast cells (Fig. 9A). The edited genes are highly expressed in all cell types (Fig. S14D). Cell proportion analysis in both LUAD and control groups showed that T lymphocytes and myeloid cells represented relatively high proportions in both groups (Fig. 9B). Significant differential expression of prognostic genes was observed in epithelial cells between LUAD and control tissues, leading to the selection of epithelial cells as a key cell cluster for subsequent analysis ($P < 0.01$; Fig. 9C). Enriched signaling pathways in epithelial cells included TWIK releasing acid-sensitive K^+ channels (TASK), ATP-sensitive potassium channels and the binding of phenylacetate to glutamine (Fig. 9D). Pseudotime analysis revealed that epithelial cells differentiated across three distinct stages and 19 clusters. Cluster 19 was predominantly localized in stage 2, while cluster 12 was primarily found in stages 1 and 3, with cluster 9 predominantly observed in stage 3 (Fig. 9E). In pseudotime, the expression of ADRB1 and IGSF10 in epithelial cells tended to increase, while the expression of CACNA2D2 initially increased and then decreased (Fig. 9F). Cell communication analysis highlighted frequent and intense interactions between epithelial and myeloid cells, suggesting a synergistic role for these two cell types in tumor microenvironment remodeling and immune regulation (Fig. 9G).

Validation of prognostic gene expression. Wilcoxon test analysis revealed notable differences in the expression levels of the four prognostic genes between LUAD and control samples, with notable downregulation in LUAD samples (Fig. 10A and B). Consistent with these findings, RT-qPCR results (Fig. 10C-F) corroborated the dataset outcomes, indicating that these four genes could potentially improve the prognosis of patients with LUAD. To validate the expression levels of the four prognostic genes, RT-qPCR was performed on clinical samples. As shown in Fig. 10C-F, the mRNA expression levels of CACNA2D2, ADRB1, IGSF10 and CA4 were significantly lower in LUAD tissues compared with control tissues (all $P < 0.01$), consistent with the bioinformatic predictions.

Discussion

The notable role of polyamine metabolism in tumor cell proliferation and malignant transformation underscores its potential as a key determinant of prognosis in LUAD (18,19). In the present study, a robust prognostic evaluation system was constructed and refined through a

triangulated approach. The resulting four-gene prognostic model (ADRB1, CACNA2D2, IGSF10 and CA4) provided moderate predictive insights for 3-, 5- and 7-year survival outcomes in both TCGA-LUAD and GSE30219 datasets. Notably, while previously reported polyamine-related models showed fluctuating performance over time (19), the current model demonstrated a degree of long-term stability, maintaining modest but consistent AUC values > 0.6 for up to 7 years. This observation suggests that the simplified four-gene signature potentially captures more fundamental biological traits associated with late-stage LUAD progression, although further optimization is required to enhance its robust predictive power.

MR analysis provided critical evidence for the causal association between these prognostic genes and LUAD risk, transcending mere transcriptomic correlation. The four prognostic genes identified, ADRB1, CACNA2D2, IGSF10 and CA4, collectively reflect the systematic impact of polyamine metabolic dysregulation. CA4 and CACNA2D2 appear to function as tumor suppressors by inhibiting Wnt signaling and modulating calcium signaling, respectively (50,58). Conversely, the sustained increase in ADRB1 may influence cell differentiation by regulating the metabolic microenvironment, while IGSF10 serves as a protective factor against metastasis (59,60). These genes likely operate within a hyperactive polyamine metabolism network where metabolites such as spermidine stabilize RNA structure and enhance translation initiation efficiency, directly regulating oxidative phosphorylation and protein stability to drive tumor initiation and growth (61,62).

