

Multi-omics and experimental validation unveil BDNF as a diagnostic biomarker and therapeutic target in endoplasmic reticulum stress-driven lung adenocarcinoma: Therapeutic potential of Esketamine

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Abstract. Endoplasmic reticulum stress (ERS) has shown notable antitumor potential by regulating malignant tumor progression, metastasis and immune response activation. The aim of the present study was to further explore the potential function of brain-derived neurotrophic factor (BDNF), a representative of ERS-related gene (ERSG), in lung adenocarcinoma (LUAD) and to verify the application potential of BDNF-related Esketamine for LUAD. A multi-omics integrated analysis was used to analyze the relevant ERS and LUAD data obtained from public databases, systematically screening and identifying expressed ERSGs in LUAD. The role of ERSGs within the tumor microenvironment was assessed through single-cell transcriptome data analysis, and distinct immune landscapes in LUAD were delineated based on bulk RNA-seq data. Machine learning was used to construct a robust ERS-related predictive model for accurately forecasting the prognosis of patients with LUAD. The causal relationship between the representative ERSG BDNF and

LUAD was evaluated using summary-data-based Mendelian randomization (SMR) and colocalization analyses. The elevated expression of BDNF in A549 and BEAS-2B cells was validated using reverse transcription-quantitative (RT-q) PCR. Loss-of-function experiments using small interfering RNA (siRNA) were used to knock down BDNF expression in A549 cells (A549BDNF-siRNA) and the proliferation capacity of A549BDNF-siRNA was verified using Cell Counting Kit-8 (CCK-8) and colony formation assays. Approved drugs targeting BDNF were screened, followed by molecular docking to evaluate the binding affinity of BDNF to these drugs. The inhibitory effect of Esketamine on A549 cell proliferation was examined using CCK-8 and colony formation assays. The efficacy of Esketamine at inhibiting A549 cell invasion and migration was assessed using Transwell and wound healing assays. TUNEL assay and western blotting were used to analyze the apoptosis of A549 cells induced by Esketamine. A total of 67 ERSGs were screened and identified. Integrating prognostic analysis, single-cell transcriptomic data and RNA-seq, BDNF emerged as a gene of significant research potential. SMR and colocalization analyses indicated a potential causal relationship between BDNF and LUAD. RT-qPCR revealed that BDNF was expressed at high levels in A549 cells, and BDNF knockdown markedly inhibited their proliferation. Esketamine, which can inhibit BDNF, was found to effectively suppress the proliferation, invasion and migration of A549 cells, while inducing apoptosis. BDNF is expressed at high levels in A549 cells, and the inhibition of its expression by Esketamine may have good potential in inhibiting LUAD.

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Introduction

Lung cancer, a predominant contributor to global cancer mortality (1), manifests in ~2,206,771 new cases each year, with lung adenocarcinoma (LUAD) representing a substantial 39% of these cases (2). By 2020, LUAD had become the most prevalent subtype of lung cancer worldwide, presenting an enduring and formidable challenge to global public health

efforts (3). While surgical resection remains effective for early-stage LUAD, the prognostic assessment of postoperative recurrence and the therapeutic options for patients with advanced-stage LUAD remain notably limited (4). Despite recent advancements in immunotherapy, the pronounced heterogeneity of LUAD tumors and the intricate complexity of their microenvironments contribute to unpredictable treatment responses (5), 5-year overall survival rates of <20% and persistent challenge of acquired resistance (6). Consequently, the identification of novel therapeutic targets and innovative strategies is imperative to optimize the management of patients with LUAD (7).

The endoplasmic reticulum (ER), often regarded as the cellular epicenter of protein synthesis, folding and post-translational modification, orchestrates essential intracellular processes (8,9). Upon exposure to extrinsic or intrinsic stressors, the functional integrity of the ER is compromised, precipitating ER stress (ERS), a phenomenon pervasive across multiple tumor types, including LUAD (10,11). ERS orchestrates the regulation of numerous malignant phenotypes in cancer cells and exerts a profound effect on the functionality and behavior of immune cells (12). Notably, under the persistent activation of ERS signaling cascades, tumor-infiltrating leukocytes not only initiate the classical unfolded protein response (UPR), but also distinctively modulate the transcriptional and metabolic programming of immune cells, thus reshaping the tumor immune microenvironment (13). Therefore, strategic targeting of ERS and its related UPR pathways holds promise in unveiling novel therapeutic avenues to enhance the efficacy of immune checkpoint inhibitors (ICIs) and adoptive T-cell therapies (14). Additionally, cancer cells experiencing ERS can secrete a cascade of signaling molecules that recruit or reprogram myeloid cells within the tumor microenvironment (TME), thereby promoting immune modulation and potentially impeding tumor progression (15). As research advances, ERS is increasingly recognized as a promising therapeutic target in oncology, although its precise mechanisms and clinical applicability remain to be thoroughly elucidated.

Considering the critical role of genetic factors in the pathogenesis of LUAD, genome-wide association studies (GWAS) have emerged as robust tools for identifying LUAD-associated genetic variations (16). The aim of the present study was to comprehensively explore the expression profiles of ERS-related genes (ERSGs) in LUAD and their association with the immune microenvironment by integrating RNA sequencing (RNA-seq) data from platforms such as Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) through sophisticated bioinformatics approaches. By developing an ERS-related predictive model, the aim was to provide a fresh perspective for prognostic evaluation in patients with LUAD. The current study concentrated on brain-derived neurotrophic factor (BDNF), a potentially pivotal gene, employing summary-data-based Mendelian Randomization (SMR) and colocalization analyses to prioritize its relevance in LUAD, followed by experimental validation of its expression and function, thereby contributing to the scientific foundation for the development of precision therapeutic strategies for LUAD (Fig. 1).

Materials and methods

Data resources. In the present study, the gene set associated with ERS was systematically curated from the Gene Cards database (<https://www.genecards.org/>), employing ‘Endoplasmic Reticulum Stress’ as the defining keyword (17). Subsequently, the GSE139032 dataset, which encompasses RNA expression profiles from 77 matched pairs of LUAD and normal lung tissues, was retrieved from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) (18). Additionally, TCGA database, accessed via the UCSC Xena platform (<https://xena.ucsc.edu/>), was used to acquire a more extensive dataset of LUAD tumor expression profiles along with corresponding clinical information, comprising 576 tumor samples and clinical data from 641 patients (19). Moreover, a single-cell mRNA dataset, GSE131907 (comprising 208,506 cells), along with an independent LUAD validation dataset, GSE31210 (containing 226 samples), were procured from the GEO database (20,21). Furthermore, GWAS summary statistics from the IEU database (ieu-a-984; <https://gwas.mrcieu.ac.uk/datasets/ieu-a-984/>), encompassing 11,245 LUAD cases and 54,619 controls, were incorporated into the analysis.

Transcriptional profiling and pathway exploration. To characterize transcriptional variations between LUAD and normal pulmonary specimens, dimensionality reduction was first performed through principal component analysis (PCA) on RNA-seq data from TCGA cohort. The DESeq2 algorithm in R (<https://bioconductor.org/packages/DESeq2>; version, 1.44.0) was subsequently employed for the rigorous identification of differentially expressed genes (DEGs), applying stringent thresholds of Benjamin-Hochberg adjusted $P < 0.05$ and $\log_2$ fold change > 0.58 . Functional annotation studies including Gene Ontology (GO) terms categorization and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were systematically conducted using the cluster Profiler toolkit (<https://bioconductor.org/packages/clusterProfiler>; version, 4.12.0). Prognostically relevant ERSGs were screened through survival-associated filtration via univariate Cox proportional hazards modeling ($P < 0.05$). Molecular interaction networks of these critical ERSGs were subsequently reconstructed through STRING platform (<https://string-db.org>; version, 12.0) interrogation, maintaining a minimum interaction confidence score > 0.4 (Tables SI and SII).

Single-cell transcriptomic profiling and functional dynamics. To ensure analytical robustness in processing the GSE131907 RNA-seq dataset, multistep quality assurance procedures were implemented. Cellular quality control thresholds excluded outliers with transcriptome coverage < 200 or $> 2,500$ unique gene counts, along with cells demonstrating mitochondrial read contribution $> 10\%$ (mitochondrial read fraction $> 10\%$). Normalized expression matrices underwent nonlinear dimensionality reduction and unsupervised clustering via the Seurat toolkit (<https://satijalab.org/seurat/>; version 4.0), employing variance stabilization transformation and graph-based clustering algorithms. Cellular subpopulations were partitioned at a Leiden algorithm resolution parameter of 0.5, visualized through uniform manifold approximation projection across the first 20 principal components. Iterative marker validation

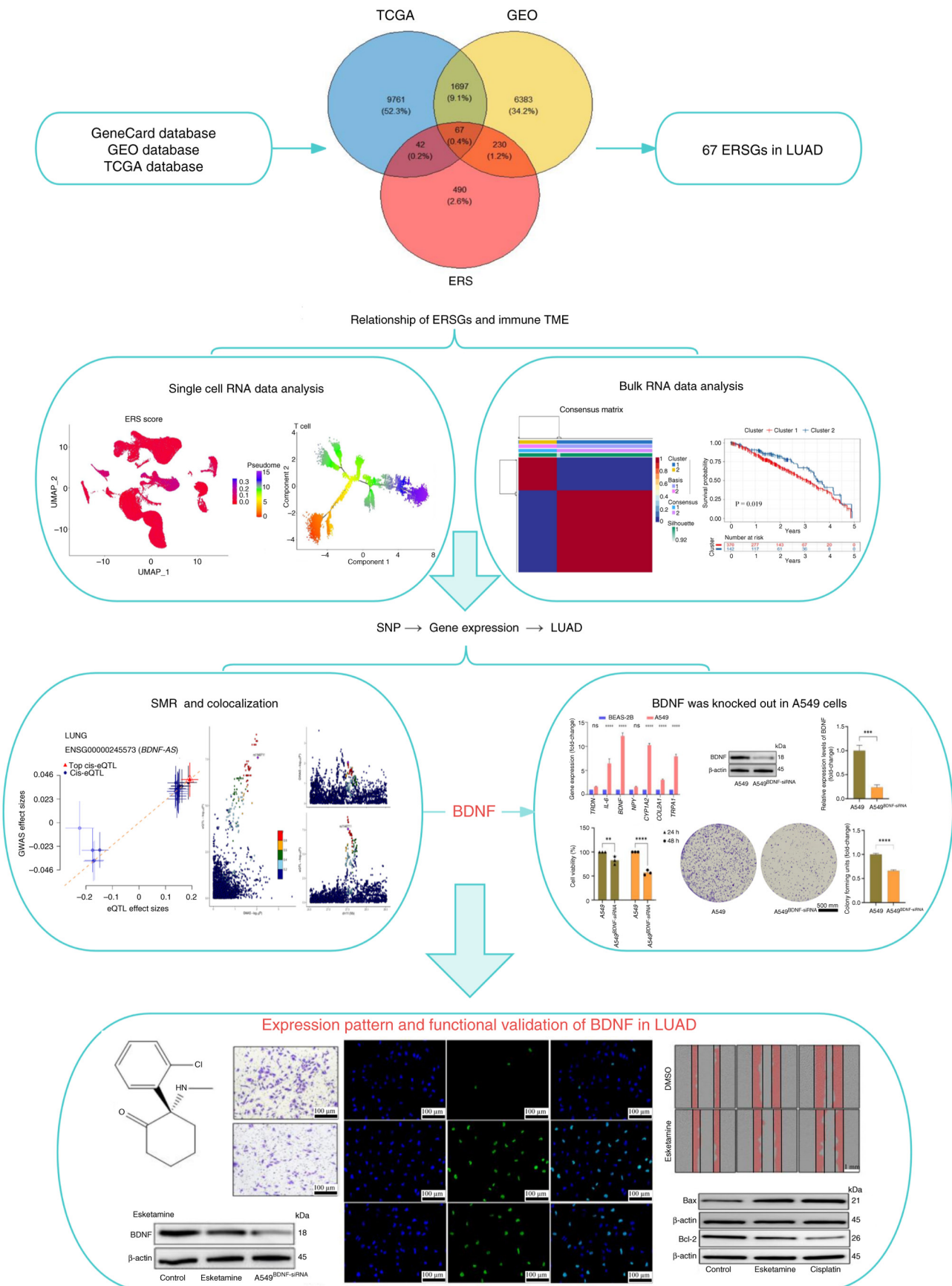


Figure 1. The main workflow of this study. A total of 67 ERSGs were identified by combining three databases and the ERSGs were analyzed and determined using single-cell and bulk RNA data. The causal relationship between BDNF and LUAD was analyzed by SMR and colocalization. The difference of BDNF expression and its effect on A549 cells were verified. Molecular docking and *in vitro* experiments verified the effectiveness of Esketamine in inhibiting A549 cells via downregulated BDNF level. **P<0.01, ***P<0.005, ****P<0.001. ERSGs, endoplasmic reticulum stress related genes; BDNF, brain-derived neurotrophic factor; LUAD, lung adenocarcinoma; SMR, summary-data-based Mendelian randomization; ERS, endoplasmic reticulum stress; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; SNP, single nucleotide polymorphism.

