



# Systematic analysis of apoptosis-related genes in the prognosis of lung squamous cell carcinoma: a combined single-cell RNA sequencing study

Peiquan Zhu, Wenxing Yang, Biao Wang, Tao Zeng, Zhi Hu, Dengguo Zhang, Ze Yang, Kaiqiang Wang, Jiangtao Pu

Department of Thoracic Surgery, Affiliated Hospital of Southwest Medical University, Luzhou, China

*Contributions:* (I) Conception and design: P Zhu; (II) Administrative support: D Zhang, Z Yang; (III) Provision of study materials or patients: W Yang, K Wang; (IV) Collection and assembly of data: B Wang, T Zeng; (V) Data analysis and interpretation: Z Hu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*Correspondence to:* Jiangtao Pu, MMed. Department of Thoracic Surgery, Affiliated Hospital of Southwest Medical University, No. 25, Taiping Street, Luzhou 646100, China. Email: pujiangtao1972@sina.com.

**Background:** Lung squamous cell carcinoma (LUSC) has a poor prognosis and lacks appropriate diagnostic and treatment strategies. Apoptosis dysregulation is associated with tumor occurrence and drug resistance, but the prognostic value of apoptosis-related genes (ARGs) in LUSC remains unclear.

**Methods:** Using univariate Cox regression, least absolute shrinkage and selection operator (LASSO) regression, and multivariate Cox regression analysis based on differentially expressed ARGs, we constructed an ARG-related prognostic model for LUSC survival rates. We conducted correlation analysis of prognostic ARGs by incorporating the dataset of normal lung tissue from the Genotype-Tissue Expression (GTEx) database. We then constructed a risk model, and the predictive ability of the model was evaluated using receiver operating characteristic (ROC) curve analysis. Non-small cell lung cancer (NSCLC) single-cell RNA sequencing (scRNA-seq) data were downloaded from the Gene Expression Omnibus (GEO) database. Subsequently, these data were subjected to single-cell analysis. Cell subgroups were determined and annotated by dimensionality reduction clustering, and the cell subgroups in disease development were identified via pseudotemporal analysis with the Monocle 2 algorithm.

**Results:** We identified four significantly prognostic ARGs and constructed a stable prognostic risk model. Kaplan-Meier curve analysis showed that the high-risk group had a poorer prognosis ( $P < 0.05$ ). Furthermore, the ROC analysis of 3-, 5- and 7-year survival rates confirmed that the model had good predictive value for patients with LUSC. Single-cell RNA sequencing showed the prognostic ARGs were enriched in epithelial cells, smooth muscle cells, and T cells. Pseudotime analysis was used to infer the differentiation process and time sequence of cells.

**Conclusions:** This study identified ARGs that are associated with prognosis in LUSC, and a risk model based on these prognostic genes was constructed that could accurately predict the prognosis of LUSC. Single-cell sequencing analysis provided new insights into the cellular-level development of tumors. These findings provide more guidance for the diagnosis and treatment of patients with LUSC.

**Keywords:** Lung squamous cell carcinoma (LUSC); prognostic model; biomarkers; apoptosis; single-cell analysis

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## Introduction

Lung cancer is the leading cause of cancer-related death, accounting for 19.4% of cancer-related deaths annually (1). Non-small cell lung cancer (NSCLC) constitutes over 80% of all lung cancer cases and includes two main types: non-squamous cell carcinoma (including adenocarcinoma, large cell carcinoma, and other cell types) and squamous cell carcinoma, which accounts for approximately 25–30% of NSCLC cases (2). Squamous cell carcinoma of the lung often develops in the central part of the lung and has the ability to grow to a large size (3). Lung squamous cell carcinoma (LUSC) is a particularly challenging disease to treat (4). Comparative results from relevant clinical trials show that the median survival of patients with advanced squamous cell carcinoma who receive first-line treatment is about 30% shorter than that of patients with other NSCLC subtypes (5,6). The tumor, node, metastasis (TNM) staging system is widely used for LUSC, but it has certain limitations because patients with the same TNM stage can have different survival outcomes (7). A few significant improvements have been achieved in molecular targeted therapy and immunotherapy for NSCLC. However, the vast majority of LUSC cases do not harbour targetable oncogenic mutations or develop resistance to the current treatment methods (8,9). Therefore, it is necessary to identify new biomarkers that can provide diagnostic and prognostic information for LUSC.

Cell apoptosis is an evolutionarily conserved, genetically regulated form of cell suicide that plays an important role in the development and maintenance of tissue homeostasis in multicellular organisms (10). Cell apoptosis is crucial for eliminating cells that have undergone mutations or transformations in the body. Therefore, cancer cells often evolve multiple efficient and diverse mechanisms to evade cell apoptosis (11). Cell apoptosis can be induced through both extrinsic and intrinsic pathways. The extrinsic pathway is stimulated by death receptors, such as Fas, tumor necrosis factor receptors, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), while the intrinsic pathway is initiated by DNA damage, energy depletion, and hypoxia, which can lead to dephosphorylation and cleavage of proapoptotic proteins (12). Genetic analysis of NSCLC has revealed inherited and somatic mutations in the *EGFR* and *P53* genes, as well as somatic mutations in the *KRAS*, *BRAF*, *ERBB2*, *MET*, *STK11*, *PIK3CA*, and *PARK2* genes. These gene mutations have led to the development of new strategies for targeted treatment (13). Exploring the targeted apoptosis mechanisms in NSCLC represents another approach aimed at selectively killing cancer cells while preserving normal cells. However, related study has shown that there is no correlation between the response to apoptosis-targeted drugs and the histological subtypes of NSCLC. The presence of apoptosis targets, such as TRAIL receptors and death-inducing signaling complex (DISC) compounds, anti-apoptotic B-cell lymphoma 2 (*BCL-2*) and inhibitor of apoptosis (IAP) family members, is important but cannot predict the response to corresponding targeted therapies (14). Moreover, patients with LUSC often have a poor prognosis. Therefore, identifying key molecules, establishing stable and effective predictive models, and implementing precision treatment are crucial for improving the prognosis of these patients.

In recent years, single-cell RNA sequencing (scRNA-seq) has developed rapidly and is a departure from the conventional “bulk” RNA sequencing (RNA-seq) methods. scRNA-seq captures the transcriptomes of individual cells and allows for unprecedented resolution to evaluate the fundamental biological properties of specific cell populations within complex multicellular ecosystems (15). By exploring the heterogeneity of cells in the tumor microenvironment at the single-cell level, we can greatly improve our understanding of the transcriptional, genetic, metabolic, and other characteristics of thousands of individual cells, thereby deepening our analysis of the cells involved in tumor progression (16). Integrating clinical pathological

### Highlight box

#### Key findings

- We constructed a prognostic model based on apoptosis-related genes associated with lung squamous cell carcinoma and integrated single-cell RNA sequencing, providing a fresh perspective on disease understanding.

#### What is known and what is new?

- Lung squamous cell carcinoma is a type of lung cancer, and apoptosis-related genes play a role in cancer.
- We constructed a prognostic model for lung squamous cell carcinoma based on apoptosis-related genes and integrated single-cell RNA sequencing, providing a fresh perspective on disease understanding.