Immune infiltration analysis revealed a critical molecular relationship between eosinophils and LUAD prognosis. A significant reduction in eosinophil abundance was observed in high-risk patients, alongside a strong positive correlation between eosinophil levels and the expression of all four prognostic genes. As eosinophils can directly kill tumor cells by releasing effector molecules such as granzymes and eosinophil cationic protein (63), the downregulation of these prognostic genes likely impairs eosinophil recruitment or survival. For instance, high expression of CA4 and IGSF10 may facilitate immune cell adhesion and reshape the tumor microenvironment to enhance antitumor immunity (64,65). CA4 functions as a tumor suppressor by inhibiting the Wnt signaling pathway through the WTAP-WT1-TBL1 axis (55). As Wnt activation is associated with immune exclusion, CA4-mediated inhibition may promote a more permissive environment for immune infiltration. Concurrently,

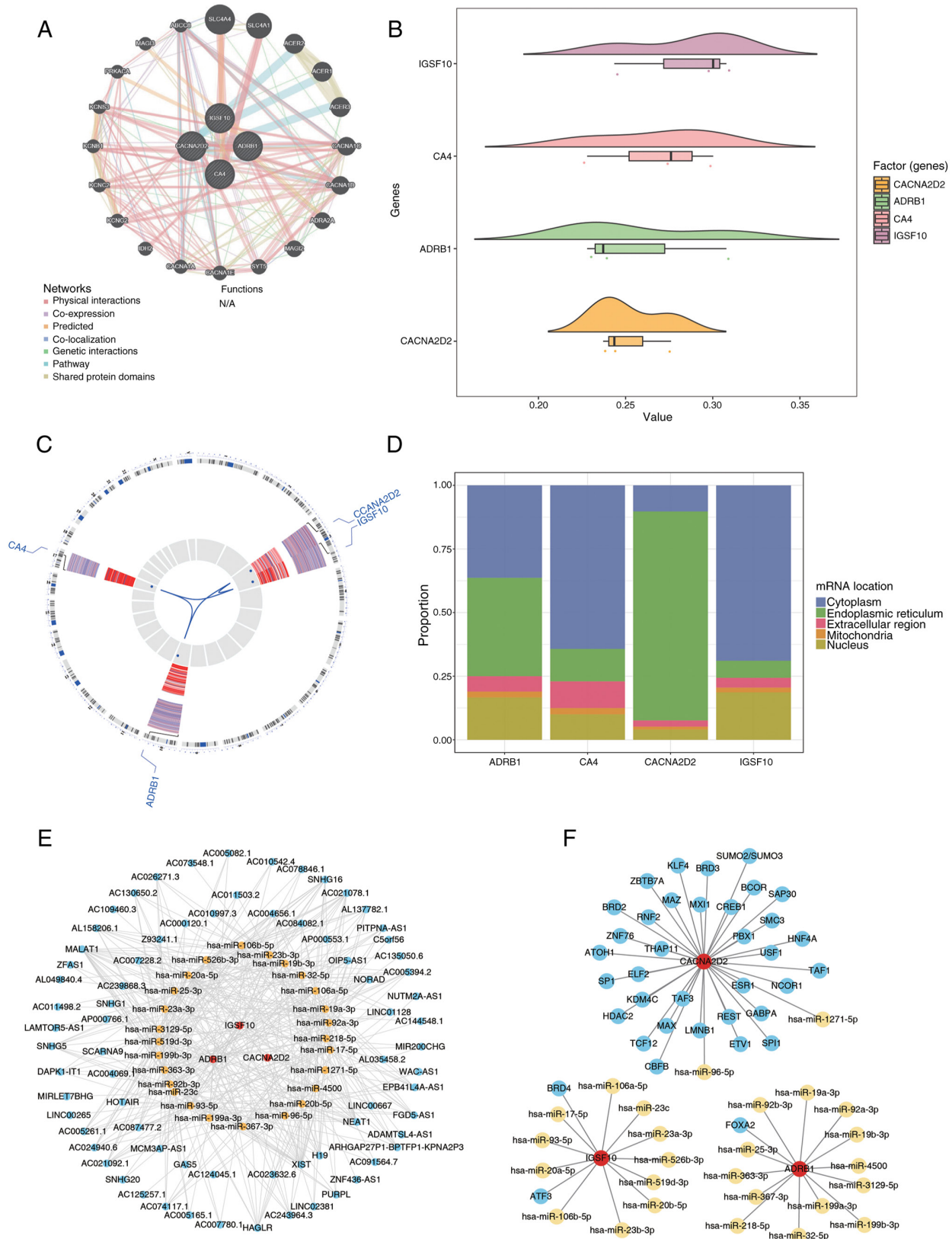


Figure 7. Prognostic gene-related genes and functional similarity analysis. (A) GeneMANIA database analysis network diagram. A total of 20 genes associated with prognostic gene functions were predicted. The large central circle represents prognostic genes, and the small outer circles represent genes correlated with prognostic genes. (B) Functional similarity cloud rain diagram. The horizontal axis denotes the similarity score, and the vertical axis represents prognostic genes. (C) Chromosomal localization of prognostic genes: The first ring shows gene names, with red indicating upregulated genes and blue indicating downregulated genes; the second ring represents the chromosomal locations of genes; the third ring displays the expression profiles of genes in samples; the fourth ring presents the $\log_2FC$ values of genes, where blue indicates $\log_2FC < 0$ and red indicates $\log_2FC > 0$. (D) Subcellular localization of prognostic genes: The horizontal axis represents prognostic genes, the vertical axis denotes the percentage and different colors correspond to distinct subcellular localizations. (E) lncRNA-mRNA-miRNA regulatory network where red represents prognostic genes, yellow represents miRNA and blue represents lncRNA. (F) TF-mRNA-miRNA network diagram where red represents prognostic genes, blue represents TF and yellow represents miRNA. FC, fold change, lncRNA, long non-coding RNAs; TF, transcription factor; miRNA/miR, microRNA.

