

ACSBG1-driven lipid reprogramming unveiled: A fatty acid metabolism-associated prognostic model for lung adenocarcinoma

PANXIN HU¹ and ANAN LI²

¹Department of Emergency Medicine, The First People's Hospital of Taizhou, Taizhou, Zhejiang 318020, P.R. China;

²Department of Cardiothoracic Surgery, The First People's Hospital of Taizhou, Taizhou, Zhejiang 318020, P.R. China

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Abstract. Lung adenocarcinoma (LUAD) remains a leading cause of cancer-associated mortality with limited prognostic biomarkers. Fatty acid metabolism (FAM) reprogramming serves a pivotal role in tumor progression; however, the functional importance of specific FAM genes, such as ACSBG1, in LUAD remains elusive. In the present study, transcriptomics data from The Cancer Genome Atlas (n=517) and Gene Expression Omnibus-GSE13213 (n=117) were analyzed to identify FAM-related differentially expressed genes (DEGs). Least Absolute Shrinkage and Selection Operator regression and Shapley Additive Explanations (SHAP) interpretable analyses established a prognostic model, and *in vitro* experiments using A549 and H1299 cell lines with ACSBG1 overexpression (OE) and knockdown. Functional assays included Cell Counting Kit-8, EdU, Transwell and apoptosis analyses. A total of 35 FAM-related DEGs were identified, which were enriched in PPAR signaling and fatty acid degradation pathways (false discovery rate <0.05). Subsequently, a seven-gene prognostic model was established, and demonstrated strong predictive power for 1-, 3- and 5-year survival (area under the curve values: 0.767, 0.769 and 0.700, respectively). SHAP analysis prioritized ACSBG1 as the dominant protective factor, and its low expression was associated with advanced Tumor-Node-Metastasis stages and poor survival. Mechanistically, ACSBG1 OE suppressed proliferation, migration and invasion, and promoted apoptosis. Immune profiling revealed ACSBG1 expression was positively correlated with the infiltration of CD4⁺ T cells, CD8⁺ T cells and B cells, suggesting its immunomodulatory potential. In conclusion, to the best of our knowledge, the present study established the first FAM-based prognostic model for LUAD, and identified ACSBG1 as a novel tumor suppressor through

dual mechanisms of metabolic regulation and immune micro-environment modulation. The risk score system established in the current study provides a clinically actionable tool for precision oncology, and ACSBG1-targeted therapy represents a promising strategy against LUAD progression.

Introduction

Lung adenocarcinoma (LUAD) is the main subtype of non-small cell lung cancer (NSCLC), accounting for ~50% of all lung malignancies and being a leading cause of cancer-related mortality worldwide (1). Despite advancements in treatment strategies, including surgical resection, chemotherapy, targeted therapy and immunotherapy, the prognosis of patients with LUAD remains unsatisfactory, with the 5-year survival rate of advanced patients being <20% (2). Current clinical challenges stem from tumor heterogeneity, limited biomarkers for early diagnosis and variable responses to immunotherapy. In addition, the traditional Tumor-Node-Metastasis (TNM) staging system cannot adequately predict individual prognosis (3). Therefore, there is a need for novel molecular markers to improve prognostic stratification and guide personalized treatment.

Fatty acid metabolism (FAM) serves a crucial role in tumor biology by facilitating membrane synthesis, energy production, and key signaling pathways for tumor proliferation and metastasis (4-7). Emerging evidence (8) has indicated that LUAD cells exhibit metabolic reprogramming, characterized by enhanced fatty acid uptake, synthesis and storage, to maintain their invasive phenotype. In addition, studies have reported that FAM-related genes (such as FABP5 and PDK1) and long non-coding RNAs (such as CCAT1 and FAM83A-AS1) are key regulators of LUAD progression, associated with poor prognosis and chemotherapy resistance (9-12). Notably, the dysregulated FAM pathway is related to the formation of an immunosuppressive tumor microenvironment and affects the efficacy of immunotherapy (13,14). However, the functional role of specific FAM genes, particularly ACSBG1, in the pathogenesis and clinical outcomes of LUAD remains insufficiently explored.

The present study aimed to systematically identify FAM-related prognostic biomarkers in LUAD and to elucidate the functional role of ACSBG1. By integrating transcriptomics data from The Cancer Genome Atlas (TCGA) and

Correspondence to: Professor Anan Li, Department of Cardiothoracic Surgery, The First People's Hospital of Taizhou, 218 Hengjie Road, Huangyan, Taizhou, Zhejiang 318020, P.R. China
E-mail: ydbxyyyk@163.com

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Gene Expression Omnibus (GEO) databases, differentially expressed FAM genes were screened, and Least Absolute Shrinkage and Selection Operator (LASSO) regression, combined with Shapley Additive Explanations (SHAP) interpretable analysis, was applied to prioritize ACSBG1 as a key prognostic determinant. Subsequently, clinical correlation analysis and *in vitro* functional analysis were conducted to verify the tumor-suppressive effect of ACSBG1.

Materials and methods

Data acquisition. LUAD RNA-sequencing data were extracted from TCGA (n=298) (<https://portal.gdc.cancer.gov/>), including 274 tumor tissues and 24 adjacent tissues. In addition, the GSE13213 (15) dataset (n=117) was obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) as a validation set. The inclusion criteria for data to be included in the present study were: i) Pathological diagnosis of LUAD; ii) complete prognostic information, whereas the exclusion criteria comprised: i) Postoperative follow-up period of <1 month or death within 1 month after surgery; ii) concurrent presence of other malignant tumors.

The R software (version 4.4.2; <https://cran.r-project.org/>) package ‘limma’ (version 3.6.9; <http://bioinf.wehi.edu.au/limma>) was used to conduct a differential expression analysis on the tumor tissues and adjacent tissues in TCGA dataset. The differentially expressed genes (DEGs) that met the criteria of $|\text{Log2FC}| > 1$ and $P < 0.05$ were selected. FAM-related genes were manually curated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/kegg/kegg1.html>) and GeneCards® (<https://www.genecards.org/>) gene sets (Table S1).

Gene Ontology (GO) and KEGG enrichment analyses. The GO analysis process is a commonly used research method in functional enrichment studies, involving large-scale functional enrichment. Gene functions encompass three categories: Molecular function (MF), biological process (BP) and cellular component (CC). KEGG pathway exploration is also widely applied in bioinformatics analysis, as it integrates extensive data on genomes, BPs and diseases. Using R packages ‘limma’ (16) and ‘clusterProfiler’ (version 4.18.4; <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>), enrichment analyses and visualization of FAM-related DEGs were conducted.

Construction of prognostic models and screening of key genes. By integrating LUAD survival information from TCGA, univariate Cox regression analysis was performed on the screened FAM-related DEGs. Using the R package ‘glmnet’ (17) (version 4.1-10; <https://cran.r-project.org/web/packages/glmnet/index.html>) for LASSO regression analysis, seven FAM-related genes significantly associated with tumor overall survival (OS) were identified. Subsequently, SHAP interpretable analysis (18) was applied to interpret the performance of the prognostic model and its outputs, highlighting the features with the greatest contribution to the prognostic model. This analysis was carried out using the ‘kernelshap’ (version 0.9.1; <https://github.com/ModelOriented/kernelshap>) R package.