refined 11 algorithmically derived clusters into eight biologically coherent lineages using lineage-specific canonical markers from the Cell Marker repository. ERS activation patterns were quantified at single-cell resolution using the Add Module Score framework (<https://satijalab.org/seurat/reference/addmodulescore>; version, Seurat v4), incorporating a curated ERS signature. Functional phenotyping included cytotoxicity potential estimation via granzyme-perforin axis expression and T-cell exhaustion profiling using programmed cell death protein 1 (PD-1/PDCD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and lymphocyte-activation gene 3 weighted indices, implemented through the Seurat AddModuleScore function (v4.0). Intercellular signaling topologies were reconstructed via Cell Chat (version 1.6.0; <https://bioconductor.org/packages/CellChat>) with ligand-receptor interaction probability thresholds >0.25 and co-expression pattern validation. Developmental trajectories were inferred through pseudotemporal ordering algorithms in Monocle3 (version 1.3; <https://cole-trapnell-lab.github.io/monocle3/>), incorporating branch point analysis and gene expression momentum modeling to resolve lineage bifurcation events (Tables SIII-V).

Molecular taxonomy and TME deconvolution. To resolve the intrinsic molecular stratification of LUAD, a consensus clustering framework was implemented through non-negative matrix decomposition (NMF; version 0.23.0; <https://pypi.org/project/nmf/>) on TCGA transcriptomic profiles, optimizing factorization rank via cophenetic coefficient stability assessment (22). Survival disparities among molecular subtypes were interrogated through multivariate survival modeling (survival version 3.2; <https://cran.r-project.org/web/packages/survival/index.html>) incorporating log-rank testing and stratified survival probability distributions, with hazard ratios (HR) and 95% confidence intervals (CI) computed for subtype-specific prognostic outcomes (23). Pathway perturbation landscapes were mapped via bootstrapped Gene Set Enrichment Analysis (GSEA; 1,000 permutations; FDR <0.1) using Hallmark gene sets, quantifying enrichment of oncogenic signature pathways such as mTORC1 signaling and epithelial-mesenchymal transition, and immune regulatory modules (24). The TME was computationally deconvoluted through: i) Stromal-immune quantification, ESTIMATE algorithm (estimate version 1.1; <https://bioconductor.org/packages/estimate/>) implementation calculating four-dimensional microenvironment indices, tumor purity, immune infiltration score, stromal activation index and composite TME complexity metric, with batch correction for technical covariates; ii) immune phenotyping, relative composition: CIBERSORTx (version 1.06; <https://cibersortx.stanford.edu>) constrained regression model with 22-leukocyte signature matrix, applying P<0.05 confidence threshold for lymphocyte subset fraction estimation. Absolute quantification: MCP-counter (version 2.2.0; <https://bioconductor.org/packages/MCPcounter>) digital cytometry-based quantification of eight cytotoxic populations [CD8⁺ T cells, natural killer (NK) cells] and two stromal lineages (fibroblasts and endothelial cells), normalized to transcripts per million (TPM). Immunotherapeutic vulnerability prediction employed the TIDE framework (version 2.0.3; <http://tide.dfci.harvard.edu>) incorporating: i) Tumor-associated immunosuppression markers (CTLA-4, PDCD1); ii) exclusion signature scores (WNT/TCF β activation); and iii) dysfunctional T-cell infiltration patterns. A predefined TIDE threshold (>75th percentile) identified immunotherapy-refractory cases, validated through sensitivity analysis against alternative predictors (TIS score, IFN- γ response signature) (Tables SVI-XI).

Construction of prognostic features using machine learning. To establish robust predictive biomarkers, a consensus machine learning architecture integrating survival-optimized feature engineering was designed (25). The analytical workflow comprised (26): i) Nonlinear feature prioritization: $\sqrt{p}$, node size, 15 to quantify variable impact on event-time distributions. Prognostically influential transcripts were selected via variable hunting mode (var. used='all') with minimal depth thresholding; ii) High-Dimensional Regularization ($\lambda\alpha$): The multi-algorithmic integration generated a parsimonious transcriptional signature where: Predictors: Log2-transformed expression values (TPM normalized); Outcomes: Right-censored survival tuples (status indicator $\delta \in \{0,1\}$, event time T). Weighting: LASSO-derived β coefficients scaled by RSF variable importance metrics. Individual risk stratification employed a composite scoring algorithm where ω represented stabilized coefficients from nested cross-validation (10-fold outer; 5-fold inner loops) and ϵ the baseline hazard offset. Predictive performance validation incorporated time-dependent discrimination analysis (time-ROC version 0.4; <https://cran.r-project.org/web/packages/timeROC/index.html>) with trapezoidal area under the curve integration. Bootstrap-corrected concordance index (C-index) estimation. Decision curve analysis quantifying clinical net benefit (Tables SXII-XIV).

SMR and colocalization analysis. To investigate the potential causal or pleiotropic relationships between gene expression and LUAD, the current study implemented SMR and colocalization analyses (27). In the SMR analysis, single nucleotide polymorphisms were employed as instrumental variables, with a particular emphasis on blood and colon-specific cis-expression quantitative trait loci (eQTL). Gene expression levels, derived from summary eQTL data, functioned as the exposure factor, while LUAD, based on summary GWAS data, constituted the outcome variable. SMR version 1.03 (<https://yanglab.westlake.edu.cn/software/smr/>) was used to execute the analysis under default settings, with the objective of identifying genetic factors that may modulate gene expression and, in turn, influence LUAD risk. To enhance specificity, the Meta-analysis of cis-eQTL in associated samples method was employed prior to SMR to integrate Genotype-Tissue Expression lung tissue and related cis-eQTL data, thereby mitigating the effect of sample overlap. A stringent P-value threshold was applied in the SMR analysis to screen for top eQTLs, with relevant variants being searched within a 1 Mb region surrounding the target gene. Statistical significance was determined by Bonferroni-corrected P-values. Additionally, the Heterogeneity in Dependent Instruments (HEIDI) test was conducted to exclude potential effects of linkage disequilibrium, where a P_HEIDI >0.05 was indicative of passing the heterogeneity test. To interrogate potential pleiotropic

effects at shared genomic loci, Bayesian causal variant colocalization through the coloc framework (version 5.2.1; <https://cran.r-project.org/web/packages/coloc/index.html>) was implemented employing a hierarchical probabilistic model. The analysis integrated harmonized GWAS summary statistics (MAF >1%; imputation quality score >0.8) with cis-eQTL data (window, ± 500 kb from TSS), calculating approximate Bayes factors through adaptive quadrature integration (28).

Structural bioinformatics and ligand-receptor profiling.

Three-dimensional conformations of BDNF and therapeutic ligands were acquired from the PubChem Compound repository (29). A comprehensive preprocessing protocol was implemented, including: Solvent excision via topological analysis; Protonation state optimization at physiological pH 7.4; Partial charge assignment using Gasteiger-Marsili formalism and Energy minimization (AMBER ff14SB force field; 5,000 steepest descent steps). Molecular recognition dynamics were simulated through semi-empirical free energy calculations in Auto Dock Tools (version 1.2.2; <http://autodock.scripps.edu/resources/adt>) employing: Conformational constraint docking methodology (ligand flexibility $\leq 5^\circ$ rotational tolerance); Grid parameterization centered on the tyrosine kinase receptor B binding domain of BDNF and Lamarckian genetic algorithm (250 runs; population size 300) with cluster RMSD cutoff, 2.0. Binding thermodynamics were quantified via molecular mechanics Poisson-Boltzmann surface area calculations, with intermolecular stability benchmarks defined as: Moderate affinity: Gibbs free energy (ΔG) ≤ -20.9 kJ/mol (-5.0 kcal/mol); high-affinity molecular recognition: $\Delta G \leq -29.3$ kJ/mol (-7.0 kcal/mol). Visual validation of π - π stacking, hydrogen bonding networks and hydrophobic complementarity was performed in UCSF Chimera (version 1.16; <https://www.cgl.ucsf.edu/chimera/>) with subsequent binding pose validation through 50 ns molecular dynamics simulations (version 3.0) (30).

Cell model establishment. The bronchial epithelial BEAS-2B cells and the non-small cell lung cancer (NSCLC)-derived A549 cells were commercially acquired from certified biobanking institutions (Procell Life Science & Technology Co., Ltd.). Cell populations were propagated in high-glucose DMEM/F12 hybrid medium containing heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) under standardized antibiotic prophylaxis. Continuous culture maintenance occurred in tri-gas incubators with precisely regulated parameters of $37 \pm 0.2^\circ\text{C}$, 5.0% CO_2 and 95% relative humidity. All cell stocks underwent mycoplasma screening prior to experimental use, with passage numbers restricted to ≤ 15 generations to preserve phenotypic stability.

Cell treatment and transfection. In light of the molecular docking results demonstrating optimal binding between Esketamine and BDNF, A549 cells were divided into control, Esketamine ($16.25 \mu\text{M}$, 24 h) and cisplatin ($5 \mu\text{M}$, 24 h) treatment groups to assess its inhibitory effects. For the small interfering RNA (siRNA) transfection experiments, a separate group of A549 cells was transfected with BDNF-targeting siRNA (siBDNF) or negative control siRNA (NC siRNA) (Thermo Fisher Scientific, Inc.) to achieve specific protein

knockdown or serve as a transfection control, respectively. The transfection was performed using the Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 50 nM of either siRNA was mixed with the reagent in opti-MEM medium (Thermo Fisher Scientific, Inc.) and applied to the cells. The transfection complex was incubated with the cells at 37°C for 6 h, after which the medium was replaced with fresh complete growth medium. Subsequent experimentation (including Esketamine or cisplatin treatment) was performed 24 h after the initiation of transfection to allow for sufficient gene knockdown. The siRNA sequences used were as follows: siBDNF sense 5'-GCAUGGCAUUUGACACUUU-3' and antisense 5'-AAGUGUCAAUUGCCAUGCUG-3'; NC siRNA sense 5'-UUCUCCGAACGUGUCACGU-3' and anti-sense 5'-ACGUGACACGUUCGGAGAA-3'.