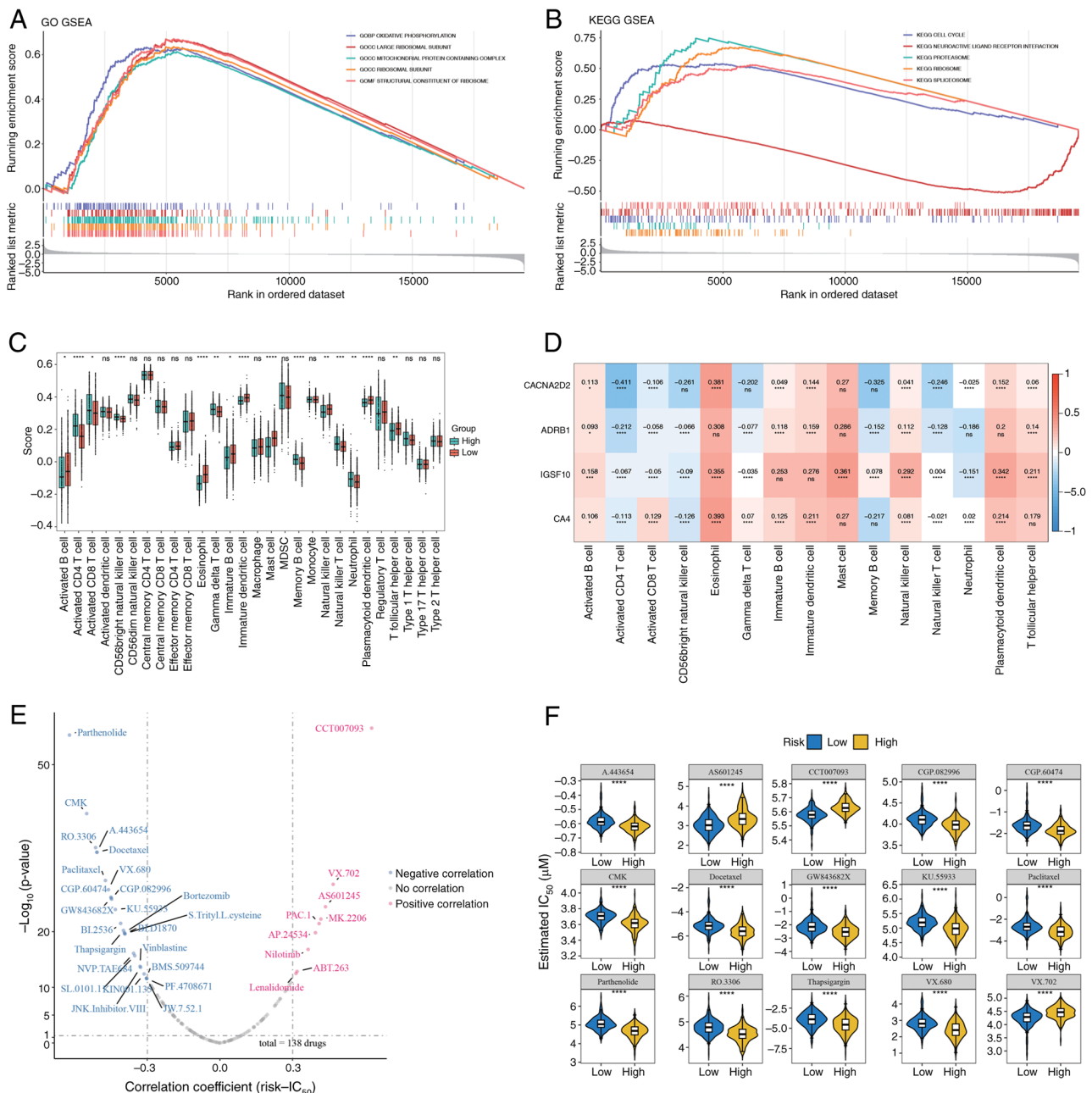


Figure 8. Enrichment analysis, immune cell infiltration and drug sensitivity analysis. (A) GSEA for GO categories, GOBP, GOCC and GOMF, in high-vs. low-risk groups. (B) GSEA for KEGG pathways. (C) Box plot showing differences in 15 immune cell populations; red bars represent high-risk and blue bars represent low-risk samples (n=513). (D) Correlation heatmap between prognostic genes and immune cells. (E) Spearman correlation analysis between risk scores and drug IC₅₀ values. (F) Differential sensitivity of 15 antineoplastic drugs between risk groups. All data are presented as median values. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 (Wilcoxon test). ns, not significant. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; GOBP, Gene Ontology Biological Process; GOCC, Gene Ontology Cellular Component; GOMF, Gene Ontology Molecular Function; GSEA, gene set enrichment analysis; MDSC, myeloid-derived suppressor cells.

as a member of the immunoglobulin superfamily, IGSF10 possesses structural domains essential for cell recognition and adhesion. Recent evidence suggests that such superfamily members are critical components of immune-associated glycopeptides that facilitate the recruitment and attachment of immune cells to specific tissues (56). Therefore, the identified gene signature provides a potential bridge between metabolic reprogramming and the suppression of the anti-tumor immune response.

At the single-cell resolution, epithelial cells emerged as a key cluster, with dynamic fluctuations in prognostic gene

expression during differentiation. Frequent and intense interactions between epithelial and myeloid cells were highlighted, suggesting a synergistic role in microenvironment remodeling. Epithelial cells appear to recruit myeloid cells via chemokines such as C-C motif chemokine ligand 2 and C-X-C motif chemokine ligand 1, which in turn create an immunosuppressive environment through the release of cytokines such as TGF- β and IL-10 (66,67). This epithelial-myeloid axis forms a pro-cancer cycle that accelerates tumor invasion, offering clear targets for subsequent experimental research and personalized immunotherapy strategies.