#### What is the implication, and what should change now?

- The significance of our study suggests the potential of using our prognostic model to guide personalized treatment for lung squamous cell carcinoma. However, further experimental and clinical research is needed to validate its effectiveness.

information and single-cell sequencing data can facilitate the identification of novel diagnostic and prognostic biomarkers as well as potential treatment-related cell types or states (17). However, scRNA-seq is relatively expensive, and therefore the available sample datasets are relatively limited. Nevertheless, the information obtained from scRNA-seq is highly valuable for exploring the characteristics of each cell subpopulation in the sample and the interactions of each cell in the tumor immune microenvironment (TIME) (18). In this study, we aimed to construct an apoptosis prognostic model associated with LUSC survival. Through bioinformatics analysis, we identified four apoptosis-related genes (ARGs) that were associated with LUSC prognosis, using these genes to construct a risk score and prognostic model. The accuracy of this model was validated in a test set, and the potential role of the risk score in guiding immunotherapy for patients with LUSC was investigated. Finally, we further elucidated the role of the prognostic-related ARGs at the cellular level in the occurrence and development of LUSC with the aim of improving the treatment outcomes and prognosis of patients with LUSC at the single-cell level. This study aims to construct a survival prognostic model for LUSC based on the differential expression of ARGs. Through the application of univariate Cox regression, least absolute shrinkage and selection operator (LASSO) regression, and multivariate Cox regression analyses, we intend to assess the correlation of prognostically relevant ARGs in LUSC. Additionally, the objectives of this study include investigating how apoptosis regulatory imbalance is associated with tumorigenesis, exploring the expression differences of apoptosis-related genes in LUSC patients, and evaluating the prognostic value of these genes. The study also aims to identify potential molecular targets to enhance diagnostic and therapeutic strategies for LUSC, ultimately improving patient survival rates. We present this article in accordance with the TRIPOD reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1712/rc>).

## Methods

### *Data source and preprocessing*

RNA-seq data and clinical information of patients with LUSC were obtained from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). Overall survival (OS) and other clinical features, including age,

gender, T stage, N stage, M stage, and clinical stage, were extracted from the downloaded cases, and missing clinical information was removed. scRNA-seq data were downloaded from the Gene Expression Omnibus (GEO) database (GSE200972) (<https://www.ncbi.nlm.nih.gov/geo/>), and four tumor tissue samples were selected for analysis. The raw data contained 23,008 genes and 8,452 cells. The “PercentageFeatureSet” function in R version 4.2.1 (The R Foundation for Statistical Computing) was used to calculate the percentage of mitochondria and RNA, and cells with a gene expression greater than 300 and less than 7,000 and with a mitochondrial content of less than 30% were selected as the basis for subsequent analysis. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### *Differential expression analysis of ARGs*

A series of bioinformatics analyses were performed in R. Specifically, the “limma” R package was used to screen for differentially expressed ARGs between tumor and nontumor tissues in TCGA cohort. For each gene, a Wilcoxon rank-sum test was executed using a for loop to calculate logFC (log<sub>2</sub> fold change) and P values. The ultimate criteria for selection are a false discovery rate (FDR) <0.05 and |log<sub>2</sub> (fold change)| >1 being the screening criteria.

### *Development and validation of the prognostic model*

In this study, an ARG-related LUSC prognostic model was constructed to predict the OS of patients with LUSC. The TCGA-LUSC cohort was divided into a training set and a testing set using the createDataPartition function, the prognostic features were constructed based on the training set, and then the predictive performance was validated in the validation set. Univariate Cox regression analysis was used to determine the prognostic ARGs in LUSC, and the results were visualized using a forest plot generated by the “forestplot” R package. The LASSO penalized Cox proportional hazards regression (implemented with R package “glmnet”) was used to reduce the genes of the model and limit the complexity of solving the problem of overfitting. Finally, four prognostic ARGs were determined via multivariate Cox regression analysis, and a 4-gene signature was constructed. In the training and testing sets, the risk score of each patient was calculated based on the regression coefficient of the ARGs. The risk score was calculated using the following formula: risk score =

expression level of gene a  $\times$  coefficient a + expression level of gene b  $\times$  coefficient b + expression level of gene c  $\times$  coefficient c + ... + expression level of gene n  $\times$  coefficient n. Patients with LUSC were divided into high-risk and low-risk groups based on the median risk value, and Kaplan-Meier survival curves were used to compare the survival between the low-risk and high-risk groups, with  $P < 0.05$  being considered statistically significant. Finally, the receiver operating characteristic (ROC) curves and area under the curve (AUC) values for both the training and testing sets were calculated and plotted using the survivalROC package. Time-dependent ROC curves were established to reflect the predictive performance of the prognostic model.

### ***Nomogram development and validation for prognostic risk prediction***

To provide a quantitative method for predicting the 3-, 5-, and 7-year survival probabilities of patients with LUSC for clinical doctors, we developed a nomogram integrating various clinical risk factors and apoptosis prognostic models. We then evaluated the calibration curve of the nomogram by plotting the predicted probability of the observed rate using the nomogram. Subsequently, 1,000 repeated samplings were used for cross-validation to construct a concordance-index (C-index) plot to illustrate the predictive ability of the model. The prognostic accuracy of the model was evaluated through sensitivity and specificity using the ROC curve. Finally, a heatmap was constructed to evaluate the correlation between prognostic features and clinical factors.

### ***Functional enrichment analysis***

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>). First, we obtained pathway enrichment information by inputting ARGs into DAVID and selected the top 10 pathways with the smallest P values for enrichment analysis in both GO and KEGG. The enriched pathways were displayed using the “ggplot” R package.

### ***Gene mutation analysis***

Tumor mutational burden (TMB) reflects the number of

mutations in cancer. We downloaded mutation data of patients with LUSC from TCGA and performed gene mutation analysis using the “maftools” R package. We calculated the optimal cutoff value for TMB data using the surv\_cutpoint function, and then compared the TMB between the high-risk and low-risk groups. Survival analysis was performed based on the TMB score, and the somatic mutations of prognostic genes were identified via the cBioPortal database(<http://www.cbioportal.org/>).

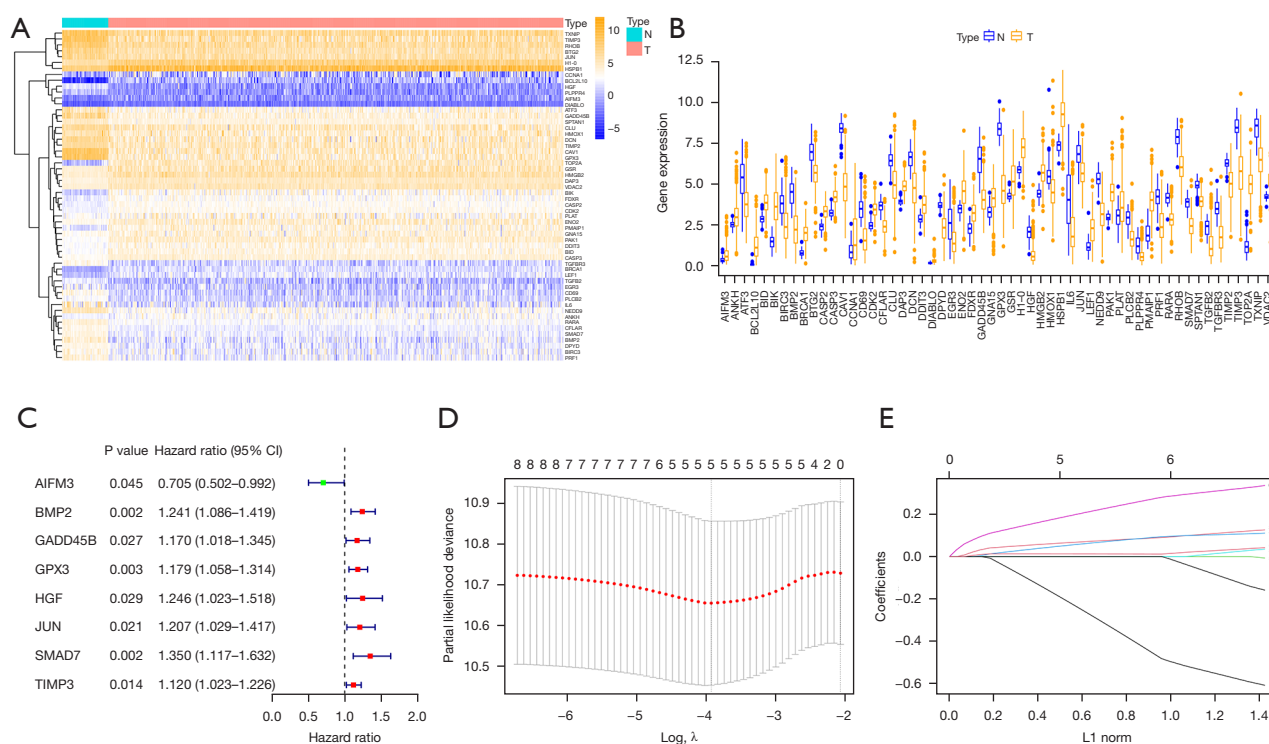
### ***Relationship of molecular patterns with TME in LUSC***

The ESTIMATE algorithm was used to evaluate whether the risk score of patients with LUSC was associated with immune and stromal components. Single-sample gene set enrichment analysis (ssGSEA) was applied to quantify the infiltration levels of 16 immune cells and 13 immune-related pathways between the two risk groups with the “GSVA” package. Immune checkpoint genes with differential expression (with  $P < 0.05$  as the threshold) were selected, and the expression differences of immune checkpoint genes between the high-risk and low-risk groups were examined.