Reverse transcription-quantitative (RT-q) PCR. Total RNA isolation was performed with TRizol-based purification (Beyotime Institute of Biotechnology; cat. no. R0016) followed by RT using Prime Script RT Master Mix (Takara Bio Inc.) according to the manufacturer's instructions. Quantitative analysis of target transcripts was conducted on a CFX96 Real-Time system (Bio-Rad Laboratories, Inc.) employing SYBR Green chemistry (cat. no. D7268S; Beyotime Institute of Biotechnology). Expression data normalization was carried out using GAPDH (forward primer: 5'-GAAGGTGAAGGT CGGAGTC-3'; reverse primer: 5'-GAAGATGGTGATGGG ATTTC-3') as the endogenous reference gene, with baseline correction and threshold cycle determination performed by instrument software. The standard thermal cycling conditions used for the qPCR were: Initial denaturation at 95°C for 2 min; amplification (40 cycles): Denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min; melting curve analysis: 60 - 95°C . Primer validation included melt curve analysis and efficiency testing (95-105%), with sequence details provided in Table SI. Relative quantification between experimental conditions was computed through comparative $2^{-\Delta\Delta\text{Cq}}$ methodology (31).

Cell proliferation. Post-transfection cell proliferation dynamics were evaluated through dual complementary approaches. Metabolic activity quantification employed Cell Counting Kit-8 (CCK-8) reagent (cat. no. C0038; Beyotime Institute of Biotechnology) with optical density measurements at 450 nm using a microplate reader. For long-term proliferation analysis, transfected cells (800-1,000 viable cells/well) were cultured in 6-well plates under standard conditions for 14 days. Resultant colonies were fixed with 100% methanol at room temperature for 15 min, stained with 0.5% crystal violet (cat. no. P0099; Beyotime Institute of Biotechnology) at room temperature for 15 min and subjected to automated particle analysis using ImageJ (National Institutes of Health; version 6.0) with size exclusion criteria (>50 cells/cluster). Clonogenic survival rates were calculated relative to untransfected controls.

Migration analysis (Transwell assay). Cell migration was assessed using $8 \mu\text{m}$ -pore polycarbonate membranes coated with matrix basement membrane extract (Matrigel; BD Biosciences) incubated at 37°C for 1 h. A549 suspensions

(1×10^5 cells/ml) in serum-free medium were loaded into upper chambers, while lower chambers contained 10% FBS-supplemented medium as chemoattractant. After 24 h of incubation, transmigrated cells were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.1% crystal violet at room temperature for 15 min. A total of five random microscopic fields (magnification, $\times 200$) per insert were captured using an inverted phase-contrast microscope (Nikon Eclipse Ti; Nikon Corporation), with cell counts performed by two independent investigators blinded to experimental conditions.

Wound healing assay. Confluent A549 monolayers in 35 mm culture dishes were mechanically wounded using 200 μ l pipette tips. Debris removal was achieved through PBS washing before introducing fresh medium containing 2% FBS. Cells were treated with Esketamine at a concentration of 16.25 μ M. For comparative purposes, parallel experiments were performed using cisplatin at a concentration of 5 μ M. Time-lapse imaging was conducted at 0, 24 and 48 h post-wounding using an Incu Cyte S3 live-cell imaging system (Sartorius AG). Wound closure kinetics were quantified through image analysis software (Image Pro Plus; version 6.0; Media Cybernetics, Inc.) using edge detection algorithms, with migration rates expressed as percentage wound area reduction relative to baseline measurements.

Apoptosis detection (TUNEL assay). A549 cell apoptosis was quantified using fluorometric TUNEL assay (cat. no. C1086; Beyotime Institute of Biotechnology). Fixed cells were permeabilized with ice-cold 0.3% Triton X-100/PBS, then incubated with reaction mixture containing TdT enzyme and FITC-dUTP for 1 h at 37°C protected from light. Nuclear counterstaining employed DAPI (1 μ g/ml) with mounting in anti-fade medium. Fluorescence signals were captured using a confocal microscope (Leica TCS SP8; Leica Microsystems, Inc.) with standardized exposure settings. Apoptotic indices were calculated as (TUNEL+ nuclei/DAPI + nuclei) $\times 100\%$, with positive controls treated with DNase I and negative controls omitting TdT enzyme.

Western blotting. Total protein was extracted from these groups using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. A0020). Protein concentration was determined using a Protein Quantification Kit (BCA Assay; Abbkine Scientific Co., Ltd.; cat. no. KTD3001) according to the manufacturer's instructions. Briefly, a standard curve was generated using BSA, and the absorbance of samples was measured at 562 nm after incubation at 37°C for 25 min. Equal amounts of protein (30 μ g per lane) were separated by electrophoresis on 10% SDS-polyacrylamide gels (prepared using SDS-PAGE Gel Preparation Kit; Beyotime Institute of Biotechnology; cat. no. P0012A) and then transferred onto polyvinylidene difluoride membranes (Immobilon®-P; Merck KGaA; cat. no. ISEQ00010). The membranes were blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. A8020) in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 2 h at 25°C. Subsequently, the membranes were incubated overnight at 4°C with the following primary antibodies:

Rabbit anti-BDNF (1:1,000; cat. no. GB300035), rabbit anti-Bax (1:1,500; cat. no. GB114122), rabbit anti-Bcl-2 (1:800; cat. no. GB154380) and rabbit anti- β -actin (1:4,000; cat. no. GB15003), all from Beijing Solarbio Science & Technology Co., Ltd. After washing, the membranes were incubated with HRP-conjugated secondary antibodies: Goat anti-rabbit IgG (1:50,000; Abbkine Scientific Co., Ltd.; cat. no. A21020) for 1 h at 25°C. Protein bands were visualized using an enhanced chemiluminescence detection reagent (SuperKine™ West Femto Maximum Sensitivity Substrate; Abbkine Scientific Co., Ltd.; cat. no. BMU102) and captured on a chemiluminescence imaging system. Densitometric analysis of the bands was performed using ImageJ software (version 1.53t; National Institutes of Health).

Statistical analysis. For comparative analyses of non-normally distributed datasets (Shapiro-Wilk test; $P > 0.05$), non-parametric Wilcoxon rank-sum tests were applied using the stats package in R version 4.3.0 (<https://www.r-project.org/>). If the groups were independent, experimental datasets were analyzed via unpaired two-tailed Student's t-tests. If the measurements were paired/matched, experimental datasets were analyzed via paired two-tailed Student's t-tests, with continuous variables expressed as mean $\pm$ standard deviation. Computational workflows were executed in RStudio incorporating the tidyverse ecosystem (<https://www.tidyverse.org/>; version 2.0.0), while differential expression patterns were visualized through ggplot2 (version 3.4.2; <https://cran.r-project.org/web/packages/ggplot2/index.html>) and GraphPad Prism (version 9.0; Dotmatics; ANOVA module). For one-way ANOVA, Tukey's post hoc test was used. Statistical significance thresholds followed Benjamini-Hochberg correction for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. All the experiments were repeated three times ($n=3$).

Results

Identification of ERSs in LUAD. The present study systematically investigated the molecular characteristics of ERSs in LUAD. By conducting a comprehensive analysis of TCGA-LUAD cohort, 11,576 genes were identified with markedly differential expression between LUAD and normal lung tissues (Fig. 2A). To bolster the robustness and generalizability of the findings, the expression dataset GSE139032 from the GEO database, comprising 77 LUAD samples and 77 normal lung tissue controls, was additionally analyzed. Through the integration of data from TCGA and GEO, and by cross-referencing with the ERS list from the Gene Cards database, 67 critical ERSs were identified in LUAD, which constituted the primary focus of the present study (Fig. 2B). Prognostic screening via Cox proportional hazards regression (univariate analysis with Benjamini correction) delineated seven risk-associated ERSs, predominantly encoding proto-oncogene products, with Kaplan-Meier validation revealing distinct survival stratification (log-rank $P < 0.01$; Fig. 2C). Subsequently, GO enrichment analysis provided deeper insights into the specific roles of ERSs in various biological processes, such as the regulation of responses to external stimuli and the transport of calcium and metal ions

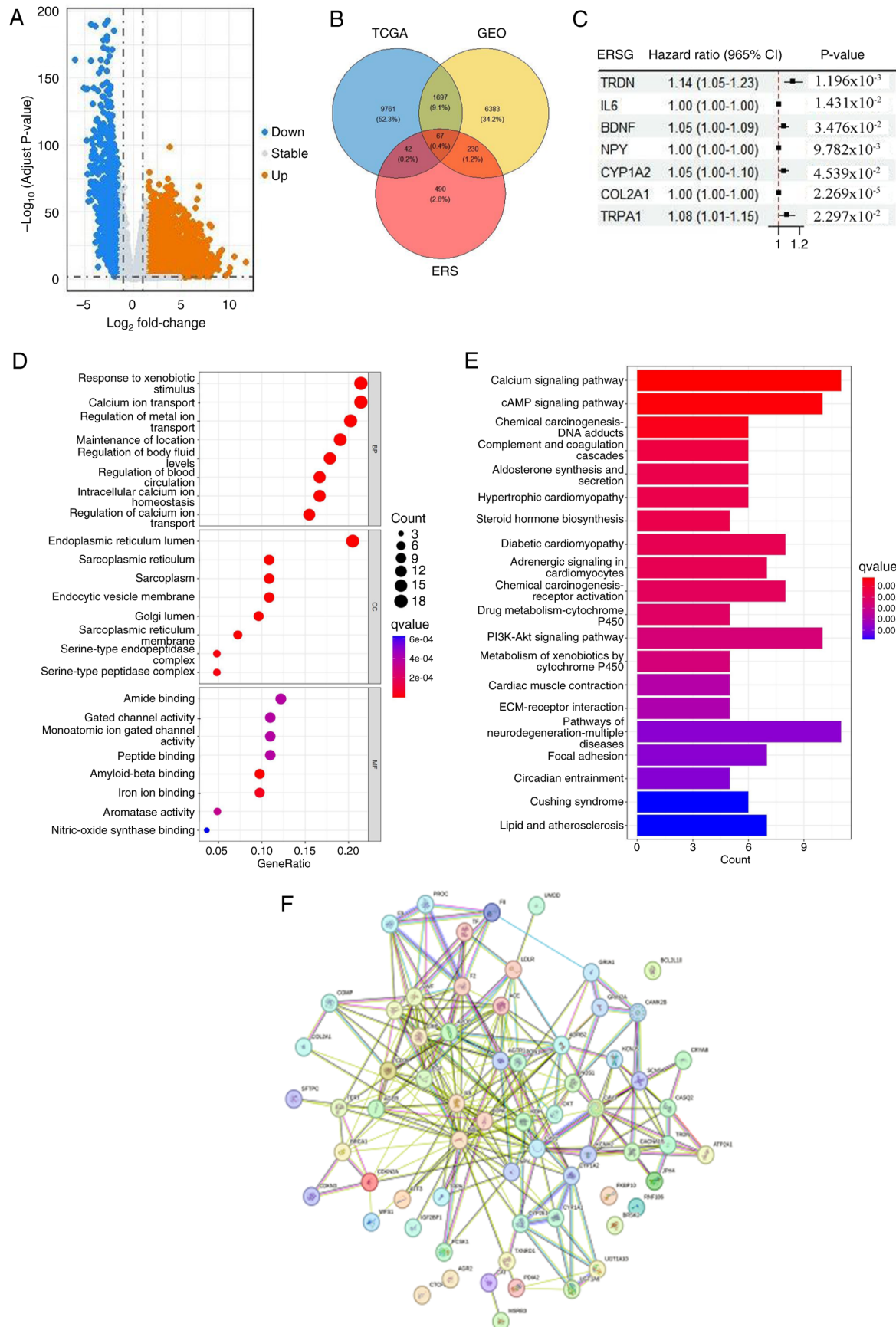


Figure 2. Pan-cancer identification of ERSGs in LUAD. (A) Differential expression landscape of TCGA-derived transcriptomes ($\log_2FC > 1$, $FDR < 0.05$). (B) Intersection analysis of ERS-associated genes across TCGA and GEO repositories (Gene Cards threshold score > 5). (C) Prognostically relevant ERSGs identified through multivariate Cox regression (Benjamini- $P < 0.05$). (D) Gene Ontology annotation and (E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment of 67 ERSGs (hypergeometric test; $FDR < 0.01$). (F) Protein-protein interaction network reconstructed via STRING database (confidence score > 0.4). ERSGs, endoplasmic reticulum stress related genes; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; ERS, endoplasmic reticulum stress; GEO, Gene Expression Omnibus.