Diagnostic value of the biomarkers in LUAD. To evaluate the predictive value of the established biomarkers, univariate and multivariate Cox regression analyses were performed to identify risk factors. The prognostic value of the signature genes was assessed using Kaplan-Meier and receiver operating characteristic (ROC) curve analyses. The GEO dataset GSE13213 was utilized to validate the prognostic signature. Based on clinicopathological parameters, a χ^2 test was conducted to examine the association between the risk score and clinical characteristics, and a nomogram was constructed. The R package ‘rms’ (version 6.8-1; <https://CRAN.R-project.org/package=rms>) (19) was employed to generate the nomogram, and calibration plots were introduced to compare the concordance between predicted and actual probabilities of 1-, 3- and 5-year survival rates.

Immune infiltration analysis of ACSBG1 in LUAD. The present study utilized the TIMER2.0 database (<https://compbio.cn/timer2/>) to analyze the correlation between the ACSBG1 gene and immune cells. By adjusting tumor purity to eliminate confounding effects, immune infiltration analysis of ACSBG1 with CD4⁺ T cells, CD8⁺ T cells, B cells, macrophages, natural killer (NK) cells and cancer-associated fibroblasts was analyzed using the Pearson method.

Cell culture and infection. The human LUAD cell line A549 [cat. no. SCSP-503; China Center for Type Culture Collection (CCTCC)] was cultured with F-12K medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The LUAD cell line H1299 (cat. no. SCSP-589; CCTCC) was cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. Both cell lines were maintained under standard culture conditions (37°C, 5% CO₂). For ACSBG1 overexpression (OE) and knockdown (KD), the pLenti-CMV-Puro vector (VectorBuilder Inc.) was used. The third-generation lentiviral packaging system was employed, consisting of the packaging plasmid psPAX2 and the envelope plasmid pMD2.G (Addgene, Inc.). Lentiviral particles were produced by co-transfecting 293T cells (CRL-3216™; American Type Culture Collection) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). In each 10-cm dish, a total of 20 µg plasmid DNA was transfected in a ratio of 2:1.5:1 (lentiviral vector:psPAX2:pMD2.G). Transfection was performed at 37°C in a 5% CO₂ incubator for 48 h. Lentiviral supernatants were collected at 48 and 72 h post-transfection, filtered through a 0.45-µm membrane, and concentrated by ultracentrifugation (100,000 × g, 4°C, 2 h). Prior to formal experiments, the optimal multiplicity of infection (MOI) and puromycin concentration were determined for each cell line via kill curve assays. Based on the results, A549 cells were infected at an MOI of 8, whereas H1299 cells were infected at a higher MOI of 15, both in the presence of 8 µg/ml polybrene. After 24 h of transduction, the medium was replaced with fresh complete medium. To establish stable polyclonal populations, the cells were subjected to puromycin selection: A549 cells were treated with 1.5 µg/ml puromycin (MilliporeSigma) for 6 days, and H1299 cells were treated with 2.5 µg/ml puromycin

for 8 days. Subsequently, the cells were maintained in medium containing a reduced concentration of puromycin (0.5 $\mu\text{g/ml}$ for A549 and 1.0 $\mu\text{g/ml}$ for H1299) for ongoing culture. All functional assays were performed ≥ 14 days after the completion of antibiotic selection to ensure stable gene expression and to avoid transient effects.

Ultimately, two distinct experimental groups were established for comparative analysis: An OE group and a KD group. For the OE group, cells were transduced with either the empty pLenti-CMV-Puro vector (OE-Control) or the same vector containing the full-length human ACSBG1 coding sequence (OE-ACSBG1). For the KD group, cells were transduced with lentivirus expressing either a non-targeting control shRNA (Sh-Ctrl) or a specific shRNA targeting ACSBG1 (Sh-ACSBG1). The shRNA target sequence for ACSBG1 was 5'-GCGCCTCAAAGAATTAATCAT-3', and the non-targeting control sequence was 5'-TTCTCCGAACGTGTCACGT-3'.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was isolated from LUAD cells with Trizol reagent (cat. no. R0016; Beyotime Biotechnology) following the manufacturer's protocol. First-strand cDNA synthesis was performed using the PrimeScript™ Reverse Transcriptase Kit (cat. no. 6210A; Takara Bio, Inc.), according to the thermal cycling conditions recommended for RT. qPCR was performed with the Accurate Genomics Fast SYBR Green qPCR Master Mix (2X) (cat. no. AG11701; Hunan Accurate Bio-Medical Technology Co., Ltd.) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The reaction protocol consisted of an initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. A melt-curve analysis was added at the end of the cycling program (95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec) to confirm amplification specificity. Relative gene expression was calculated using $2^{-\Delta\Delta C_q}$ method (20). The primer sequences were as follows: ACSBG1 gene (human), forward (5'-3') GCACAGTATTTGCAGGTGGC, reverse (5'-3') CCA TGATGACATTGGCGCAG; and GAPDH (housekeeping gene; human), forward (5'-3') GATTCCACCCATGGCAAA TTC, and reverse (5'-3') CTGGAAGATGGTGATGGGATT. Five independent biological replicates were assessed.

Western blot analysis. Total cellular proteins were extracted from cultured LUAD cells using RIPA lysis buffer (cat. no. P0013B; Beyotime Biotechnology) supplemented with 1X protease inhibitor cocktail (cat. no. P1005; Beyotime Biotechnology). The protein concentration was determined using a BCA Protein Assay Kit (cat. no. P0010S; Beyotime Biotechnology) according to the manufacturer's instructions. Protein lysates were mixed with 5X SDS loading buffer (cat. no. P0015; Beyotime Biotechnology) and denatured at 95°C for 10 min. Equal amounts of protein (20 $\mu\text{g/lane}$) were separated by SDS-PAGE on 4-20% gradient gels and subsequently transferred onto 0.22- μm PVDF membranes (MilliporeSigma). The membranes were blocked with 5% skim milk in TBS-0.1% Tween for 1 h at room temperature, followed by incubation overnight at 4°C with the following primary antibodies: Rabbit polyclonal anti-ACSBG1 (1:1,000; cat. no. 16077-1-AP; Proteintech Group, Inc.) and rabbit monoclonal anti-vinculin (1:1,000; cat. no. ab129002;

Abcam). After washing, the membranes were incubated for 1 h at room temperature with a DyLight® 800-conjugated goat anti-rabbit secondary antibody (1:15,000; cat. no. A23620; Abbkine Scientific Co., Ltd.). The blots were then washed and visualized using an Odyssey DLx Imaging System (LI-COR Biosciences). Protein band intensities were semi-quantified using the integrated Image Studio™ Lite software (version 5.2; LI-COR Biosciences).

Cell proliferation assays

Cell counting kit (CCK)-8. A549 and H1299 cells were seeded in a 96-well plate (6,000 cells/well), with five replicate wells per cell line. The plate was incubated under standard culture conditions (37°C and 5% CO_2) for 5 consecutive days. CCK8 assays were performed on days 1-5. Subsequently, 10 μl CCK-8 reagent (cat. no. C0037; Beyotime Biotechnology) was added to each well and the cells were further incubated for 1 h under standard conditions. Finally, the optical density was measured at 450 nm using a microplate reader for analysis.