### ***Processing of single-cell sequencing data***

The NSCLC scRNA-seq dataset, GSE200972, was downloaded from the GEO database, containing 19 tissue samples from four patients with multiple primary lung cancer (1 squamous carcinoma and 3 adenocarcinoma cases). We selected four tumor samples for subsequent analysis. The samples were integrated using the anchors method in the R package “Seurat”. To exclude cell data with genes detectable in only 3 or fewer cells and cell data with fewer than 300 genes detected in 1 cell while retaining those with gene counts between 300 and 7,000, mitochondrial content less than 10%, blood cell content less than 1%, and a total transcript count less than 100,000. The “FindVariableFeatures” function in R was used to identify the top 3,000 highly variable genes, and the top 10 genes were displayed. Principal component analysis (PCA) was performed on single-cell samples, and the top 13 principal components (PCs) were selected for subsequent analysis. The samples were subjected to overall dimensionality reduction analysis via the uniform manifold approximation and projection (UMAP) algorithm. Different cell clusters were manually annotated using the R package “singleR”, the CellMarker database, and references as auxiliary annotations.





**Figure 1** The differential expression of ARGs in LUSC was analyzed, and genes associated with the prognosis of LUSC were screened using regression algorithms. (A) Heat maps of 56 differentially expressed ARGs. (B) Boxplot of 56 differentially expressed ARGs in LUSC and normal lung tissue. (C) Forest maps of 8 prognostic ARGs were obtained via univariate Cox analysis. (D) Ten-time cross-validation for tuning parameter selection in the LASSO model. (E) Distribution plot of gene coefficients generated with  $\log(\lambda)$  in the LASSO model. ARG, apoptosis-related gene; LUSC, lung squamous cell carcinoma; LASSO, least absolute shrinkage and selection operator.

### Construction of a pseudotemporal analysis trajectory

Pseudotemporal analysis was performed using the Monocle 2 algorithm through branching trajectory analysis. Gene expression levels and dispersion were filtered and sorted to exclude noise and irrelevant genes in the single-cell data. The “orderCells” function in R was used to sort cells and determine their position on the trajectory. Trajectories were constructed for two clusters, and differentiation trajectories of cells in different states were plotted. Subsequently, the expression of prognostic genes in the two clusters was visualized, and the pseudotemporal trajectories of several prognostic genes in T cells were observed.

### Statistical analysis

All statistical analyses were performed using R software version 4.2.1. A P value  $<0.05$  was considered statistically significant. Univariate Cox, LASSO regression analysis, and multivariate Cox analyses were used to select key

ARGs associated with survival. Survival variations between different groups were compared using Kaplan–Meier curves. The predictive ability of this model was tested using ROC analysis.

## Results

### Identification of the differentially expressed genes in LUSC

We obtained RNA-seq data and clinical follow-up data from 502 LUSC samples and 51 normal lung tissue samples in TCGA dataset. Differential expression analysis was performed to determine the expression levels of ARGs in tumor and normal samples, and 56 differentially expressed genes were identified (Table S1), most of which were enriched in tumor samples (Figure 1A,1B). Univariate Cox regression was used to screen ARGs, and 8 prognostic-related ARGs were identified (Figure 1C). LASSO Cox regression analysis and 10-fold cross-validation were used to determine the optimal regularization parameter  $\lambda$  to

obtain the best model performance (Figure 1D,1E). Finally, multivariate analysis was performed to identify four ARGs (*BMP2*, *GPX3*, *JUN*, and *AIFM3*) that were most relevant to the prognosis of patients with LUSC. Kaplan-Meier analysis was used to analyze the impact of the high and low expression of these four genes on prognosis, and the three most significant genes (*BMP2*, *GPX3*, and *JUN*) were identified (Figure S1A-S1C). We found that patients with a high expression of *BMP2*, *GPX3*, and *JUN* had a worse prognosis than did those with low expression. Correlation analysis revealed a close relationship between these genes (Figure S1D-S1F), suggesting a possible synergistic effect on prognosis.

### Construction of a risk score based on prognostic ARGs

TCGA cohort was randomly divided into a training set and a testing set, and based on the median risk cutoff value of patients, they were further divided into high-risk and low-risk groups (Figure 2A,2B). The risk score was calculated as follows: risk score =  $(-0.411 \times \text{expression level of } AIFM3) + (0.084 \times \text{expression level of } BMP2) + (0.0838 \times \text{expression level of } GPX3) + (0.258 \times \text{expression level of } JUN)$ . The patients in the training and testing sets were sorted from left to right based on their increasing risk scores. The results showed that as the risk score increased, the risk of death increased and the survival time decreased. The trend of survival status and survival time in the testing set was consistent with that in the training set, and the decrease in survival time was significantly correlated with the increase in risk score (Figure 2C,2D). Similarly, Kaplan-Meier survival curves showed that patients in the high-risk group had a lower survival rate than those in the low-risk group (Figure 2E,2F). The predictive performance of the prognostic risk score model was evaluated using time-dependent ROC curves and the AUC (Figure 2G,2H). The results showed that the risk score model had good predictive performance for patients with LUSC, as confirmed by mutual verification between the training and testing sets.

### Screening of independent prognostic factors and construction of a nomogram

To identify independent prognostic factors, univariate and multivariate Cox analyses were performed on clinical features and risk scores. We found that risk score and tumor stage were independent prognostic factors for patients, and a nomogram model was constructed to improve its