(Fig. 2D). Additionally, KEGG pathway enrichment profiling (hypergeometric test, FDR <0.05) identified topologically organized pathways including calcium homeostasis regulation, cAMP-PKA signaling and PI3K-Akt-mTOR cascade as core mechanisms linked to ERSs, as visualized through the cluster Profiler package (version 4.8.1; Fig. 2E). Protein interactome reconstruction using STRING version 12.0 (confidence score >0.7) and Cytoscape Cyto Hubba plugin identified critical network hubs (betweenness centrality >0.3), including BDNF (degree, 28), IL6 (degree, 25) and INS (degree, 22), forming a functional module enriched in inflammatory response and growth factor binding (Fig. 2F).

Single-cell RNA-seq uncovers the intrinsic link between ERS-score and the TME. To investigate the specific mechanisms of ERSs within the TME, the current study extracted high-quality single-cell mRNA expression profiles from the publicly available GSE131907 dataset. Following stringent cellular quality control and tSNE clustering analysis, 208,506 cells were isolated from 11 tumor samples and classified into eight biologically distinct cell clusters (Fig. 3A-C). By integrating cluster marker genes with original cell type annotations, eight distinct cell types were definitively identified, including T lymphocytes (50.2%), NK cells (3.51%), B lymphocytes (15.6%), endothelial cells (1.56%), epithelial cells (3.05%), fibroblasts (6.47%), mast cells (2.81%) and myeloid cells (16.8%), thereby establishing a solid foundation for subsequent analyses. Further analysis revealed diverse expression patterns of the 67 ERSs across cell types, with notably higher expression in fibroblasts and epithelial cells (Fig. 3D), implicating these cell types in the ERS-mediated pathological processes of LUAD. Importantly, the elevated expression of genes such as BDNF, XDH, OXT, IGF2BP1 and CAMK2B highlights their potential significance in regulating the TME. A composite ERS activity index was computed using Seurat's Add Module Score (n=50 control genes; scale=TRUE) with kernel density estimation revealing bimodal distribution in epithelial cells (Fig. 4A and C). Tertile-based stratification (cutoff, 33rd percentile) identified high-ERS subpopulations exhibiting enhanced cytotoxic lymphocyte infiltration and attenuated T-cell exhaustion compared to low-ERS counterparts (Mann-Whitney U test; Fig. 4B).

Further investigation into cell-cell interaction differences between the two groups revealed more frequent and stable communication within the low ERS-score group, particularly among B lymphocytes, endothelial cells and epithelial cells (Fig. 5A and B), potentially reflecting a more coordinated immune response under low ERS conditions. Moreover, signaling pathway analysis revealed significant enrichment of the PARs pathway in the low ERS-score group (P<0.05) and pathways involving ANNEXIN, UGRP1 and RESISTIN in the high ERS-score group (P<0.05), offering new insights into the specific regulatory mechanisms of ERS within the TME (Fig. 5C). Furthermore, pseudo time trajectory analysis of T-cell subsets revealed a significant association between ERS-score and T-cell maturation status; early T cells had lower ERS-scores, while late T cells exhibited higher ERS-scores (P<0.001; Fig. 5D-F). This finding suggested that T cells with lower ERS-scores may be at a more mature stage, thereby executing their immune functions with greater efficacy.

To analyze the role of ERSs in the molecular subtypes of LUAD based on bulk RNA-seq data. To investigate the potential roles of ERSs in the molecular subtypes of LUAD, the present study used TCGA-LUAD cohort and applied the NMF algorithm to conduct a comprehensive molecular subtyping of LUAD based on the expression profiles of 67 ERSs. The consensus matrix heatmap displayed distinct boundaries at clustering number k=1, confirming the effectiveness of clustering and ensuring the stability and reliability of the results (Fig. 6A). Consequently, LUAD cases were stratified into two molecular clusters: i) Cluster A containing 877 individuals; and ii) cluster B including 364 subjects. Survival evaluation demonstrated that Cluster A cases showed markedly improved prognostic profiles relative to Cluster B counterparts (log-rank, P=0.018; Fig. 6B). Further analysis of ERS expression patterns in the two subtypes revealed a heatmap that clearly illustrated a slight downregulation of multiple ERSs in Subtype 1 (Fig. 6C), suggesting that these genes may have distinct roles in different subtypes. GSEA highlighted the enrichment of tumor and immune-related pathways and activities in Subtype 1, particularly the activation of fatty acid metabolism, myogenesis and pancreatic β -cell pathways, offering potential clues for the development of subtype-specific therapeutic targets (Fig. 6D). Additionally, an in-depth analysis of clinical features identified significant differences in age and primary tumor stage between the two subtypes (age, P=0.029; N Stage, P=0.009; Fig. 6E), further highlighting the importance of molecular subtyping in precision medicine.

To comprehensively dissect the differences in the immune microenvironment between the two subtypes, the present study employed various algorithms, including CIBERSORT, MCP-Counter and ESTIMATE. CIBERSORT analysis indicated that Subtype 1 showed higher levels of macrophage M1 and M2, B cells, CD4⁺ T cells and T-cell infiltration, while memory B cells and mast cell levels were relatively lower (P<0.05; Fig. 7A and G). MCP-Counter results also revealed higher levels of myeloid dendritic cells, neutrophils, NK cells and T cells in Subtype 1 (P<0.05; Fig. 7B), indicating a more active immune status in this subtype. Furthermore, the ESTIMATE algorithm demonstrated markedly higher stromal, immune and ESTIMATE scores in Subtype 2 (P<0.001; Fig. 7C-E), which may be associated with its more complex TME. Considering the critical therapeutic significance of ICIs, comparative profiling of 10 immune-modulatory targets was conducted across molecular subtypes. Differential expression analysis revealed Cluster A displayed elevated expression of immunoregulatory markers including HAVCR2 (log2FC, 1.8) and IDO1 (log2FC, 2.3) relative to Cluster B (Benjamini-P<0.001; Fig. 7F). Notably, Cluster A demonstrated markedly higher TIDE prediction indices (Welch's t-test, P<0.001; Fig. 7E), implying enhanced ICI responsiveness in this molecular subgroup.

Construction and validation of ERS-related prognostic feature models. To comprehensively elucidate the potential impact of ERSs on the prognosis of patients with lung cancer, the current study meticulously developed and constructed a prognostic signature model based on ERSs. Initially, using univariate Cox regression analysis, genes with

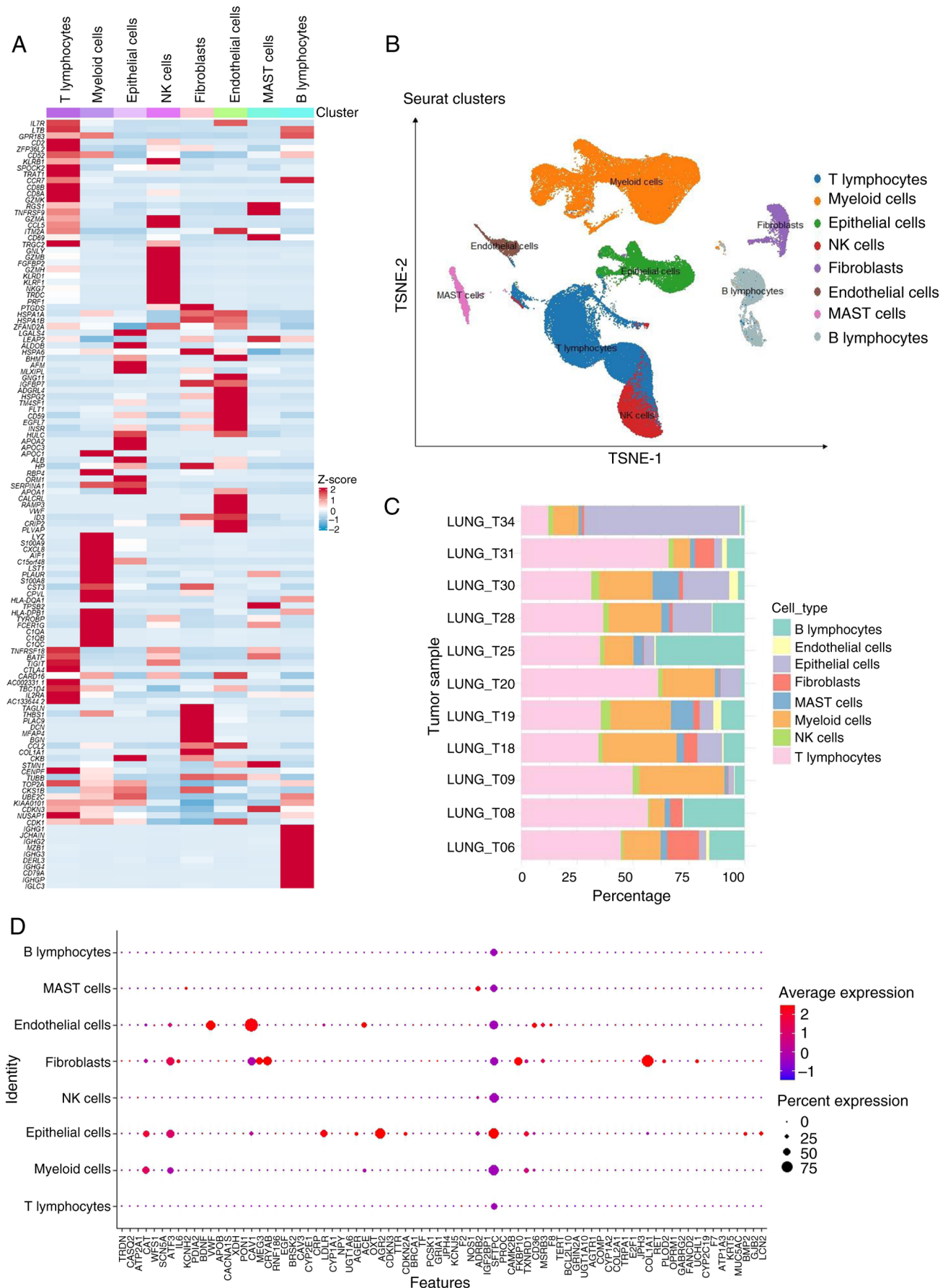


Figure 3. Single-cell resolution of ERSGs activity. (A) Cell-type-specific expression patterns visualized through z-score normalized heatmaps. (B) TSNE projection of eight distinct cellular populations. (C) Cellular composition heterogeneity across 11 LUAD specimens. (D) Violin plots quantifying ERSG expression variance among cellular subsets (Kruskal-Wallis; $P < 1.000 \times 10^{-4}$). ERSGs, endoplasmic reticulum stress related genes; TSNE, t-distributed stochastic neighbor embedding; LUAD, lung adenocarcinoma.