EdU assay. Cell proliferation was assessed using the BeyoClick™ EdU Cell Proliferation Kit (cat. no. C0078S; Beyotime Biotechnology). The two LUAD cell lines were seeded in 6-well plates with a seeding density of 50% and incubated with EdU-containing medium (10 μM) for 2 h to allow EdU incorporation into replicating DNA. Cells were then fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. After washing with PBS, the cells were incubated with Click reaction buffer containing Alexa Fluor 594 azide for 30 min in the dark, followed by nuclear counterstaining with Hoechst 33342 at room temperature for 20 min. EdU-positive cells exhibiting red fluorescence were quantified under fluorescence microscopy to determine the proliferation rate. This method utilizes click chemistry technology, offering high sensitivity and experimental efficiency for cell proliferation analysis.

Colony formation assay. For the colony formation assay, cells were quantified using a hemocytometer and plated at a density of 1,000 cells/well in 6-well plates containing complete growth medium supplemented with 10% FBS. Following a 14-day incubation period under standard culture conditions (37°C, 5% CO_2), cell colonies were fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently stained with 0.1% crystal violet solution (cat. no. G1062; Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. Colony quantification was performed by capturing high-resolution images using an M7000 inverted phase-contrast light microscope (x40 magnification) and manually counting colonies in three randomly selected fields per well, with colonies defined as cell clusters containing ≥ 50 cells. The colony formation efficiency (%) was calculated as: (number of colonies/number of cells seeded) $\times 100$.

Transwell migration and invasion assays

Migration assay. The cell migration assay was performed in 24-well plates using Transwell chambers (pore size, 8 μm ; cat. no. 3395; Corning, Inc.). For each treatment group, three chambers were used according to the manufacturer's protocol. Briefly, 5×10^3 LUAD cells/well in serum-free medium were seeded into the upper chamber, whereas complete medium

supplemented with 20% FBS was added to the lower chamber as a chemoattractant. After 24 h incubation at 37°C with 5% CO₂, non-migrated cells on the upper membrane surface were removed with a cotton swab. Migrated cells on the lower surface were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystal violet for 15 min at room temperature. Quantitative analysis was performed by counting migrated cells in three randomly selected fields under an M7000 optical microscope (x200 magnification), with results expressed as the mean number of migrated cells per field.

Invasion assay. Matrigel (cat. no. 354230; Corning, Inc.) was mixed with basal medium at a 1:7 ratio and used to uniformly coat the upper chamber surface at 37°C for 30 min. LUAD cells (10,000 cells/well) were seeded into the same upper chamber, where hydrolytic enzymes secreted by the cells facilitated detachment from the Matrigel, enabling migration through the membrane pores. Following a 48-h incubation under standard culture conditions (37°C, 5% CO₂), the chambers were retrieved, and residual non-invasive cells on the upper surface were removed by washing with PBS and mechanical swab abrasion. Invasive cells on the lower membrane were then fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.01% crystal violet for 20 min in the dark at room temperature. Images of three randomly selected fields per chamber were captured and analyzed using an M7000 inverted light microscope (x20 objective) to quantify cell invasion activity.

Wound healing assay. A549 and H1299 cells were cultured in 6-well plates until they reached ~95% confluence. A sterile 100-μl pipette tip was then vertically aligned to the plate surface to generate uniform linear scratches through the cell monolayers. To eliminate confounding effects of cellular proliferation on wound closure, cells were incubated at 37°C (5% CO₂) in serum-free medium supplemented with 1 μg/ml mitomycin C (cat. no. M5353; MilliporeSigma). Migration dynamics were monitored at 0, 12, 24 and 36 h post-scratching using phase-contrast light microscopy (x10 magnification). Quantitative analysis of wound width was performed via ImageJ software (version 1.5.4; National Institutes of Health), with closure rates calculated based on the temporal reduction of the denuded area. Wound closure rate (%) = $[(A_0 - A_t)/A_0] \times 100$; A₀ represents the cell-free scratch area immediately after scratching (0 h); A_t represents the remaining cell-free scratch area measured at a specific time point.

Flow cytometric analysis of apoptosis. Flow cytometric analysis of apoptosis was performed using a commercial Annexin V-FITC/PI apoptosis detection kit (cat. no. C1062S; Beyotime Biotechnology). For each experimental group, the assay was performed in triplicate. Approximately 1 × 10⁶ cells were resuspended in binding buffer and underwent dual staining with FITC-conjugated Annexin V and PI for 15 min under light-protected conditions at 25°C. Flow cytometric quantification was conducted using an Agilent NovoCytte Advanteon Dx VBR system (IVD-CE certified; Agilent Technologies, Inc.) coupled with NovoExpress software (v1.6.2; Agilent Technologies, Inc.), with fluorescence compensation adjusted according to unstained and single-stained controls.

Quantitative analysis of apoptotic subpopulations (viable: Annexin V⁻/PI⁻; early apoptotic: Annexin V⁺/PI⁻; late apoptotic/necrotic: Annexin V⁺/PI⁺) was achieved using threshold parameters optimized for Annexin V/PI signal discrimination.

Statistical analysis. Continuous variables were compared using the Wilcoxon rank-sum test (for non-normally distributed data) or unpaired Student's t-test (for normally distributed data). Survival curve divergences in Kaplan-Meier analyses were evaluated using a two-sided log-rank test. All statistical analyses and graphical visualizations were performed using R software version 4.4.1 and GraphPad Prism 10.1 (Dotmatics). All experiments were independently repeated at least three times, unless otherwise stated. P < 0.05 was considered to indicate a statistically significant difference.

Results

Screening and functional enrichment analysis of FAM-related DEGs. RNA expression data from 298 cases in the LUAD group were collected from TCGA database. After intersecting with FAM-related genes, 35 DEGs were screened out (Fig. 1A), including 19 upregulated genes and 16 downregulated genes (Fig. 1B). GO functional and KEGG pathway enrichment analyses were performed on the aforementioned DEGs.

The results of GO functional enrichment analysis showed that in the BP category, the DEGs were mainly involved in 'fatty acid metabolic process', 'organic acid biosynthetic process', 'carboxylic acid biosynthetic process', 'sulfur compound metabolic process' and 'fatty acid biosynthetic process'. In the CC category, the DEGs were mainly related to the 'mitochondrial matrix', 'peroxisome' and 'microbody'. In the MF category, the DEGs were mainly associated with 'lyase activity', 'carbon-oxygen lyase activity' and 'oxidoreductase activity, acting on the CH-CH group of donors' (Fig. 1C). The KEGG signaling pathways were mainly concentrated in the 'PPAR signaling pathway', 'fatty acid degradation', 'fatty acid metabolism', 'glycolysis/gluconeogenesis' and 'adipocytokine signaling pathway' (Fig. 1D).