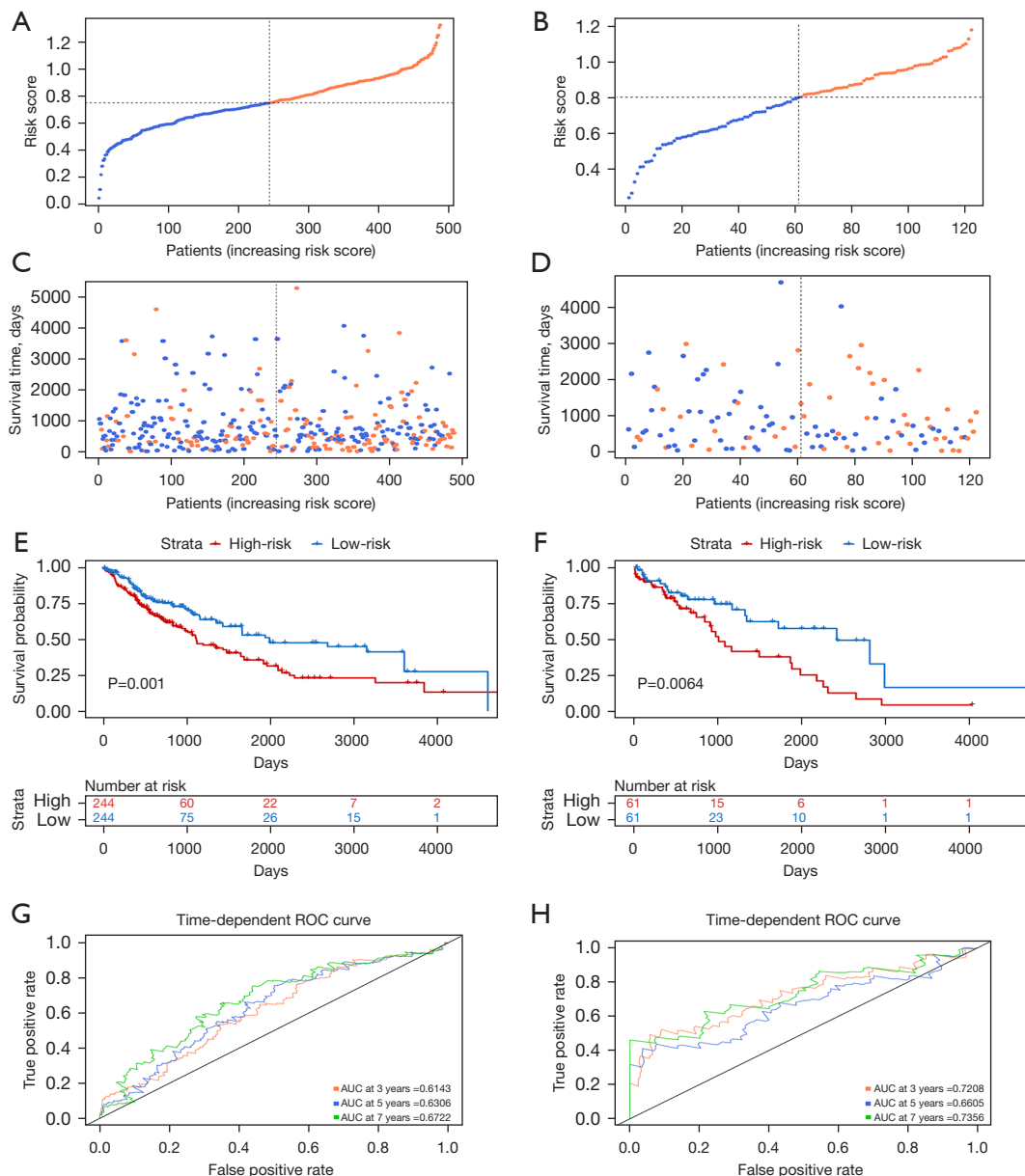
predictive ability (Figure 3A). By constructing calibration curves, it was found that the calibration curves of the 3-, 5-, and 7-year calibration points were relatively well matched with the standard curves, indicating that the model had good predictive performance (Figure 3B). The time-dependent ROC curves and C-index indicated that the nomogram based on the independent prognostic factors had better prognostic predictive performance for LUSC than did the single clinical features (Figure 3C,3D). Finally, we linked the risk score with clinical factors, such as tumor stage, and the results showed that patients with high-risk scores had shorter survival times (Figure 3E). In conclusion, our study results indicated that the risk score of prognostic genes can predict patient prognosis.

### Functional analysis of differentially expressed ARGs

To clarify the biological functions and important pathways of differentially expressed ARGs, we performed GO enrichment analysis to enrich the functions and pathways related to biological process (BP), cell component (CC), and molecular function (MF), and displayed the top 10 pathways with the smallest P values. We found that regulation of the apoptotic process was significantly enriched in BP (Figure 4A), cytoplasmic components such as mitochondrial outer membrane were significantly enriched in CC (Figure 4B), and protein complexes such as cysteine-type endopeptidase were significantly enriched in MF (Figure 4C). Similarly, KEGG enrichment analysis was performed using the same GO enrichment analysis method, and other pathways such as the apoptosis pathway and cancer pathway were enriched (Figure 4D). The detailed results of GO and KEGG enrichment analysis can be found in Table S2.

### TMB of prognostic biomarkers associated with ARGs in LUSC samples

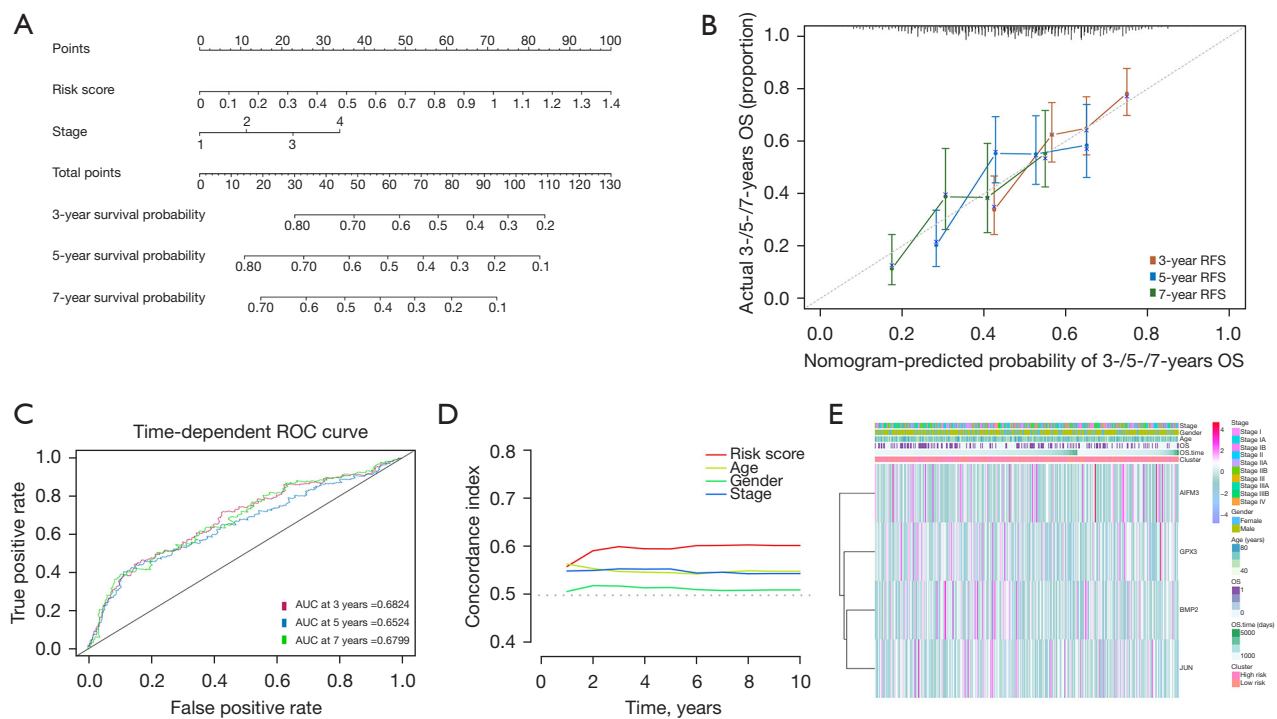
A high TMB indicates the presence of mutations in tumor cells, which may make them more susceptible to attack by the immune system, thereby increasing the effectiveness of immunotherapy. To investigate the significance of tumor mutations in LUSC, we downloaded LUSC somatic mutation data and calculated TMB scores, comparing the genomic mutation differences between the high-risk and low-risk groups. The mutation rates in *TP53*, *TTN*, *CSMD3*, *MUC16*, and *RYR2* were higher than or equal to 35% in patients with LUSC in both risk groups. Interestingly, the likelihood of these gene mutations was