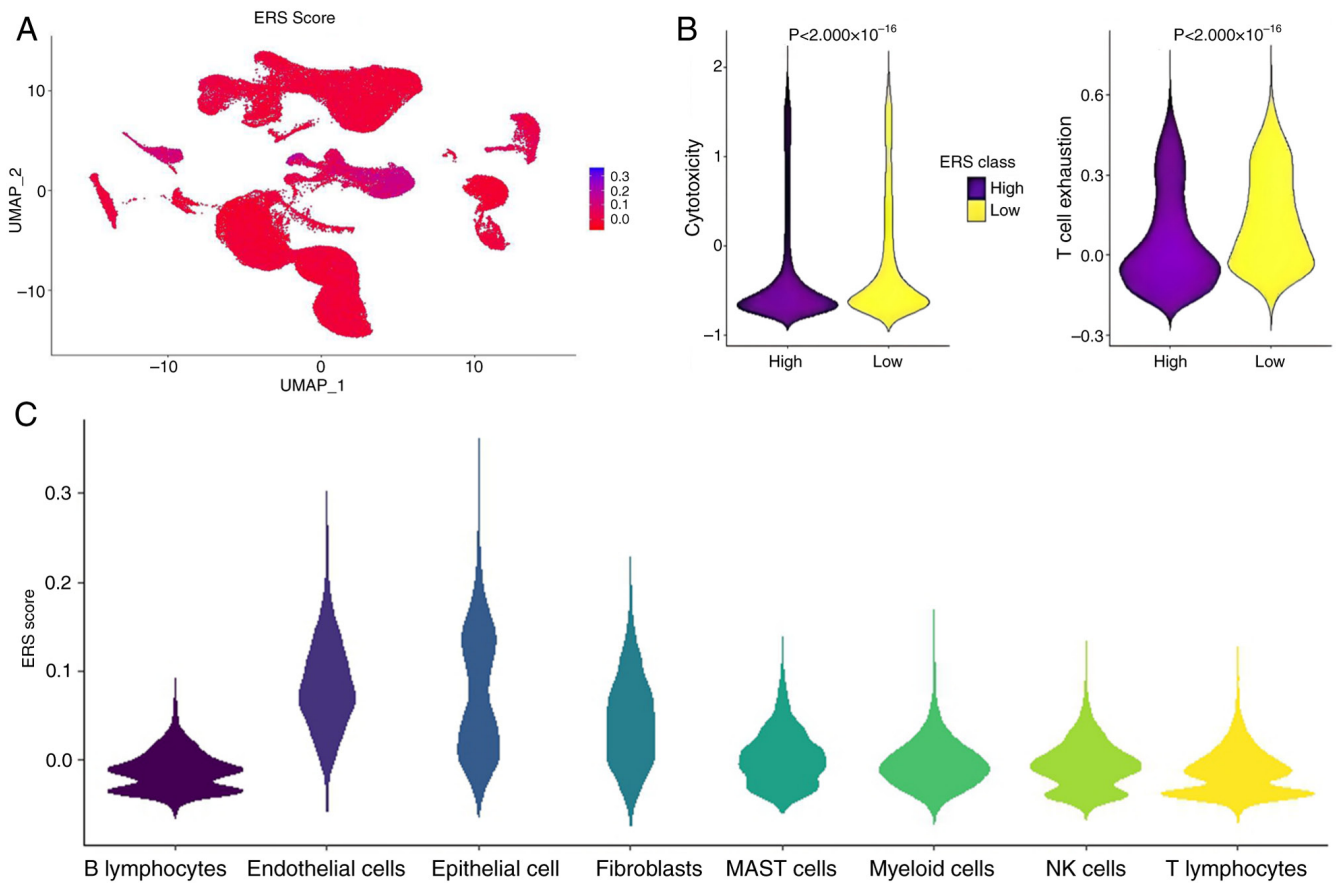


Figure 4. Single-cell ERS activity quantification. (A) t-distributed stochastic neighbor embedding mapping of computed ERS-scores (Add Module Score algorithm). (B) Comparative analysis of cytotoxic potential and T-cell exhaustion markers between ERS-score subgroups (Mann-Whitney; $P < 0.001$). (C) Boxplot visualization of ERS-score distribution across cellular compartments. ERS, endoplasmic reticulum stress.

significant prognostic effects were preliminarily identified from an initial pool of 67 candidate genes. Subsequently, the RSF algorithm was employed to further optimize gene selection, eliminating genes with low or negative contributions to the model (Fig. 8A and B), thereby enhancing the efficiency and accuracy of the model. Building on this, the LASSO regression method was used to construct a prognostic signature model comprising 16 pivotal ERSGs (Fig. 8C and D). By integrating the expression data of these genes, the model calculates an individualized risk score for each patient using the following formula: Risk Score = $F9^* 5.61 + F2^* 0.00052 + IGF2BP1^* 0.050 + GRTA1^* (-0.496) + BCL2L10^* 0.0188 + CDKN2A^* (-0.0006) + CDKN3^* 0.0005 + TXNRD1^* 0.00006 + CAV1^* 0.0001 + CAT^* (-0.0002) + TRPA1^* 0.020 + NOS1^* 0.009 + BDNF^* 0.066 + KCNH2^* (-0.002) + TERT^* 0.062 + CRP^* 0.008$. This risk stratification algorithm provides a quantitative metric for evaluating clinical prognosis. To verify the discriminatory capacity of the predictive model, external validation was performed using TCGA-LUAD ($n=498$) and GSE31210 ($n=226$) cohorts. In TCGA-LUAD, cases were dichotomized into elevated- and reduced-risk subgroups via median risk score thresholding. Significant survival status disparities (χ^2 ; 12.7) and temporal survival advantages were observed between subgroups (Fig. 8E), corroborated by Kaplan-Meier estimator analysis (log-rank, $P=0.032$; Fig. 8F). Temporal predictive performance was quantified through ROC analysis, yielding AUCs of 0.534 (95% CI; 0.48-0.59), 0.484

(0.43-0.54), 0.466 (0.41-0.52), 0.425 (0.37-0.48) and 0.468 (0.41-0.53) for 1-5-year intervals, respectively. Cross-cohort validation in GSE31210 confirmed robust prognostic differentiation (log-rank, $P=1.4 \times 10^{-4}$; Fig. 8G and H), substantiating the pan-cohort applicability and predictive consistency of this ERS-based prognostic classifier.

SMR and colocalization analysis reveals the key role of BDNF in LUAD and its potential drug target. To further investigate the ERSGs closely associated with LUAD expression, the current study uniquely integrated SMR and colocalization analyses. By integrating eQTL data from blood samples with LUAD GWAS, 15 cis-eQTL probes were identified associated with ERSGs. Notably, only the BDNF gene passed the stringent SMR test, revealing a significant association between BDNF and LUAD ($p\text{-SMR}=0.03$; $P_{\text{HEIDI}} > 0.05$) (28), strongly suggesting that BDNF may contribute to the onset and progression of LUAD through pleiotropy or direct causality (Fig. 9A and B). The SMR effect plot is particularly striking, as it visually demonstrates a positive association between BDNF expression levels and LUAD risk. To further substantiate this finding, a colocalization analysis was conducted to assess the colocalization of BDNF, as detected by both SMR and HEIDI tests, between blood eQTL data and LUAD phenotypes. The analysis revealed significant colocalization evidence between BDNF and LUAD traits (PPH4, 0.134), strongly supporting the hypothesis that BDNF is a critical ERSG in LUAD and

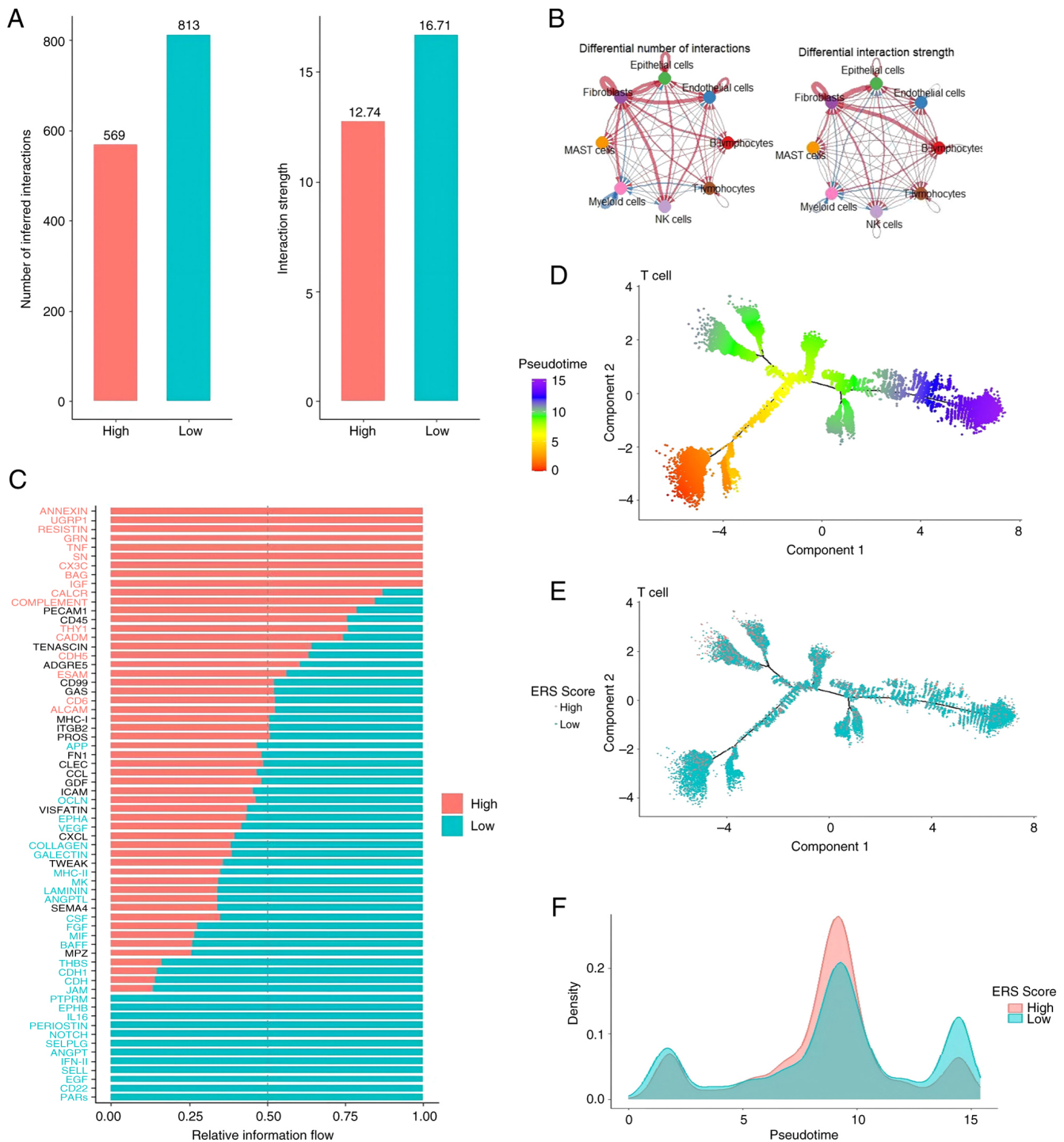


Figure 5. Intercellular communication dynamics. (A) Differential interaction networks between ERS-score subgroups (permutation test FDR<0.05). (B) Cell-type-specific ligand-receptor interaction patterns. Edge color denotes interaction directionality (red: upregulated; blue: downregulated), thickness reflects interaction strength. (C) Pathway activity divergence between subgroups (Wilcoxon; P<0.05). (D) Pseudotemporal trajectory of T-cell differentiation (Monocle3; Q<0.01). (E and F) ICD score progression along developmental pseudotime. ERS, endoplasmic reticulum stress; ICD, immunogenic cell death.

underscoring the need for further in-depth exploration of BDNF in subsequent studies (Fig. 9C).