Construction and evaluation of a prognostic risk model. Univariate Cox analysis showed that seven FAM-related genes were significantly associated with the prognosis of patients with LUAD (Fig. 2A). After further LASSO regression analysis, a prognostic risk model for LUAD was constructed (Fig. 2B). After integrating the seven genes and weighting their multivariate Cox regression coefficients, the risk score formula was obtained: Risk score = $(-0.149 \times \text{ALDH2 exp}) + (-0.916 \times \text{ACSBG1 exp}) + (-0.028 \times \text{PTGDS exp}) + (0.128 \times \text{PTGR1 exp}) + (-0.310 \times \text{LTA4H exp}) + (0.143 \times \text{CA2 exp}) + (0.293 \times \text{SMS exp})$. Using the median risk value as the cut-off, patients were divided into high-risk and low-risk groups (Fig. 2C). The Kaplan-Meier survival curves in both the training set (TCGA) and test set (GSE13213) showed that the survival rate of patients in the high-risk group was significantly lower than that in the low-risk group (P < 0.05; Fig. 2D and E). The time-dependent ROC curve was used to evaluate the predictive performance of the prognostic model for the 1-, 3- and 5-year prognosis of patients. The area under the curve (AUC) values for 1, 3 and 5 years were 0.767, 0.769 and 0.700, respectively (Fig. 3C). The

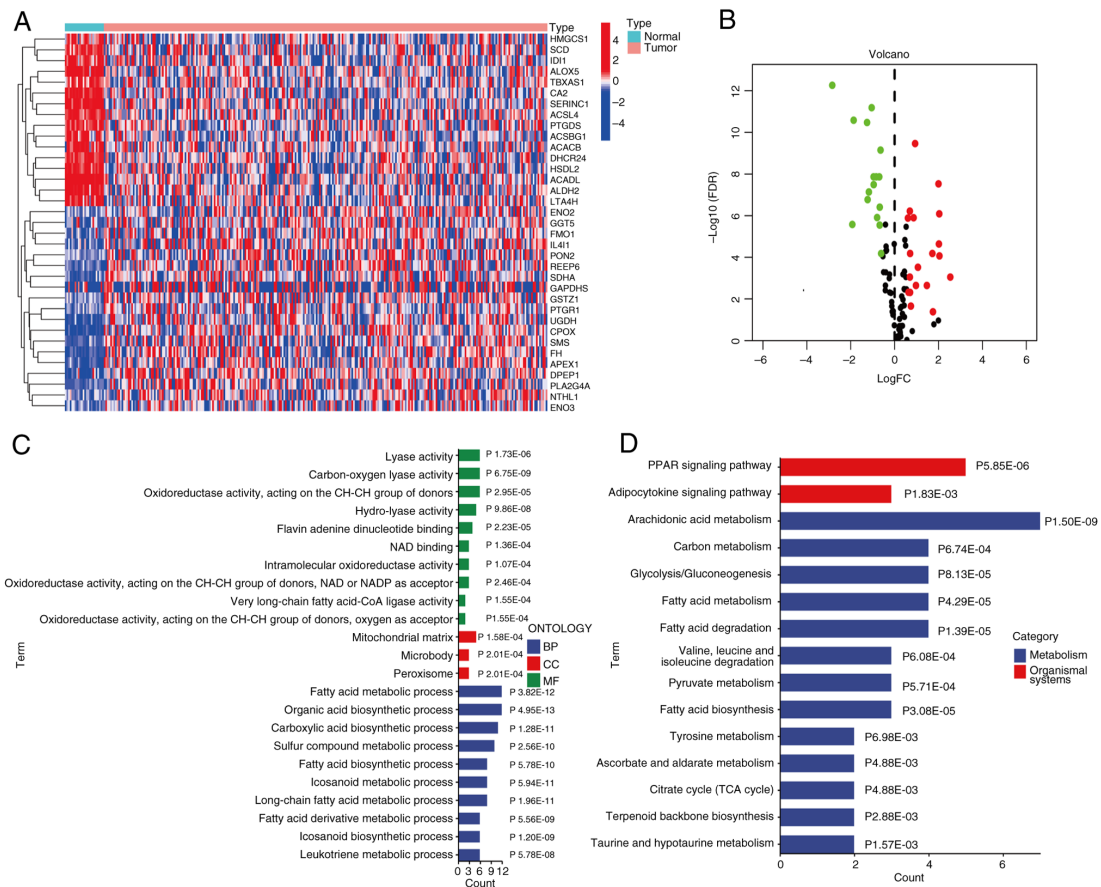


Figure 1. Screening and functional enrichment of differentially expressed genes related to fatty acid metabolism in lung adenocarcinoma. (A) Heat map. (B) Volcano map. Red dots indicate upregulated genes and green dots indicate downregulated genes. Results of (C) Gene Ontology and (D) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. BP, biological process; CC, cellular component; FC, fold change; FDR, false discovery rate; MF, molecular function.

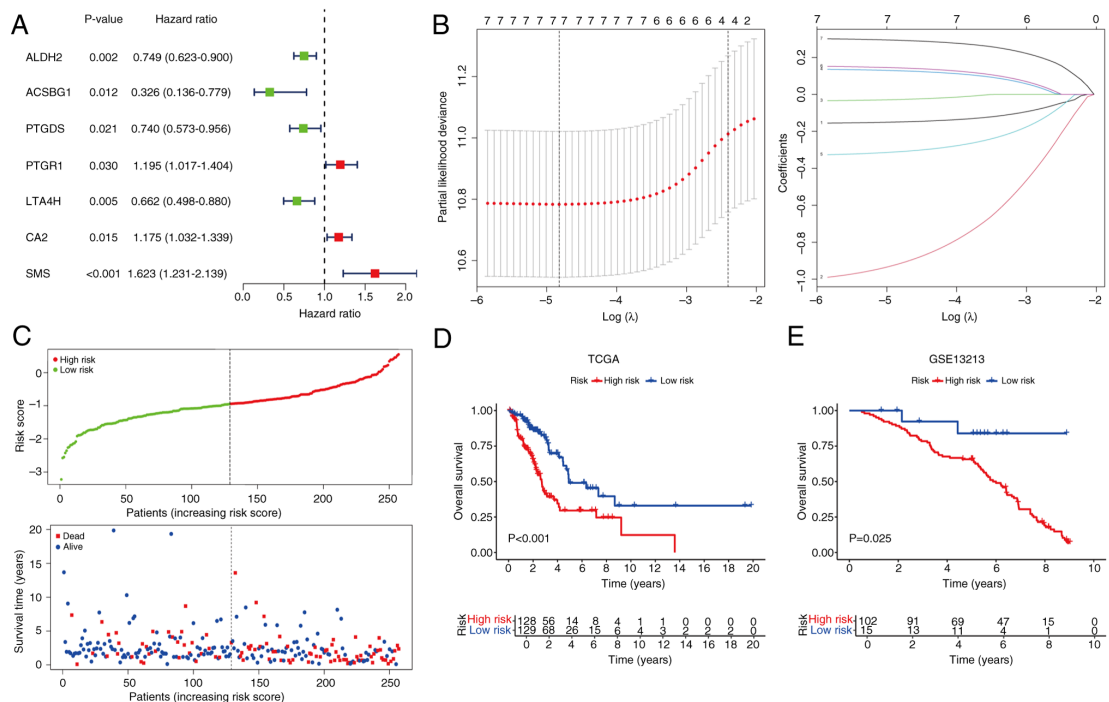


Figure 2. Construction and evaluation of prognostic models. (A) Forest plot showing the overall survival-related fatty acid metabolism genes in LUAD. (B) Least Absolute Shrinkage and Selection Operator regression analysis. (C) Distribution of risk scores in patients with LUAD. Kaplan-Meier curves show the difference in survival between low- and high-risk patients in (D) TCGA and (E) GSE13213 datasets. LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas.