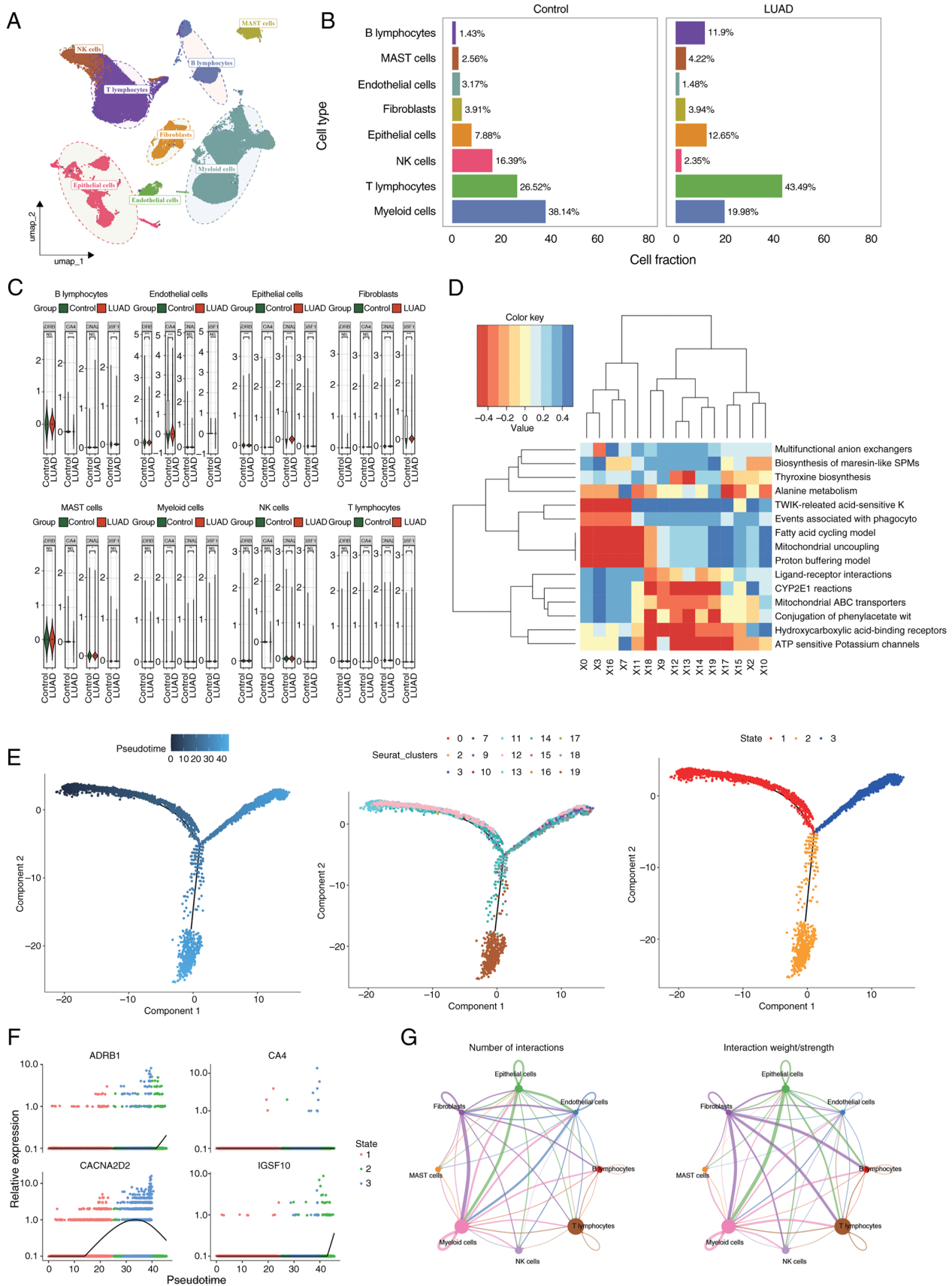


Figure 9. Significant enrichment of signaling pathways and identification of key cell clusters. (A) Annotated UMAP clustering diagram: Eight cell types were annotated according to marker genes, including epithelial cells, fibroblasts, endothelial cells, T lymphocytes, NK cells, B lymphocytes, myeloid cells and mast cells. (B) Proportion chart of each cell type in LUAD and control groups. (C) Violin plot of prognostic gene expression between the LUAD and control groups. (D) Enrichment analysis heatmap of cells. (E) Proposed temporal trajectory diagram: trajectory diagram of different cell subpopulations and their differentiation stages. (F) Expression of prognostic genes in different temporal stages. (G) Quantitative diagram of cell communication interactions and probability intensity values of cell communication interactions. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant. NK, natural killer; LUAD, lung adenocarcinoma; UMAP, Uniform Manifold Approximation and Projection.