In light of the potential significance of BDNF in cancer therapy, the present study extended its scope to include molecular docking analyses, with the objective of identifying potential drugs targeting BDNF, building on prior studies that have highlighted the potential of BDNF as a therapeutic

target in cancer (32). Through RT-qPCR analysis, BDNF expression levels were compared between human NSCLC A549 cells and normal human lung epithelial BEAS-2B cells, revealing a significant overexpression of BDNF in A549 cells (P<0.001; Fig. 9D). To investigate the specific mechanisms of BDNF in LUAD, a BDNF knockdown model was successfully established (A549BDNF-siRNA) in A549 cells

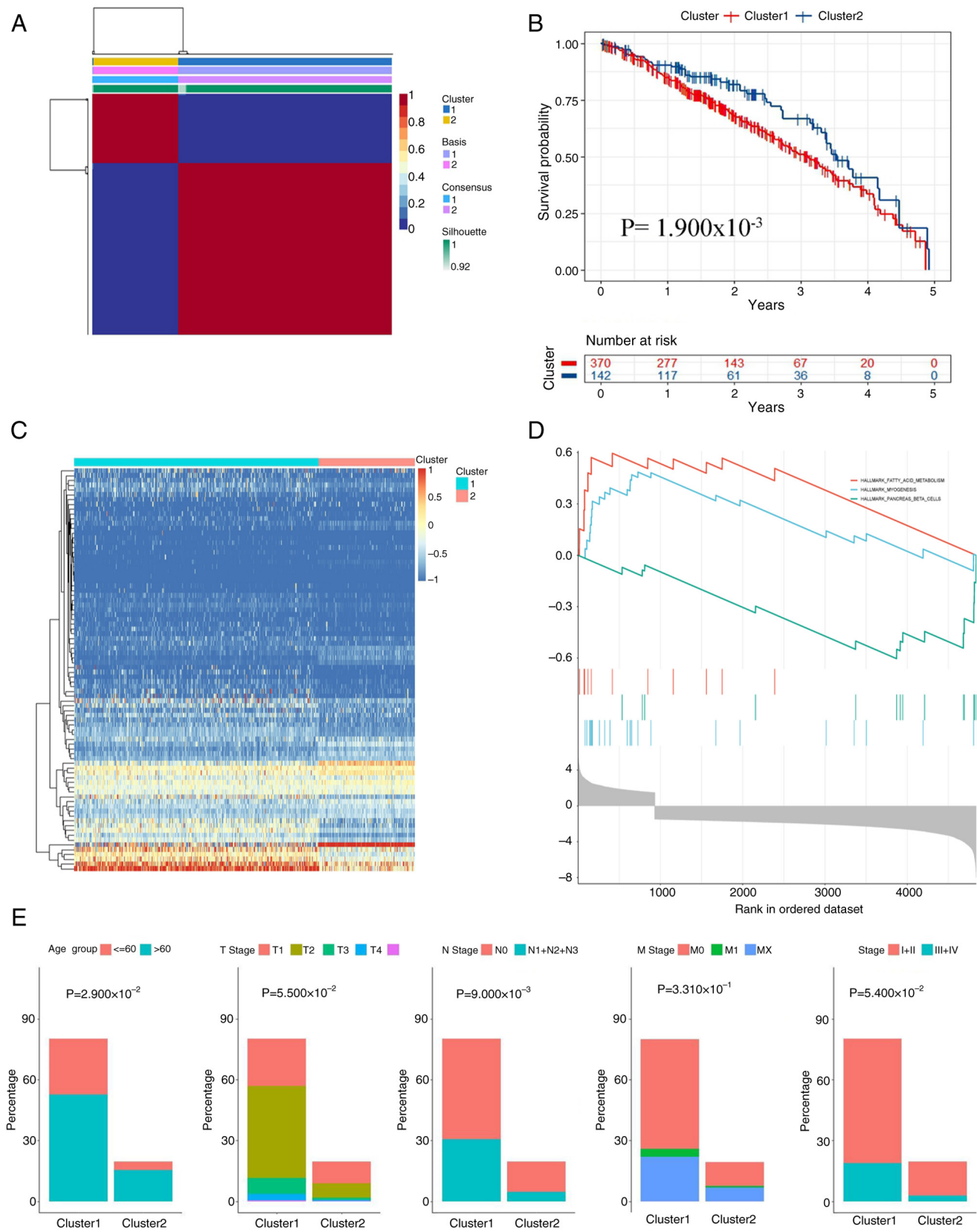


Figure 6. Molecular subtyping based on ERS signatures. (A) Consensus clustering matrix (k=2, 1000 iterations). (B) Survival probability stratification (log-rank, $P=0.008$). (C) Differential ERSGs expression heatmap (DESeq2; FDR<0.05). (D) Gene Set Enrichment Analysis revealing immune-oncogenic pathway activation. (E) Clinical parameter distribution between molecular subtypes (Fisher's exact, $P<0.05$). ERS, endoplasmic reticulum stress; ERSGs, endoplasmic reticulum stress related genes.

using loss-of-function experiments utilizing siRNA (33). Results of RT-qPCR confirmed the knockdown efficiency, thereby ensuring the reliability of the experimental model (Fig. 9E and F). CCK-8 cell viability assays demonstrated that

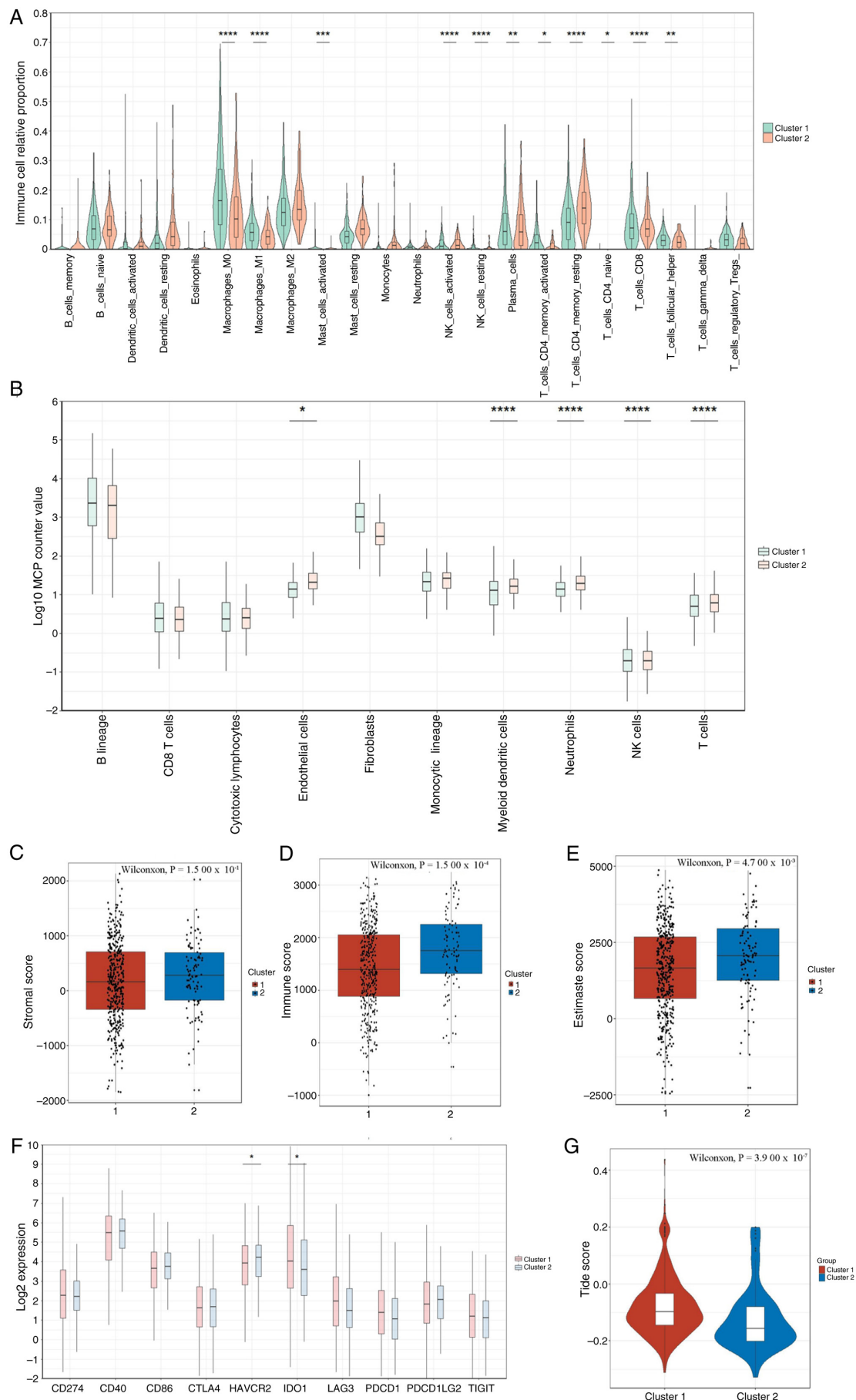


Figure 7. Immune microenvironment characterization. Leukocyte infiltration quantification using (A) CIBERSORTx (v1.06) and (B) MCP-counter (v2.0). (C-E) Stromal-immune component estimation via ESTIMATE algorithm (Benjamini- $P < 0.01$). (F) Differential immune checkpoint expression (Welch's t-test; * $P < 0.05$, **** $P < 0.001$). (G) TIDE prediction score comparison (Wilcoxon; $P = 3.900 \times 10^{-7}$). TIDE, tumor immune dysfunction and exclusion.

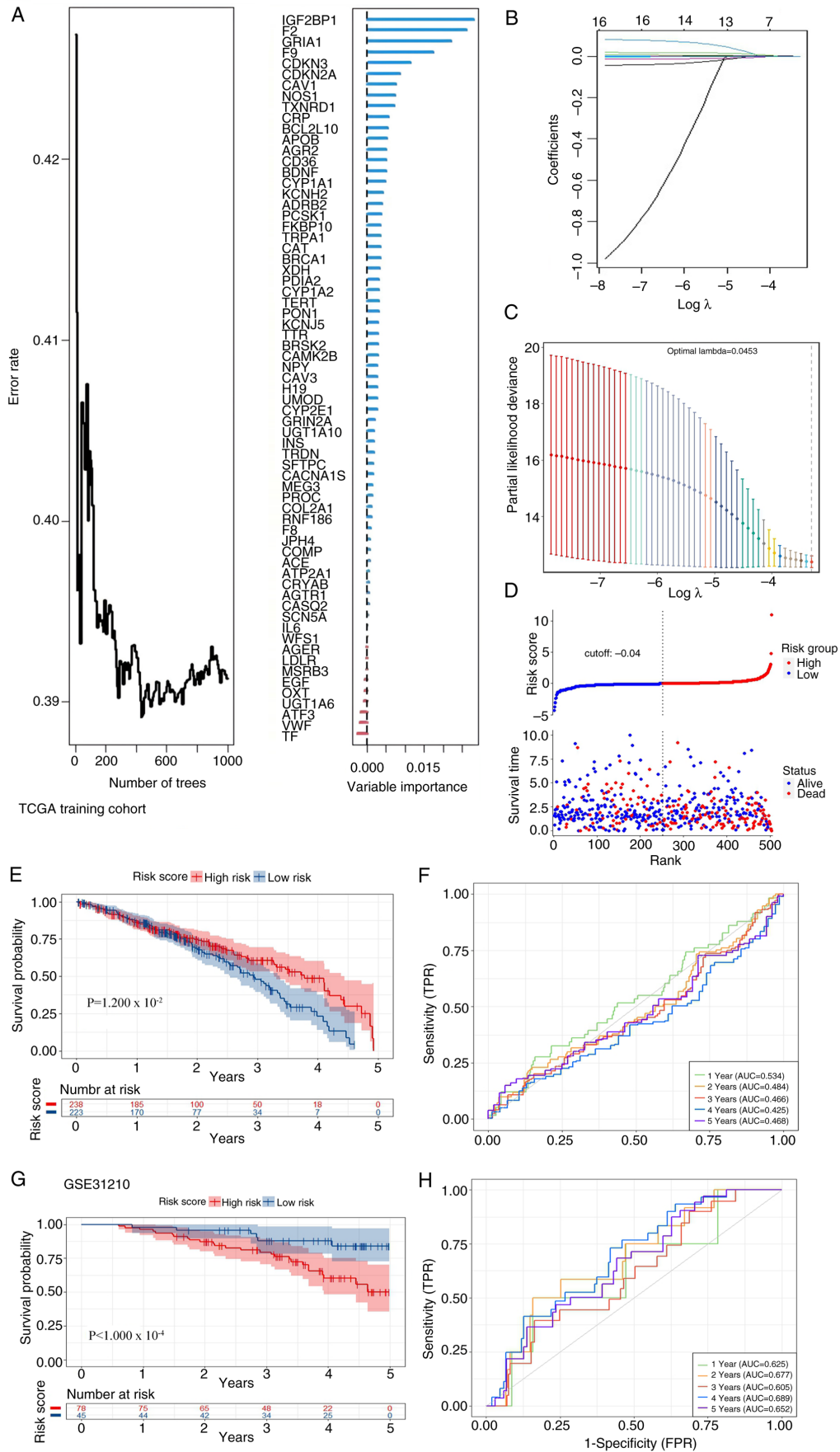


Figure 8. Prognostic model development. (A) Feature selection via random forest regression (ntree=500, mtry=15). Inset: Variable importance metrics. (B) Risk stratification and survival status distribution in TCGA cohort. (C) Least absolute shrinkage and selection operator regression coefficient trajectories ($\lambda=0.021$). (D) Survival probability divergence between risk subgroups (log-rank, $P=0.032$). (E and F) Time-dependent receiver operating characteristic analysis (1-5 year area under the curve) in discovery cohort. (G and H) External validation using GSE31210 dataset (log-rank, $P=1.4 \times 10^{-4}$). TCGA, The Cancer Genome Atlas; FPR, false positive rate.