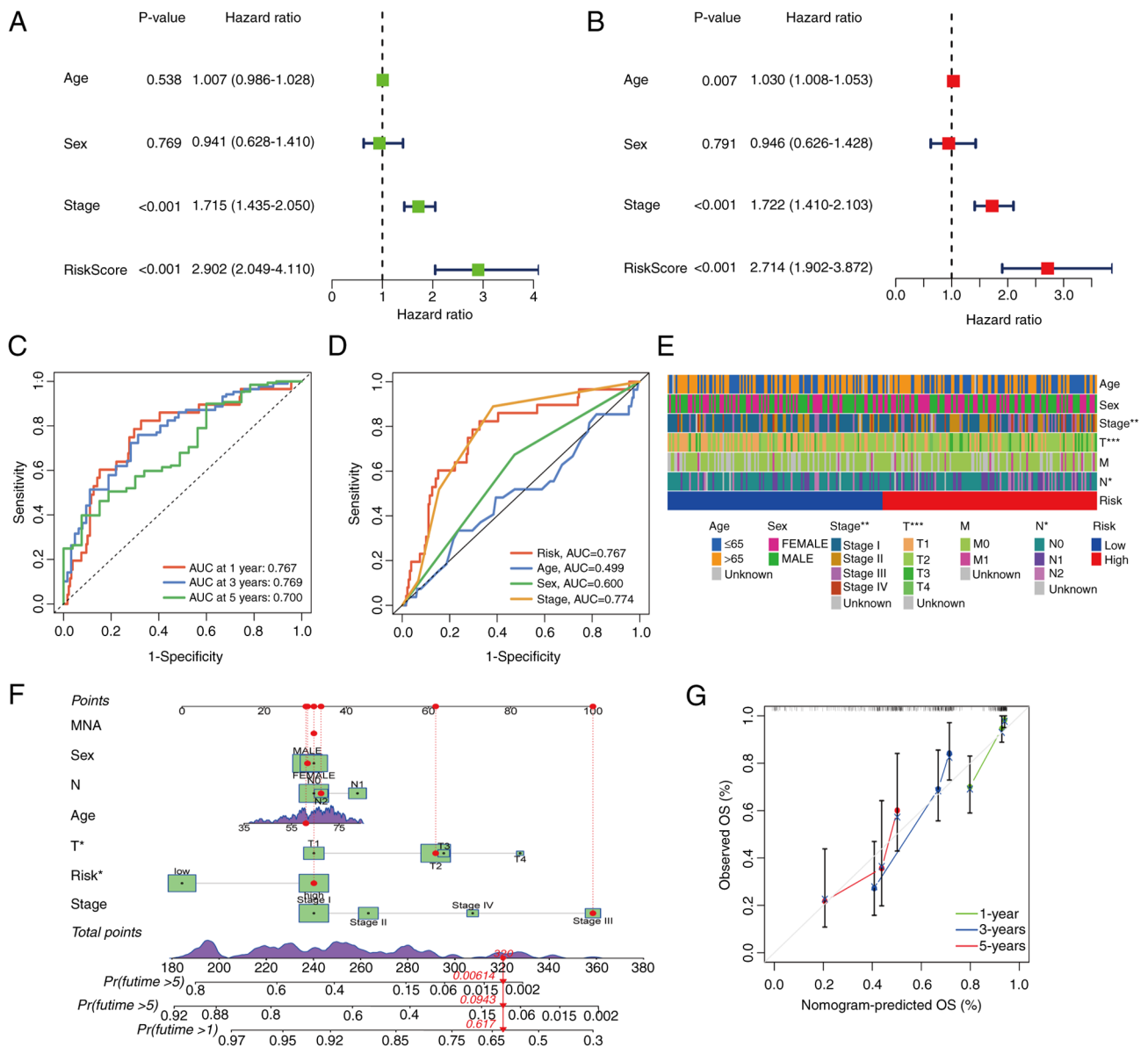


Figure 3. Verification of the accuracy of the prognostic scoring model. (A) Univariate and (B) multivariate independent prognostic analyses. (C) ROC curves for 1-, 3- and 5-year OS. (D) ROC curves for clinical characteristics and risk scores. (E) Heat map of clinically relevant features. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (F) Nomogram integrates multiple clinicopathological characteristics, including sex, age, T stage and risk grade, to provide individualized predictions of patient survival probability at different time points. (G) Calibration plot predicting the agreement between observed and predicted rates of OS at 1, 3 and 5 years. AUC, area under the curve; OS, overall survival; ROC, receiver operating characteristic.

forementioned evaluation results indicate that the risk score model has good sensitivity and specificity in predicting the prognosis of LUAD.

Analysis of the association between the independent prognostic risk model and clinical characteristics. The risk scores of the seven model genes were combined with the clinical data (age, sex, stage and grade) of LUAD samples from TCGA. Univariate Cox analysis showed that stage and risk score were risk factors for the prognosis of patients with LUAD ($P < 0.05$; Fig. 3A). Multivariate Cox analysis also revealed that stage and risk score were reliable independent prognostic factors for LUAD ($P < 0.05$; Fig. 3B). The ROC curve indicated that stage (AUC=0.767) and risk score (AUC=0.774) had good predictive values for the prognosis of LUAD (Fig. 3D). Furthermore, the χ^2 test demonstrated that there were differences in the

distribution of tumor stage, T stage, N stage and survival status between the high- and low-risk groups (Fig. 3E). Subsequently, a nomogram was constructed that incorporated age, sex, pathological grade, as well as T and N stages (Fig. 3F). The calibration curves for 1-, 3- and 5-year survival indicated that the nomogram could accurately predict the OS of patients with LUAD (Fig. 3G).

Interpretation of the prognostic model and screening of the prominent contributing features using SHAP analysis. The SHAP algorithm is used to determine the importance of each variable on the prediction results of a prognostic model. The importance of each variable in descending order is shown in Fig. 4A. ACSBG1 had the strongest predictive value across all prediction levels, followed by LTA4H, CA2, SMS, ALDH2 and PTGR1.