**Figure 2** Construction of a risk model based on apoptosis-related genes associated with prognosis in LUSC. (A,B) The blue dots represent patients in the low-risk group, while the orange dots may represent patients in the high-risk group. The distribution of risk scores for patients in the training and testing groups. (C,D) The blue and orange dots reflect the distribution of survival time among patients as the risk score increases. The survival status of patients in the training and testing groups. (E,F) Kaplan-Meier analysis of the training and testing groups showing different OS between the high- and low-risk groups. (G,H) Time-dependent ROC curve predicting the OS rate for the training and testing groups. ROC, received operating characteristic; AUC, area under the curve; LUSC, lung squamous cell carcinoma; OS, overall survival.

greater in the low-risk group compared to the high-risk group (Figure 5A,5B). To investigate the impact of TMB status on the prognosis of patients with LUSC, survival

analysis was performed on different TMB subgroups, and it was found that the prognosis of patients with high TMB was better than that of patients with low TMB (Figure



**Figure 3** Establishment and validation of the nomogram prediction model. (A) Nomogram combining risk score and other clinical factors to predict 3-, 5-, and 7-year OS of patients in TCGA-LUSC dataset. (B) Calibration plot of the nomogram predicting 3-, 5-, and 7-year survival probabilities. (C) Time-dependent ROC curve predicting 3-, 5-, and 7-year survival of patients with LUSC. (D) The concordance index indicated that the predictive accuracy of the risk model was superior to other clinical parameters. (E) Heat map of four prognostic ARGs combined with different clinical features. OS, overall survival; RFS, relapse-free survival; ROC, received operating characteristic; AUC, area under the curve; TCGA, The Cancer Genome Atlas; LUSC, lung squamous cell carcinoma; ARGs, prognostic apoptosis-related genes.

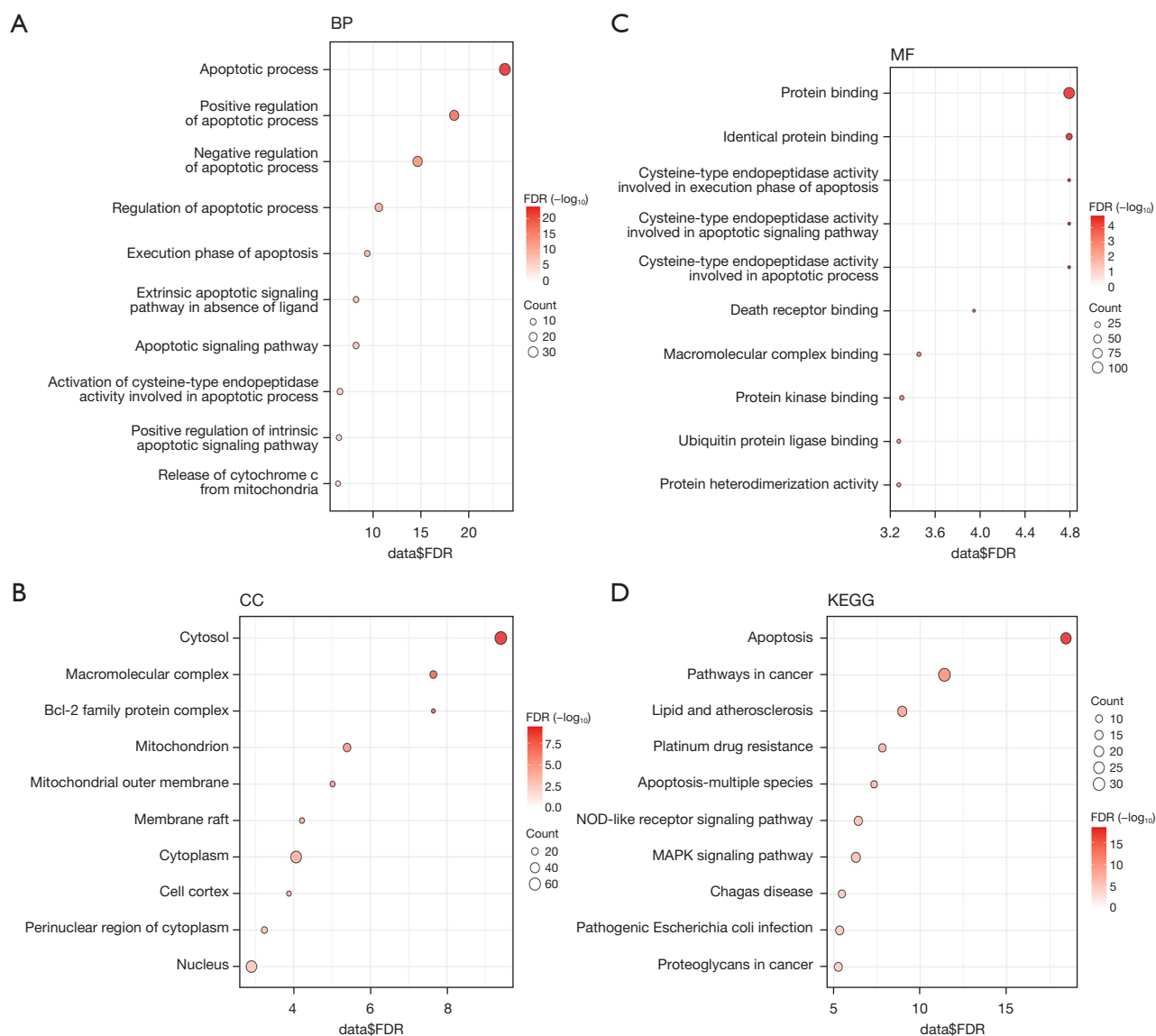
5C). Subsequently, we combined TMB with risk scores for survival analysis, and the results showed that patients with high TMB and low risk scores had the best prognosis (Figure 5D). Finally, we examined the mutation rates of the four genes in the prognostic model and found that a segment of DNA sequence in the *AIFM3* gene was duplicated, leading to an increase in copy number, which may contribute to the occurrence and development of tumors and an increase in the expression of other genes. However, the mutation rates of these genes were all very low (Figure 5E).

#### Assessment of tumor immune infiltration and immune checkpoint

The correlation between risk scores and tumor stromal and immune scores was analyzed, and the results showed that as the risk score increased, the abundance of tumor stroma and immune infiltration also increased (Figure 6A, 6B). This implied a positive correlation between risk

scores and the degree of tumor stromal and immune cell infiltration. Therefore, an increase in risk score may lead to changes in the tumor microenvironment and immune response, promoting tumor stromal and immune cell infiltration. To further understand the characteristics of the tumor microenvironment and its impact on tumor treatment, samples were divided into high-risk and low-risk groups, and the differences in tumor microenvironment score and tumor microenvironment evaluation indicators between different risk groups were compared. We found that both the stromal score and immune score in the high-risk group were higher than those in the low-risk group (Figure 6C). Previous studies have shown that the immune microenvironment has a significant impact on various aspects of tumor occurrence and development, treatment response, and the identification of new therapeutic targets (19,20). Furthermore, our study showed that as the risk score increased, the abundance of immune infiltration also increased. We used the ssGSEA algorithm to demonstrate