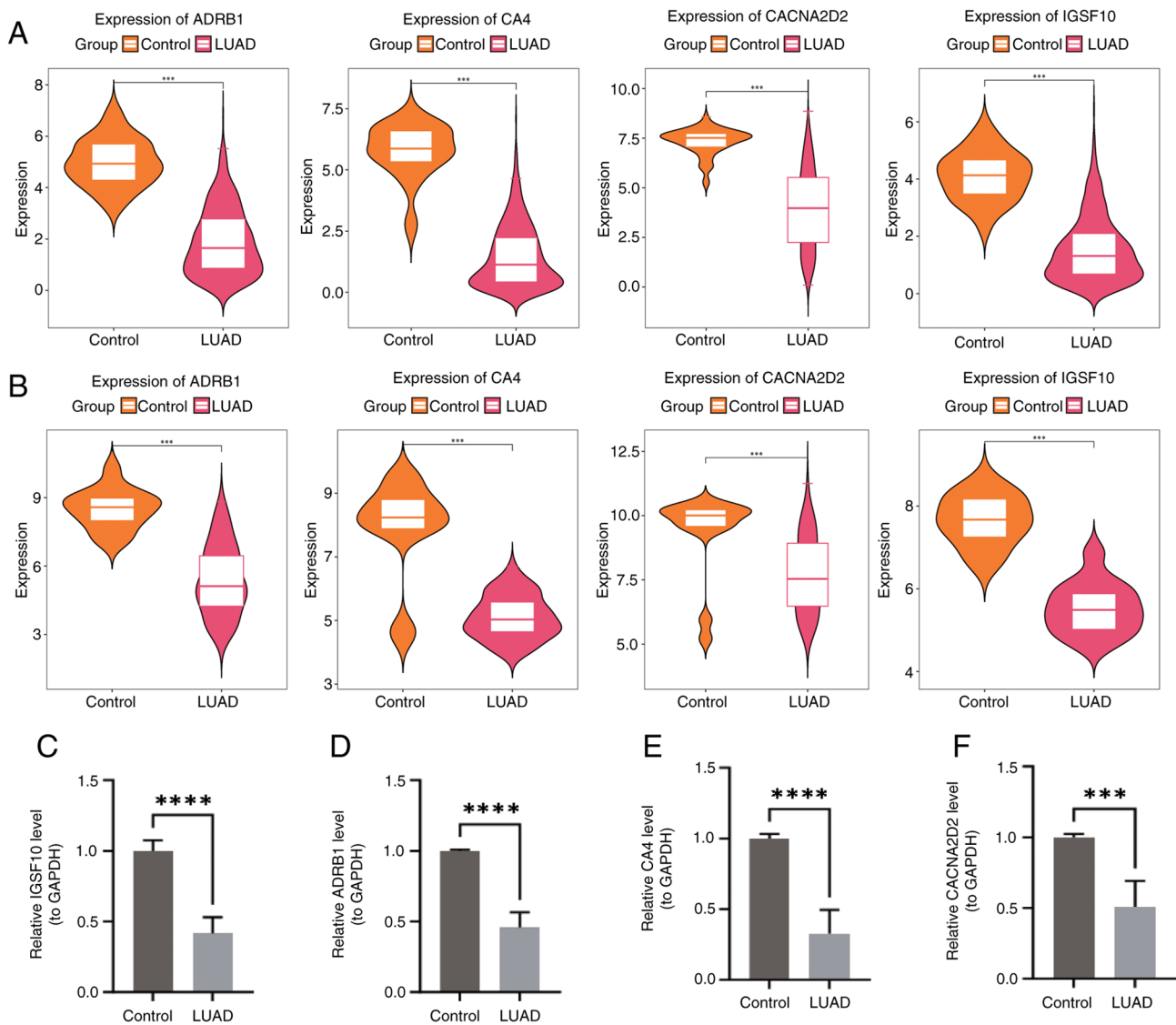


Figure 10. Validation of prognostic gene expression in clinical samples. (A-B) Differential expression of four prognostic genes in TCGA training and validation sets. (C-F) RT-qPCR validation of CACNA2D2, ADRB1, IGSF10 and CA4 mRNA levels in 5 pairs of LUAD and adjacent control tissues collected from Hunan Cancer Hospital (Changsha, China). Data are expressed as mean $\pm$ SD of three technical replicates (n=5 patients per group). The internal reference gene was GAPDH. Statistical analysis was performed using Wilcoxon signed-rank test with *** P <0.001 and **** P <0.0001. TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR.

Beyond molecular mechanisms, the present study identified marked differences in the sensitivity to 15 chemotherapy drugs between risk groups. Specifically, high-risk patients exhibited lower IC_{50} values for drugs such as Parthenolide, CMK and RO-3306, indicating higher potential sensitivity. The CDK1 inhibitor RO-3306, which blocks cell cycle progression, may be particularly effective in high-risk groups characterized by uncontrolled cell cycles due to polyamine dysregulation (68). Furthermore, the regulatory network analysis suggested that oncogenic molecules, such as miR-106b-5p and lncRNAs such as HOTAIR or MALAT1, precisely modulate the expression of the prognostic genes (69,70). These findings provide a biological foundation for selecting individualized therapeutic strategies based on risk stratification.

Despite these insights, several limitations exist. The clinical validation via RT-qPCR was based on a small sample size (n=5), which limits the statistical robustness and universality of the experimental findings. Future research should

focus on multi-center collaborations to collect more comprehensive clinical samples for large-scale validation. Moreover, subsequent work should clarify the regulatory mechanisms of ADRB1 and CACNA2D2 through knockdown or overexpression experiments in cell lines to confirm their effects on LUAD proliferation and metastasis. Functional experiments should also be conducted to clarify the core regulatory molecules involved in epithelial-myeloid interactions, thereby enhancing the reliability and translational value of the prognostic model.