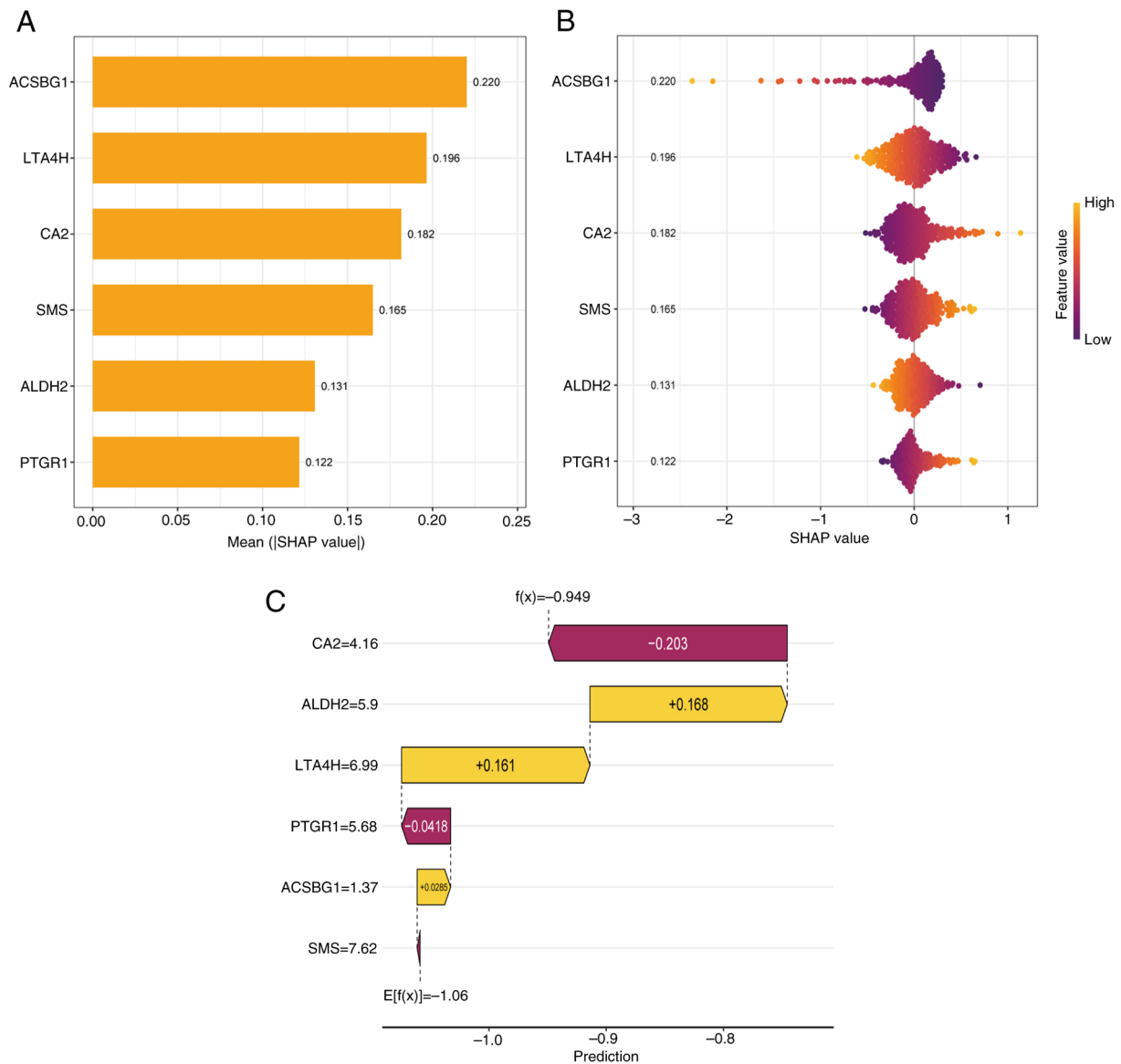


Figure 4. SHAP interpretable analysis of the contribution degree and direction of each gene feature on the prediction results of the prognostic model. (A) Feature importance bar chart shows the overall influence intensity of each gene feature on the model output, ranked by the average absolute SHAP value. The longer the bar, the more important the feature. (B) Feature dependency swarm plot: Each point represents a sample, showing the distribution relationship between the specific gene feature value and its SHAP value. The more dispersed the points or the more gradient of colors, the stronger the nonlinear effect of the feature on the prediction result. (C) Local explanation waterfall chart: Taking a single sample as an example, it shows how each gene feature pushes its predicted value from the baseline to the final output. The yellow bar represents the positive contribution that increases the prediction risk, and the purple bar represents the negative contribution that reduces the risk. SHAP, Shapley Additive Explanations.

In addition, in order to detect the positive and negative associations between the predicted values and the target results, SHAP analysis was employed to reveal the proportion of risk factors in the prognostic model. As shown in Fig. 4B, the horizontal position indicates whether the influence of the value is associated with higher or lower predictions, and the color represents the gene expression level, with purple indicating low expression and orange representing high expression. As the SHAP value of ACSBG1 increased, the gene expression decreased, suggesting that this gene is a protective factor.

In Fig. 4C, the ordinate represents the gene expression level, and the abscissa represents the prediction value of the feature. $E[f(x)]$ denotes the baseline prediction value. It can be observed that CA2, PTGR1 and SMS are classified into the low-risk group, whereas the predicted values of ACSBG1, LTA4H and

ALDH2 were higher than the baseline levels; therefore, these genes were classified as the high-risk group.

Immune-related analysis. The results of immune-related analysis revealed that the expression of ACSBG1 was correlated with the infiltration of CD4⁺ T cells, CD8⁺ T cells, B cells, M2 macrophages, cancer-associated fibroblasts and NK cells (Fig. 5). However, it is worth noting that the correlation between these immune cells and ACSBG1 was relatively weak.

Cell infection and validation. After lentiviral transduction, stable cell lines were established via puromycin selection for 4 weeks, including the OE-Ctrl, OE-ACSBG1, Sh-Ctrl and Sh-ACSBG1 groups. RT-qPCR and western blot analysis were performed to determine the mRNA and protein expression