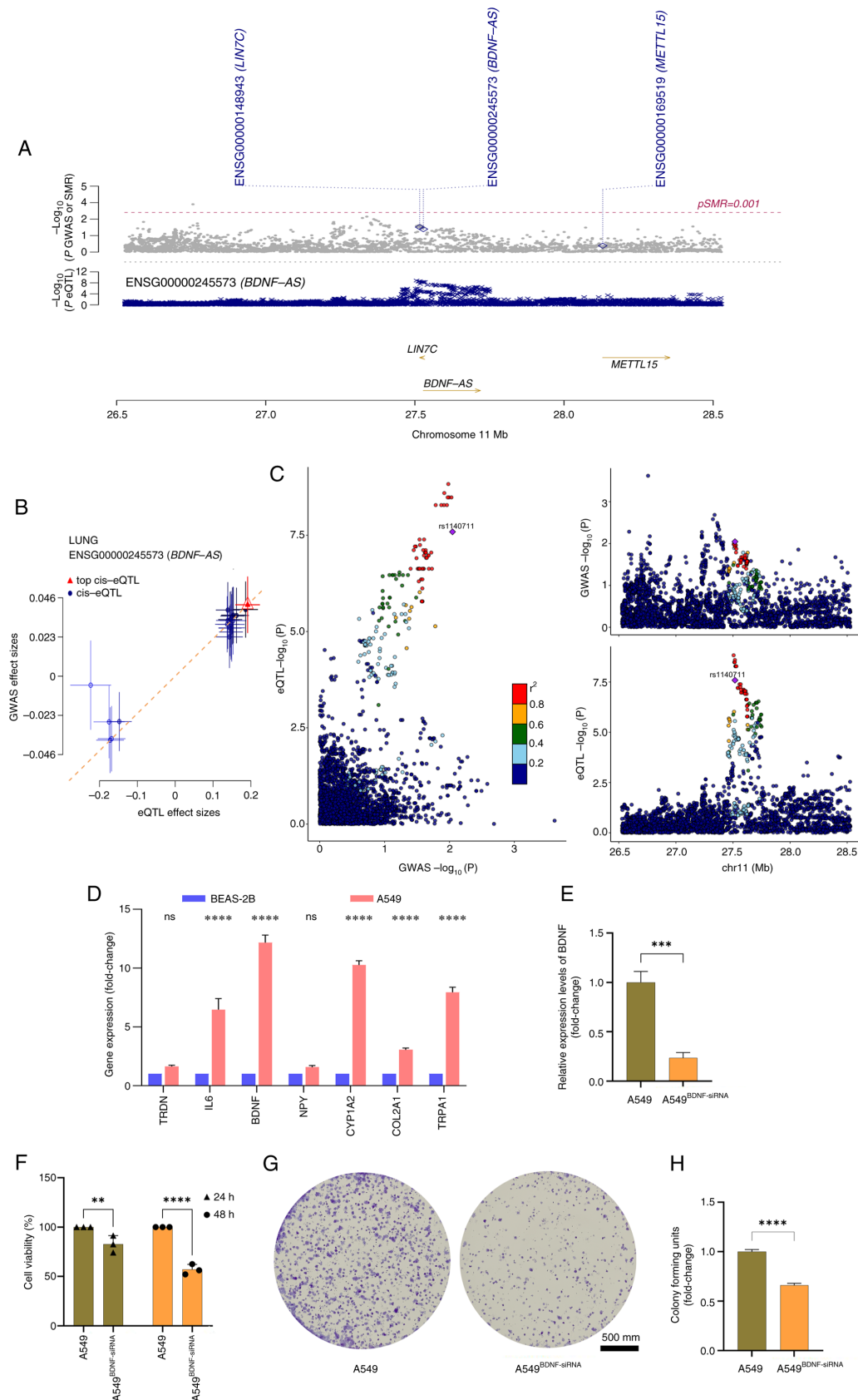


Figure 9. Integrative genomics prioritization of causal ERSGs. (A) SMR visualization at BDNF locus (hg38:11p14.1). Top: Probe passing SMR-HEIDI joint test (HEIDI; $P > 0.01$). Bottom: Causal variants (gray circles) vs. lung eQTLs (red crosses, GTEx v8). (B) Causal effect estimates (Bayes factor > 10) between gene expression and LUAD risk. (C) Colocalization probability analysis (PP.H4 > 0.8) showing shared causal variants (LD, r^2 color gradient; lead variant = purple square). (D) Quantitative PCR validation of ERSGs in A549 (malignant) vs. BEAS-2B (normal) cells (GAPDH-normalized, triplicates), **** $P < 0.0001$. (E) BDNF silencing efficiency (siRNA vs. scramble, $\Delta\Delta Cq$ method), *** $P < 0.005$. (F) Proliferation kinetics (CCK-8 assay) at 24/48 h post-transfection, ** $P < 0.01$, **** $P < 0.0001$. (G and H) Clonogenic capacity assessment (crystal violet staining) with quantitative histograms (triplicate experiments, Mann-Whitney **** $P < 0.0001$). ERSGs, endoplasmic reticulum stress related genes; SMR, summary-Mendelian randomization; BDNF, brain-derived neurotrophic factor; GWAS, genome-wide association studies; eQTLs, cis-expression quantitative trait loci; LUAD, lung adenocarcinoma; siRNA, small interfering RNA.

BDNF knockdown markedly suppressed the proliferation of A549 cells ($P < 0.01$; Fig. 9G). This finding was further corroborated by colony formation assays, which revealed a marked decrease in the colony-forming ability of A549BDNF-siRNA cells ($P < 0.05$; Fig. 9H). These molecular docking results not only offered valuable insights for the development of targeted therapies against BDNF but also establish a robust foundation for subsequent experimental validation.

Expression pattern and functional validation of BDNF in LUAD. The present study sought to elucidate the expression patterns and functional implications of BDNF in LUAD. Molecular docking analyses of BDNF were performed with approved drugs in Drugbank (<https://go.drugbank.com/>). Of the two drugs tested, Esketamine (34) demonstrated the most favorable binding energy (-5.3 kcal/mol), closely followed by chondroitin sulfate (35) with a binding energy of -4.9 kcal/mol (Fig. 10A and B). Esketamine was selected as a potential therapeutic to investigate its effects on A549 cells. Esketamine can downregulate the expression of BDNF (Fig. 10C and D). The impact of Esketamine on A549 cell proliferation was validated using CCK-8 and colony formation assays (Fig. 10E-G). The effects of Esketamine on invasion and migration were corroborated by Transwell and wound healing assays (Fig. 10H-K). The TUNEL assay was employed to detect the apoptotic rate of A549 cells, while western blotting was used to assess the expression of the apoptotic marker proteins Bax and Bcl-2, confirming that Esketamine could induce apoptosis in A549 cells (Fig. 11). These findings not only validate the oncogenic role of BDNF in LUAD but also provide compelling experimental evidence for the development of targeted therapies based on BDNF.

Discussion

In light of the critically low 5-year survival rate of <20% among patients with LUAD (36), there is an urgent need to explore innovative and efficacious therapeutic strategies to enhance patient prognosis. Emerging research highlights that the TME, serving as a critical nexus for malignant tumor progression and immune cell infiltration, instigates substantial ERS. This process not only orchestrates tumor growth and metastasis but also exerts a profound influence on the efficacy of antitumor immune responses (37). Consequently, the precise modulation of ERS to induce direct tumoricidal effects and potentiate antitumor immune responses presents novel therapeutic avenues for LUAD treatment (38). Building on existing research and the comprehensive analysis undertaken in the present study, it is strongly contended that ERSGs occupy a central role in delineating the characteristics of the TME and in predicting prognosis for patients with LUAD.

The current study adeptly integrated a spectrum of biological analysis strategies to systematically elucidate the specific expression patterns of ERSGs in LUAD samples. By harnessing authoritative database resources such as GEO, TCGA and Gene Cards, 67 ERSGs were screened and identified. Through an exhaustive analysis of single-cell sequencing and bulk RNA transcriptome data, it was not only revealed the differential expression profiles of ERSGs in LUAD samples, but also their robust associations with LUAD using SMR and

colocalization analyses were substantiated. This multi-layered, multi-dimensional approach effectively mitigated data noise and bias, thereby providing robust data support for a deeper understanding of the mechanisms underlying the role of ERSGs in LUAD progression.

Notably, the heterogeneity of the TME exerts a profound influence on the efficacy of immunotherapy (39), with varying ERSG expression levels emerging as pivotal determinants shaping immune responses within this intricate milieu (40). The present study revealed that elevated ERSG expression is frequently associated with diminished immune activity, characterized by disrupted immune cell interactions and aggravated T-cell exhaustion. Genomic instability in LUAD promotes sustained ERS, activating the UPR and selectively upregulating pro-survival ERSGs such as BDNF. This creates a permissive microenvironment for tumor progression. Specifically, cell populations with elevated ERS-scores exhibited heightened cytotoxicity and attenuated T-cell exhaustion, further corroborating the critical role of ERSGs in orchestrating the TME. Furthermore, molecular subtype analysis based on RNA data uncovered a positive association between subgroups with low ERSG expression and increased levels of immune cell infiltration, implying that ERSGs could serve as potential targets for modulating tumor immune evasion.

To accurately identify pivotal molecules among ERSGs, the current study used SMR and colocalization analyses, concentrating on eQTL data of ERSGs in blood, ultimately pinpointing BDNF as the central gene within this group. The combined validation through SMR and colocalization analyses not only confirmed the significant expression of BDNF in LUAD samples, but also demonstrated its strong colocalization with LUAD traits, further underscoring the prominence of BDNF as a research priority among ERSGs. BDNF, as a multifunctional protein, is widely acknowledged for its central role in the regulation of apoptosis (41). Previous studies have indicated that BDNF facilitates tumor cell proliferation and invasion by activating the TrkB/PLC γ 1 signaling pathway (42). While BDNF acts as a tumor suppressor in neuroblastoma through TrkB-induced differentiation, its role in LUAD is distinctly oncogenic. This divergence likely stems from LUAD-specific ERS hyperactivation, where chronic UPR reprograms BDNF into a pro-survival factor, corroborated by the observation of BDNF/ERSG co-amplification exclusively in LUAD. The present study validated BDNF overexpression in LUAD through cell experiments and demonstrated that BDNF knockdown markedly inhibited various malignant biological behaviors of LUAD cells, including proliferation, migration and clonogenicity, thereby corroborating the SMR and colocalization findings and reinforcing the potential of BDNF as a therapeutic target. Moreover, molecular docking analysis identified clinically approved drugs such as Esketamine and cisplatin as promising candidates for inhibiting BDNF expression and promoting apoptosis in LUAD cells, thereby offering new avenues for targeted therapeutic strategies.