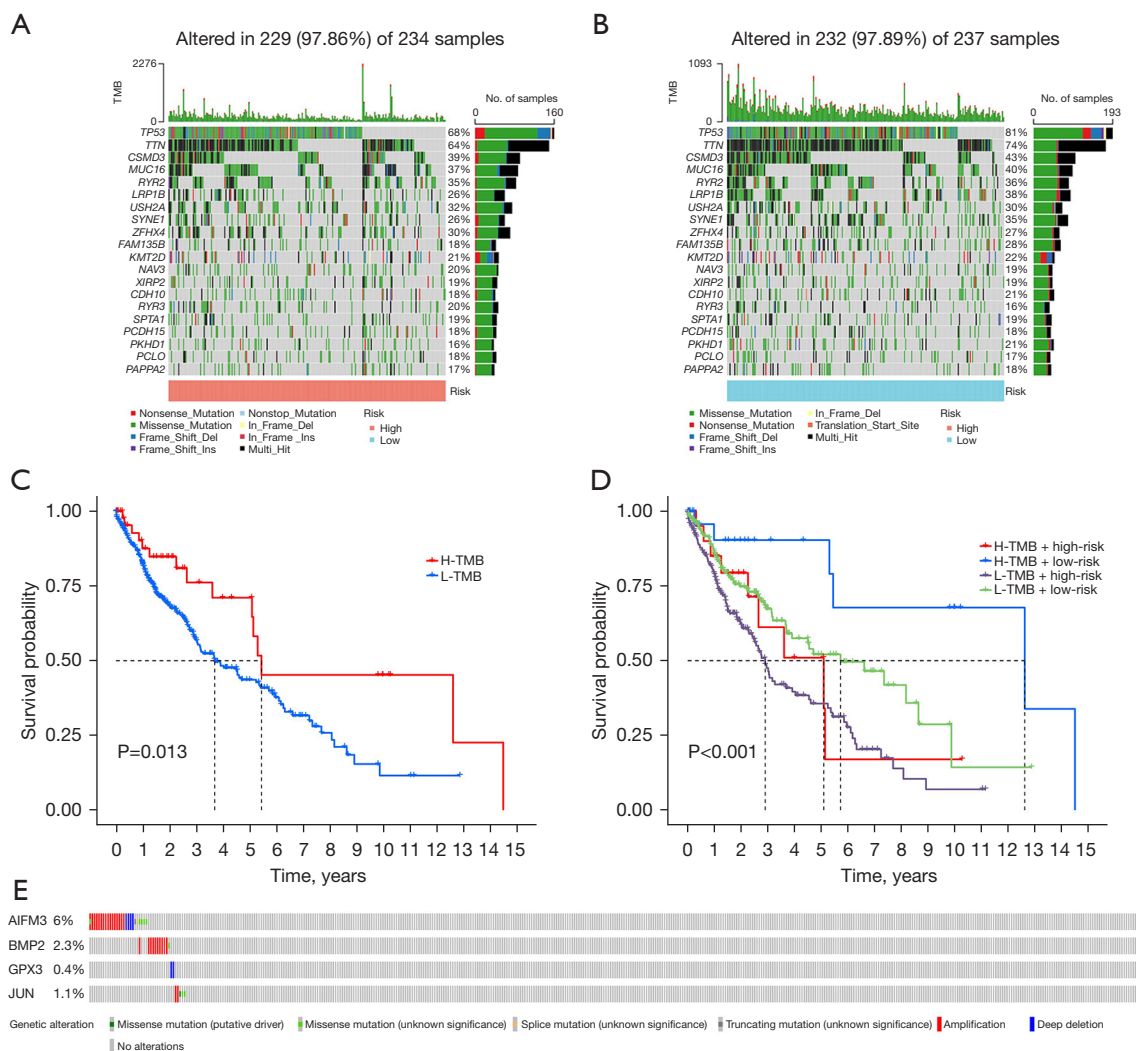
**Figure 4** Function analysis of differentially expressed ARGs. (A-C) GO functional analysis of differentially expressed ARGs (BP, CC, MF). (D) KEGG pathway analysis of differentially expressed ARGs. FDR, false discovery rate; BP, biological processes; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; ARG, apoptosis-related gene; GO, Gene Ontology.

the enrichment levels of different immune cells in the tumor microenvironment of high- and low-risk groups in order to understand the mechanisms of tumor immune escape and identify potential targets for immunotherapy. We found that compared to the low-risk group, the high-risk group had higher immune cell infiltration and more immune-related functions and pathways (Figure 6D,6E). Next, we evaluated the expression differences of immune checkpoints between the high- and low-risk groups (Figure

6F). The results showed that there were differences in the expression of 35 immune checkpoints between the two risk subgroups, including TNFRSF4, CTLA4, and CD200.

#### Quality control and filtering of scRNA-seq data

First, we filtered out the unqualified cells and used the filtered cells for subsequent analysis (Figure 7A). We performed PCA on the four single-cell samples and

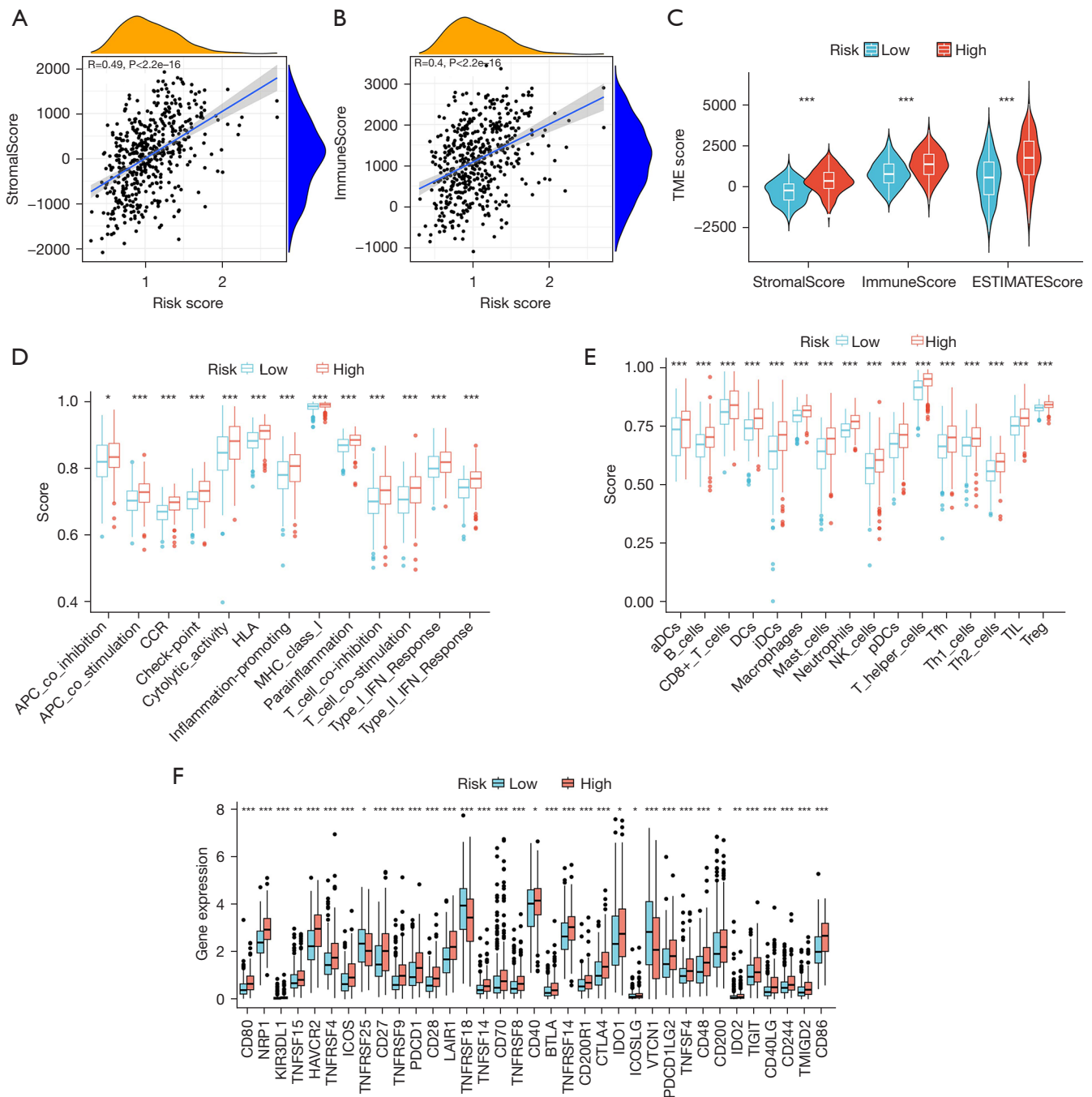


**Figure 5** Waterfall plot of high-risk and low-risk groups and prognostic gene somatic mutations. (A,B) Waterfall plot of somatic mutations in the high-risk and low-risk groups. (C) Survival curves of the high-TMB group and low-TMB group. (D) Survival curves based on TMB and risk score. (E) Study of mutation rates of four prognostic genes using the cBioPortal database. TMB, tumor mutational burden; H, high; L, low.