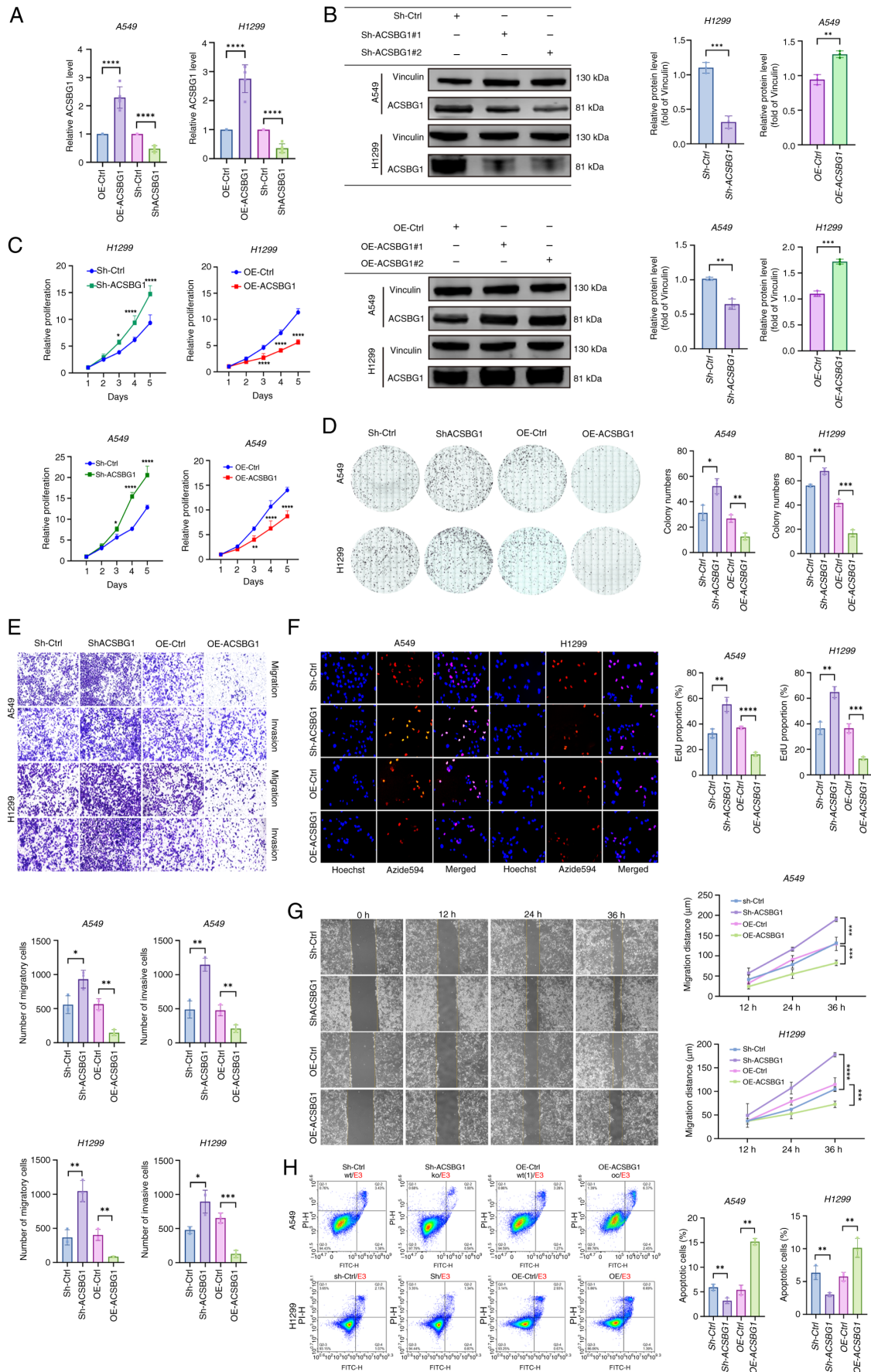


Figure 5. Functional role of ACSBG1 in modulating cellular phenotypes. (A) mRNA and (B) protein expression level verification post-transfection. (C) Cell Counting Kit-8 assay was used to analyze the proliferation of cells. (D) Colony formation assay verified the colony-forming ability of cells (x40 magnification). (E) Transwell assays verified the migration and invasion of cells (x200 magnification). (F) EdU assay was used to analyze the proliferation of cells (x200 magnification). (G) Wound healing assays verified the migration of the cells (x10 magnification). (H) Annexin V-FITC/PI cell apoptosis assay verified the apoptosis rate of cells. Ctrl, control; OE, overexpression; Sh, short hairpin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

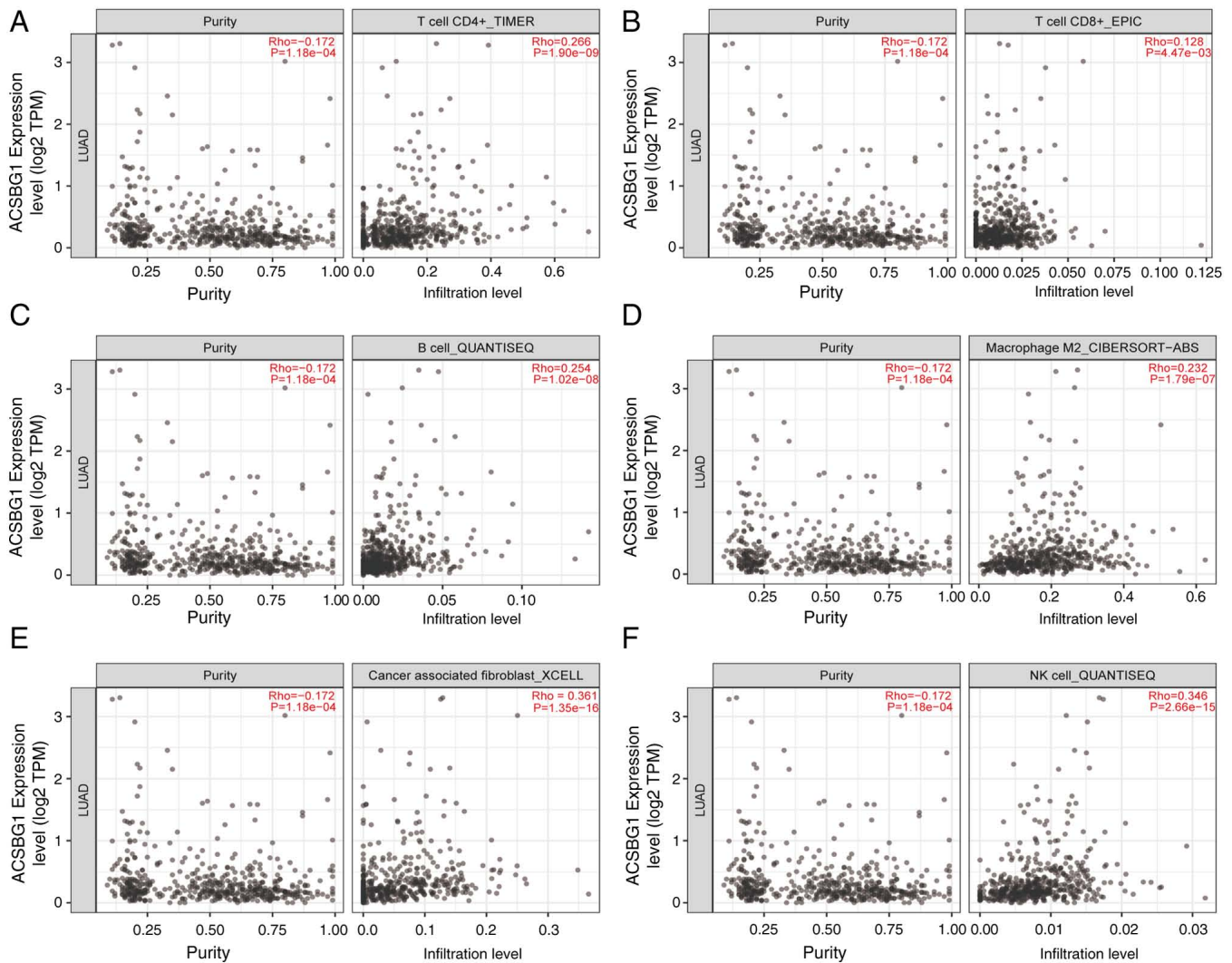


Figure 6. Immune infiltration analysis of the ACSBG1 gene in LUAD. (A) CD4⁺ T cells, (B) CD8⁺ T cells, (C) B cells, (D) M2 macrophages, (E) cancer-associated fibroblasts and (F) NK cells. LUAD, lung adenocarcinoma; NK, natural killer; TPM, transcripts per million.

levels of ACSBG1 in A549 and H1299 LUAD cell lines. The experimental results clearly demonstrated that, compared with in their respective control groups (OE-Ctrl or Sh-Ctrl), both the mRNA and protein levels of ACSBG1 were significantly upregulated in the OE-ACSBG1 group and downregulated in the Sh-ACSBG1 group (Fig. 6A and B). According to the experimental results, shACSBG1# and OE-ACSBG1# were selected for use in further experiments.

ACSBG1 inhibits the proliferation of LUAD cells in vitro. As determined by CCK-8 assay, the OE of ACSBG1 suppressed the proliferation of A549 and H1299 LUAD cells, whereas its KD promoted cell proliferation (Fig. 6C). The experimental results showed that statistically significant differences between the OE-ACSBG1/OE-Ctrl groups and the Sh-ACSBG1/Sh-Ctrl groups regarding cell proliferation rates were only observed after 72 h of culture.