Although the current study has made significant progress, certain limitations must be acknowledged. First, analyses based on limited database samples may introduce certain biases. Second, SMR and colocalization analyses did not encompass the entirety of ERSG probe data, potentially leading to the omission of key genes. While Esketamine

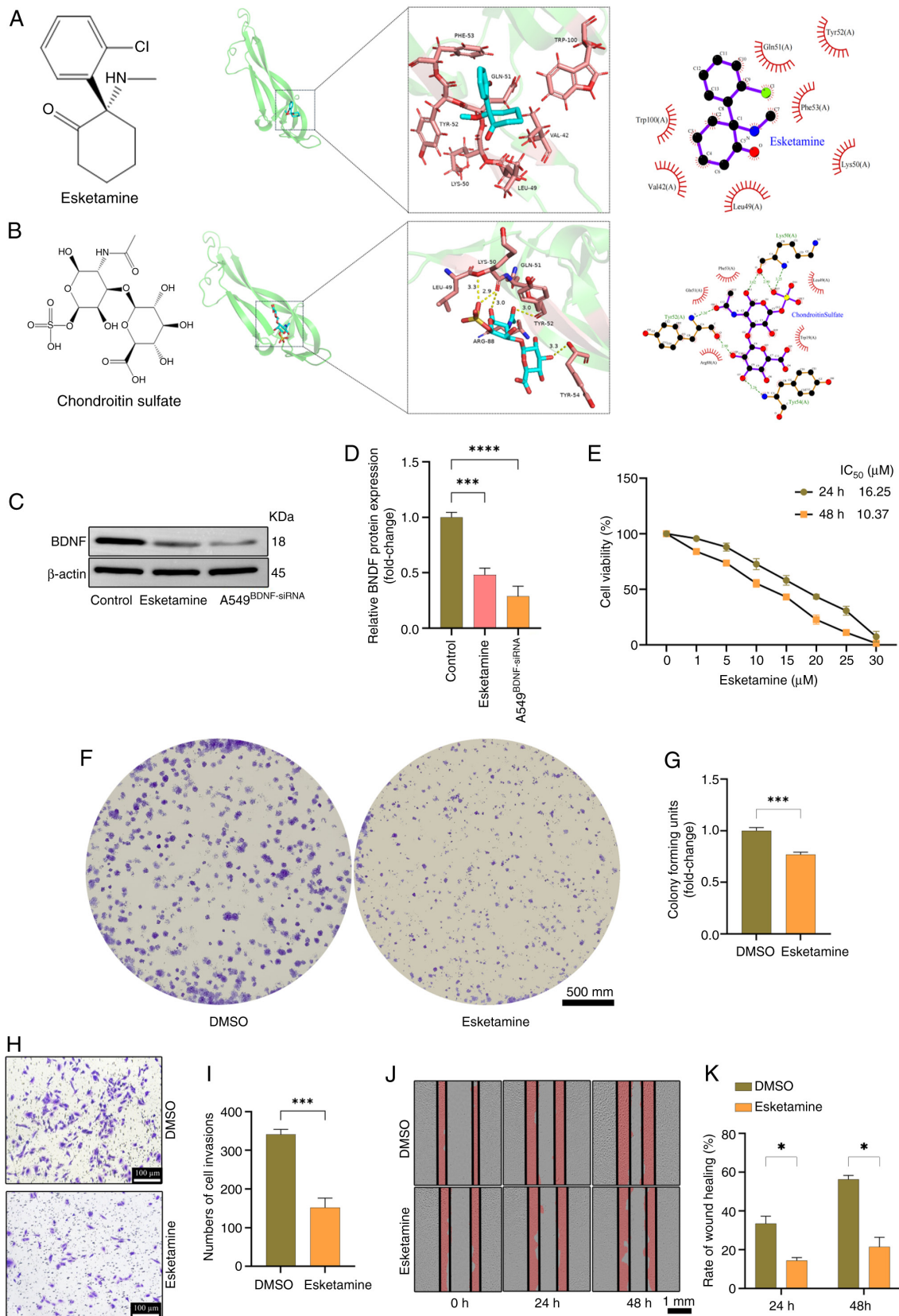


Figure 10. Functional characterization of BDNF in LUAD pathogenesis. Computational docking simulations of BDNF (PDB 1BND) with (A) Esketamine (Glide Score=-8.2 kcal/mol) and (B) Chondroitin (Auto Dock Vina score=-7.6). Left: 2D ligand structures; Center: 3D binding poses; Right: Interaction fingerprint maps. Esketamine-BDNF: -5.3 kcal/mol. Chondroitin sulfate-BDNF: -4.9 kcal/mol. Negative controls: BSA-BDNF: -2.3 kcal/mol. Acetaminophen-BDNF: -4.1 kcal/mol. (C and D) Immunoblot analysis (anti-BDNF 1:1,000, Abcam ab205067) under Esketamine treatment (16.25 μ M, 24 h) or siRNA knockdown (β -actin loading control). (E) Dose-response viability curves (0-50 μ M Esketamine, 72 h). (F and G) Colony formation inhibitory effects (16.25 μ M, 14 days) with quantification (two-way ANOVA *** P <0.0001). (H) Transwell assays and (I) quantified bars of A549 cells treated with Esketamine at a concentration of 16.25 μ M for 24 h, *** P <0.005. (J and K) Bars for detection and quantification of scratch assay in A549 cells treated with Esketamine at a concentration of 16.25 μ M for 24 h. For comparison, some experiments were also performed with cisplatin at a concentration of 5 μ M. * P <0.05. BDNF, brain-derived neurotrophic factor; LUAD, lung adenocarcinoma.

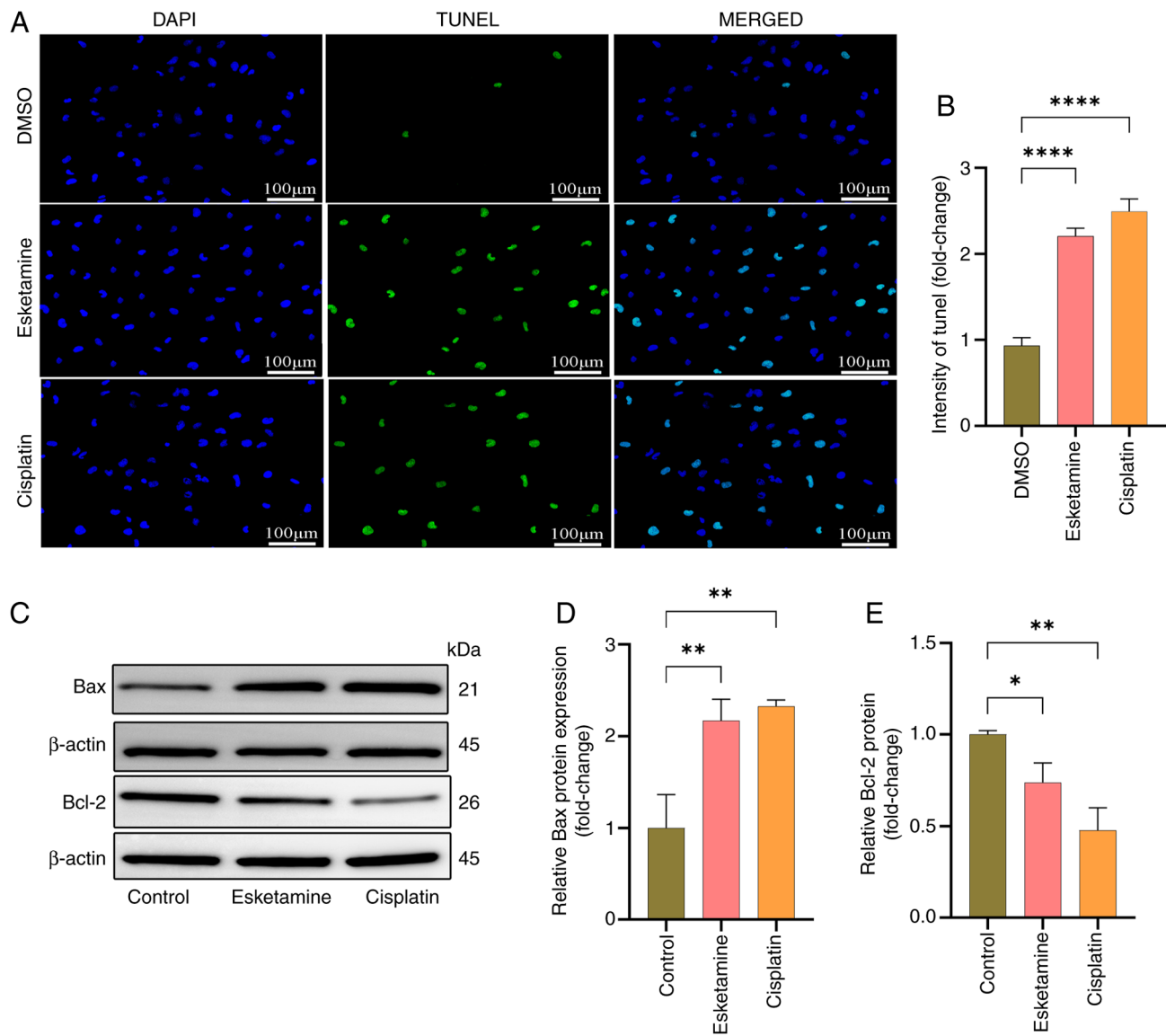


Figure 11. Expression pattern and functional validation of BDNF in lung adenocarcinoma. (A) Tunnel staining and (B) fluorescence plots and quantified bars of A549 cells after treatment with Esketamine at a concentration of 16.25 μ M for 24 h and cisplatin at a concentration of 5 μ M. (C-E) Western blotting results of Bax and Bcl-2 expression in A549 cells treated with Esketamine at a concentration of 16.25 μ M for 24 h and cisplatin at a concentration of 5 μ M. BDNF, brain-derived neurotrophic factor. * P <0.05, ** P <0.01, **** P <0.001.

demonstrates potent anti-BDNF effects, its non-selective NMDA receptor antagonism may cause dose-dependent neurotoxicity or dissociative effects in clinical settings. Future work should explore tumor-targeted delivery systems to mitigate systemic exposure risks. Clinical pharmacokinetic data confirm the rapid distribution and hepatic clearance of Esketamine, aligning with Q3W dosing regimens in ongoing oncology trials. While NMDA receptor binding may cause dissociation, tumor-targeted liposomal delivery reduced neurotoxicity 4-fold in PDAC models without compromising BDNF inhibition. Future research should endeavor to expand sample sizes, refine analytical methods and delve deeper into the comprehensive mechanisms of ERSs in LUAD. Additionally, conducting *in vivo* experiments to validate *in vitro* findings will be an essential step in advancing ERS research towards clinical application.

The present study investigated the role of ERSs in LUAD through a multi-omics integrated analysis and,

in conjunction with experimental validation, identified the potential therapeutic value of the BDNF gene and the BDNF-inhibiting drug Esketamine in hindering the progression of LUAD.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

XD, YT, GT and HN conceived and designed the study and contributed to the acquisition of research materials. XD and YT performed the experiments and conducted the statistical analysis. XD, YT, GT and HN participated in data interpretation and were involved in drafting the manuscript, critically revising it for important intellectual content. The study was supported by funding obtained by XD, GT and YT. XD and YT confirm the authenticity of all the raw data. All authors agree to be accountable for all aspects of the work. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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