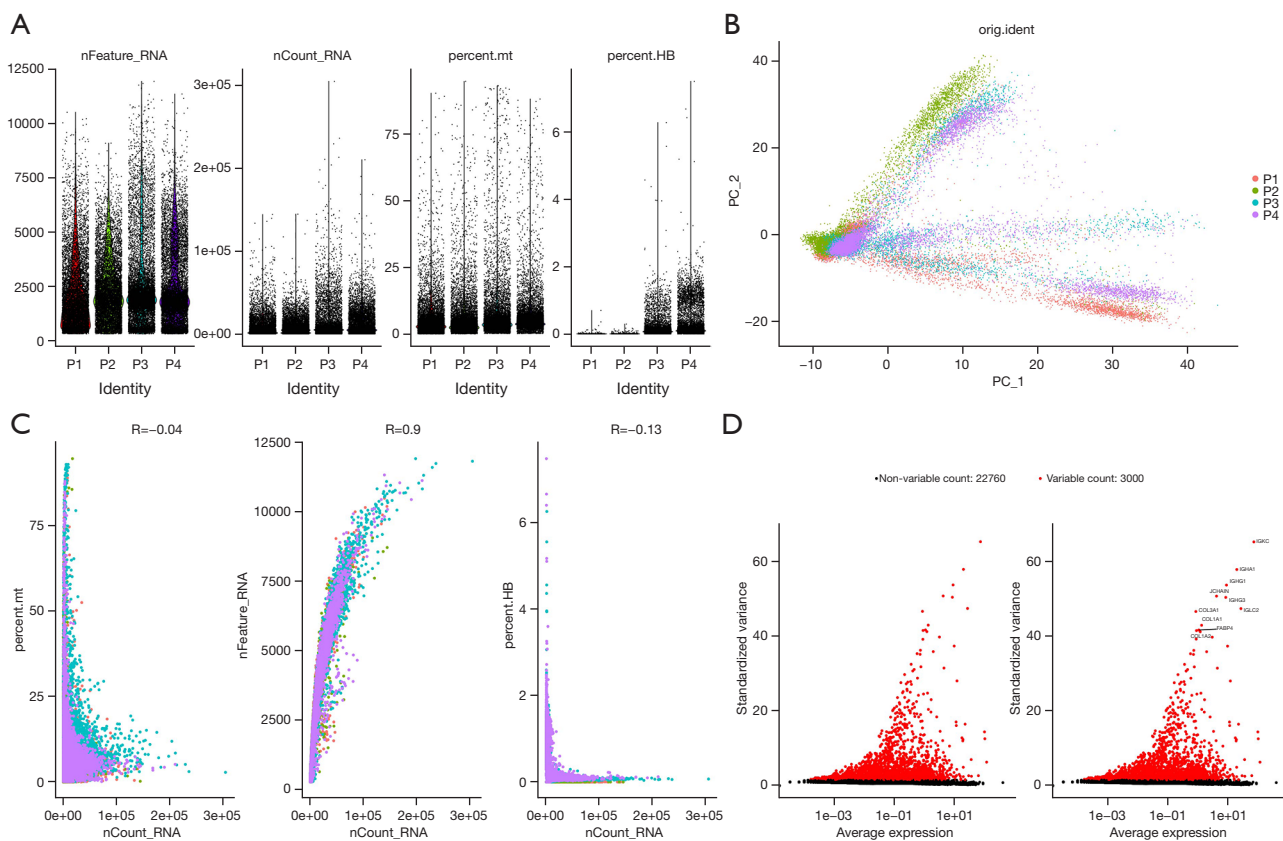
found that the four NSCLC samples had a high degree of aggregation (*Figure 7B*), which suggested that the transcriptional differences between these four samples were relatively small. We then conducted a correlation analysis of several quality control indicators and found that the proportion of mitochondria and hemoglobin did not increase with the increase of RNA molecules, while the number of transcriptional genes increased with the increase of RNA molecules (*Figure 7C*), indicating that our quality control was relatively good and removed low-quality cells. Subsequently, we used differential analysis to screen out 3,000 highly variable genes (*Figure 7D*).

**Identification and localization of LUSC cell subtypes**

We used the UMAP algorithm to divide the core cells into 16 independent cell clusters (*Figure 8A*). In order to explore the heterogeneity within the tumor, we calculated the proportion of each cell in the four samples separately (*Figure 8B,8C*). The results showed that the proportions of different cells were different in each sample, indicating the presence of different cell subpopulations within the tumor, which is consistent with the characteristics of tumor heterogeneity. We identified marker genes using the “singleR” package in R, the CellMarker database, and other references (21) and then annotated the different



**Figure 6** Analysis of TME and immune-related characteristics in the high-risk and low-risk groups. (A) Correlation between risk score and stromal score. (B) Correlation between risk score and immune score. (C) Evaluation of the tumor microenvironment using TME score. (D) Box plots showing the scores of various immune-related functions in the high-risk and low-risk groups. (E) Box plots showing the scores of multiple immune cells in high-risk and low-risk groups. (F) Expression of immune checkpoints in the high-risk and low-risk groups. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ . TME, tumor microenvironment; APC, antigen-presenting cell; CCR, co-stimulatory cell receptor; HLA, human leukocyte antigen; MHC, major histocompatibility complex; IFN, interferon; NK, natural killer; TIL, tumor-infiltrating lymphocytes; Treg, regulatory T cell.