By co-labeling live cells with blue Hoechst dye and proliferating cells with red Alexa Fluor 594 azide, the proliferative status of cells within the field of view can be visualized. Fig. 6F shows representative images of the proliferation process in A549 and H1299 cells. OE of ACSBG1 significantly

suppressed the proliferation of LUAD cells compared with that in OE-Ctrl cells, whereas KD of ACSBG1 led to a marked increase in their proliferation rate compared with in the sh-Ctrl group.

Validation of colony-forming ability. The colony formation assay reflects the proliferation capacity of tumor cells. In A549 and H1299 cell lines, compared with in the Sh-Ctrl group, the Sh-ACSBG1 group exhibited significantly enhanced colony-forming ability, whereas the OE-ACSBG1 group showed a significant reduction in colony formation compared with that in the OE-Ctrl group (Fig. 6D). These results are consistent with those from CCK-8 and EdU assays.

Validation of cell migration and invasion. The current study evaluated the impact of ACSBG1 on the migratory and invasive phenotypes of LUAD cells using Transwell invasion and migration assays. Compared with in their respective control groups (OE-Ctrl or Sh-Ctrl), OE of ACSBG1 significantly inhibited the migratory and invasive capacities of LUAD cells, whereas KD of ACSBG1 markedly promoted these phenotypes

(Fig. 6E). The wound healing assay indicated statistically significant differences in wound healing capacity at the 36-h time point, with the expression level of ACSBG1 exhibiting an inverse association with the wound healing capacity of LUAD cells (Fig. 6G).

Validation of apoptosis rate. In LUAD cells, the Sh-ACSBG1 group exhibited significantly reduced apoptosis rates compared with those in the Sh-Ctrl group (quadrants 2 and 4 represent the early and late apoptosis rates), whereas the OE-ACSBG1 group exhibited significantly increased apoptosis rates compared with those in the OE-Ctrl group (Fig. 6H).

Discussion

LUAD, as the main subtype of NSCLC, has long presented a marked challenge in clinical treatment due to its high early recurrence rate and poor prognosis (21-25). Studies have shown that fatty acid metabolic reprogramming serves a crucial role in the occurrence, development and treatment resistance of LUAD. Tumor cells create a metabolic microenvironment conducive to their own proliferation and metastasis by upregulating fatty acid synthesis pathways [such as the acetyl-CoA conversion process, which is mediated by genes such as fatty acid synthase (FASN) and acetyl-CoA carboxylase], enhancing lipid uptake and inhibiting fatty acid oxidation (26-28). In EGFR-mutated LUAD, FASN-mediated palmitoylation modification promotes resistance to targeted therapy, whereas stearoyl-CoA desaturase 1 inhibitors can reverse gefitinib resistance (29). Moreover, abnormal lipid metabolism is closely associated with the immunosuppressive microenvironment. High lipid accumulation can markedly affect the therapeutic efficacy of immune checkpoint inhibitors by regulating the fatty acid oxidation of tumor-associated macrophages and the infiltration of CD8⁺ T cells (30-33). This indicates that constructing a prognostic model related to FAM is not only biologically reasonable but also holds promise for providing new targets for personalized treatment.

In the present study, a prognostic risk model based on FAM genes was successfully constructed, and ACSBG1 was screened as the core regulatory factor. This risk scoring model demonstrated stable predictive performance in both the training set and the independent validation set (AUC values for 1/3/5 years were >0.7). SHAP interpretability analysis revealed that ACSBG1 made the highest contribution to prognostic prediction. Clinical cohort analysis showed that the high-risk group was significantly associated with TNM stage, stage classification and shortened OS. Downregulation of its expression may lead to defects in lipid antigen presentation, thereby creating an immunosuppressive microenvironment. These findings provide a novel perspective for an in-depth understanding of the interaction between metabolic reprogramming and tumor immune escape.

Given the importance of ACSBG1, the current study further investigated the role of this gene in LUAD. As a member of the acyl-CoA synthetase family, the ACSBG1 gene serves a vital role in the activation of long-chain fatty acids. It catalyzes the combination of fatty acids with coenzyme A to generate acyl-CoA, providing substrates for β -oxidation and phospholipid synthesis (34). Notably, it has been shown that the abnormal expression of ACSBG1 is closely related

to metabolic reprogramming in cancer (35). For example, in breast cancer, low expression of ACSBG1 is associated with increased lipid accumulation and poor prognosis (35). It has been demonstrated that breast cancer cells with ACSBG1 OE acquire proliferative advantages in nutrient-deprived microenvironments by enhancing fatty acid uptake and oxidation. This metabolic reprogramming provides substantial acetyl-CoA to the tricarboxylic acid cycle, thereby promoting the synthesis of ATP and phosphocreatine (35). However, the function of ACSBG1 in LUAD remains controversial. Although an earlier study suggested that it may limit tumor growth by maintaining lipid homeostasis, direct experimental evidence is lacking (36). In the current study, lentiviral plasmid infection technology was used in LUAD cells to induce ACSBG1 OE and KD, and functional experiments were conducted to verify ACSBG1 as a tumor suppressor gene inhibiting the progression of LUAD. The experimental results showed that the expression levels of ACSBG1 were negatively associated with the progression of LUAD, which was highly consistent with the tumor suppressor phenotype observed clinically.

It is worth noting that although the present study comprehensively analyzed the role of ACSBG1 in LUAD, there are still some limitations to be addressed. Specifically, the observed correlations between ACSBG1 expression and immune cell infiltration do not establish causality. The analysis was based on bioinformatics associations from public databases and lacks experimental validation of direct mechanistic links between ACSBG1 and immune modulation. Further studies, such as co-culture assays or *in vivo* models, are needed to confirm whether ACSBG1 directly influences immune cell recruitment or function. In-depth exploration of the potential molecular mechanisms and downstream signaling pathways affected by ACSBG1 will also contribute to a more comprehensive understanding of its clinical relevance and potential therapeutic targets. In addition, more internal clinical samples are required to validate the results.

In conclusion, by integrating the transcriptome data of LUAD from TCGA and GEO databases, 35 FAM-related DEGs were screened out, and a prognostic risk model based on seven key genes was constructed. Through LASSO regression and SHAP interpretability analyses, ACSBG1 was revealed to be the most predictive protective factor in the model. Experimental verification showed that high expression of ACSBG1 significantly inhibited the proliferation, migration and invasion of LUAD cells, and promoted cell apoptosis. Furthermore, the low expression of ACSBG1 was weakly correlated with a reduction in CD4⁺ and CD8⁺ T-cell infiltration, suggesting that it may affect the prognosis of patients by regulating the tumor immune microenvironment. Clinical analysis confirmed that the 5-year survival rate of patients in the high-risk group was significantly lower than that in the low-risk group, and the risk score was independently associated with TNM staging. To the best of our knowledge, the present study is the first to reveal the molecular mechanism of ACSBG1 as a novel tumor suppressor gene, providing metabolism-related biomarkers and potential therapeutic targets for the prognostic stratification and targeted therapy of LUAD.

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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

AL conceptualized the study, reviewed and edited the manuscript, conducted data collection, wrote the original draft of the manuscript and investigated the research background. PH developed the methodology, performed formal analysis, conducted project administration and supervised the study. AL and PH confirm the authenticity of all the raw data. All authors read and approved the final 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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