While the present study provides a robust causal framework using MR and single-cell mapping, the lack of direct functional validation should be acknowledged as a limitation. To further elucidate the molecular mechanisms of the identified polyamine-related signature, future research should focus on several experimental directions. First, loss-of-function and gain-of-function assays (such as short hairpin RNA-mediated knockdown or plasmid-based overexpression) of ADRB1, CACNA2D2, IGSF10 and CA4

should be performed in LUAD cell lines to evaluate their direct impact on cell proliferation, migration and invasion. Second, to bridge the gap between metabolism and signaling, dual-luciferase reporter assays and immunoprecipitation should be employed to investigate the regulatory cross-talk between polyamine pathways and calcium signaling. Finally, validation of the protein-level expression of these genes via immunohistochemistry is needed in an expanded clinical cohort with conduction of *in vitro* drug sensitivity testing to confirm the translational potential of a risk model in guided therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HY collected, analyzed and interpreted the data, contributed to conception, design and drafted the manuscript. JC and LZ performed the experiments and supervised the present study. JZ designed, revised, supervised the present study and edited the manuscript. HY and JZ were involved in conceptualization and funding acquisition. All authors read and approved the final version of the manuscript. JC and LZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hunan Cancer Hospital (approval no. SBQLL-2022-127). Informed consent was obtained in writing from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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