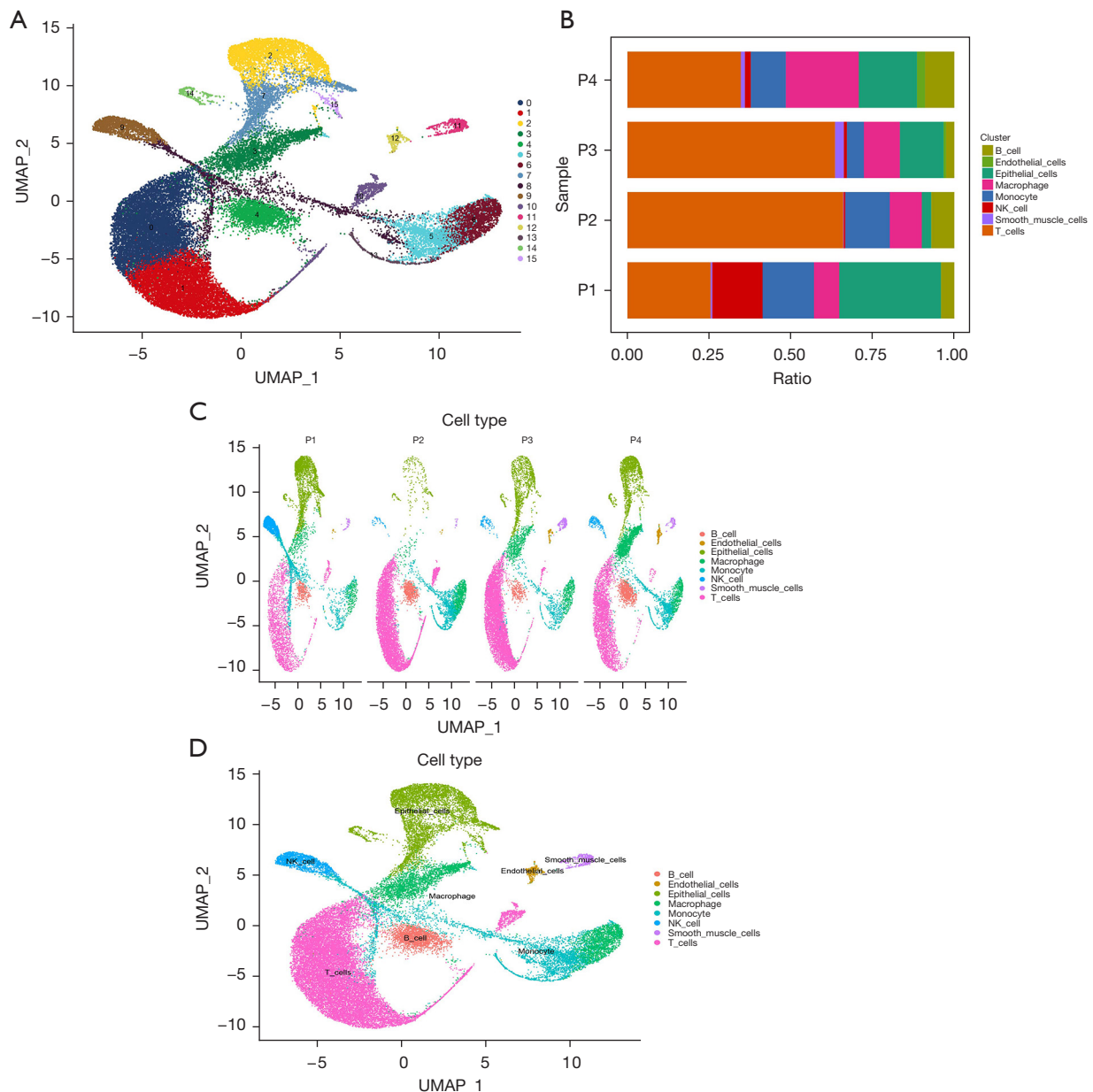
**Figure 7** Quality control and filtering analysis of single-cell RNA sequencing data from non-small cell lung cancer samples. (A) The distribution of the number of detected genes, sequencing depth, mitochondrial gene content, and HB content across the four samples. (B) Principal component analysis-based preliminary dimensionality reduction of the data from four samples. (C) There was a negative correlation ( $R=-0.04$ ) between sequencing depth and mitochondrial gene content, a positive correlation ( $R=0.9$ ) between sequencing depth and the number of intracellular genes, and a negative correlation ( $R=-0.13$ ) between sequencing depth and intracellular HB content in the four samples. (D) The volcano plot shows the genes that are active in all samples, with the names of top 10 genes being given in the red-marked highly variable genes. HB, hemoglobin; PC, principal component; scRNA-seq, single-cell RNA sequencing.

clusters (Figure 8D). Subsequently, the upregulated, significantly upregulated, downregulated, and significantly downregulated genes in the marker genes of the 16 clusters were displayed (Figure 9A). Similarly, we visualized the marker genes of the annotated 8 cell types (Figure 9B) to better characterize the differences in their functions and characteristics. We localized four apoptosis-related prognostic genes associated with LUSC within individual cell subtypes to understand the biological characteristics and functions of these cell subtypes. Furthermore, this localization could provide clues for further research, such as that exploring the interactions and regulatory mechanisms of these prognostic genes in different cell subtypes and their relationships with other BPs. This localization of cell subtypes can provide insights into the mechanisms

of LUSC occurrence and development. We observed the distribution of several apoptosis-related prognostic genes in these 8 cell clusters (Figure 9C), and it was found that *BMP2* was significantly upregulated in epithelial cells, *GPX3* was expressed at a higher level in smooth muscle cells, and *JUN* was expressed in all cell subtypes, but relatively higher in T cells. To further understand the specific expression of prognostic genes in the 8 cell clusters, we visualized the gene expression by drawing a heatmap (Figure 9D).

#### Identification of lung cancer subpopulations via single-cell trajectory analysis

Temporal analysis aims to model the time information of single cells. It can reveal the temporal trends and expression

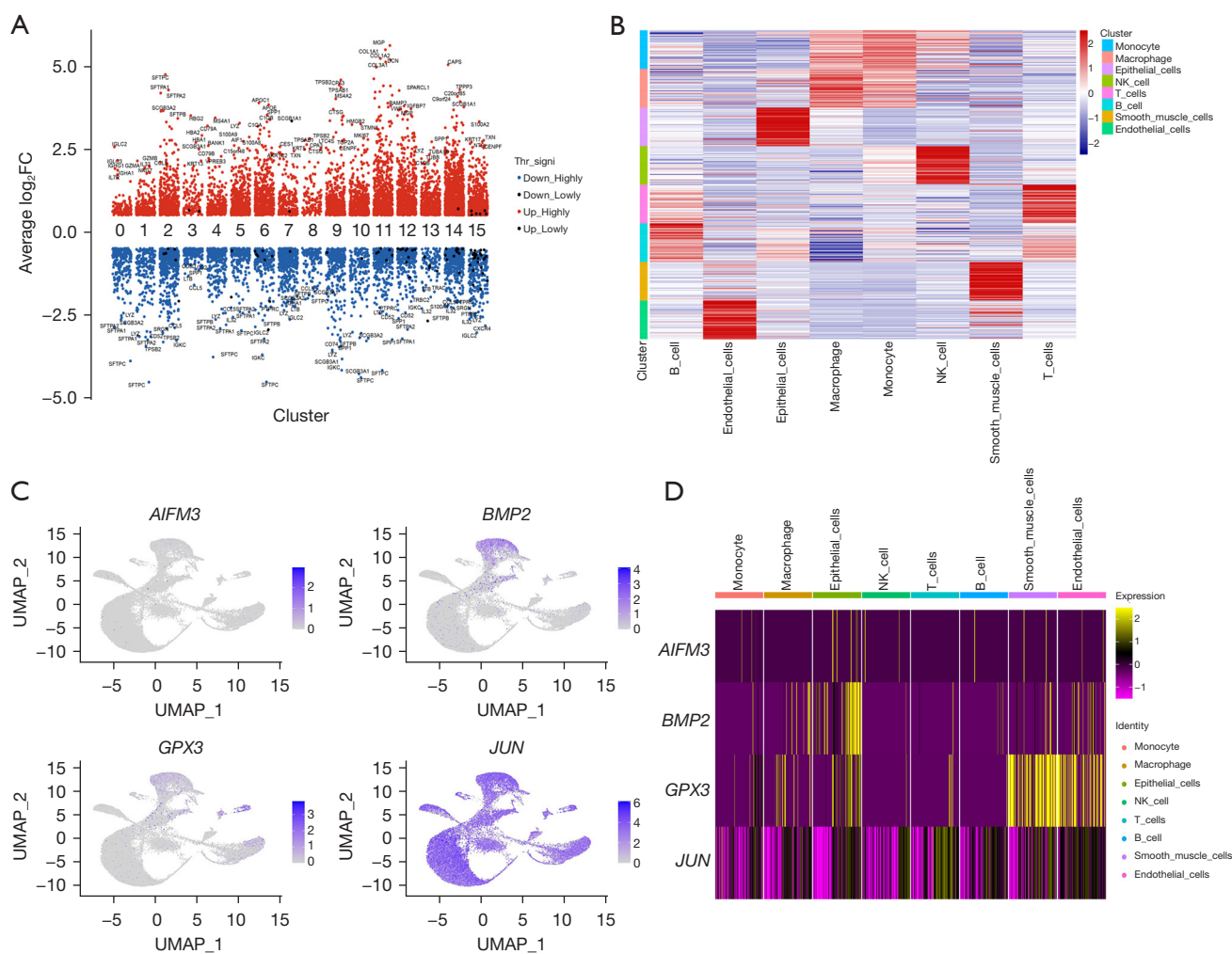


**Figure 8** Cluster assignment, proportions, and annotated distribution of cell types in non-small cell lung cancer samples. (A) Clustering of cells using the UMAP algorithm. (B) The proportion of cells in the four samples. (C) The distribution of cells in each of the four samples. (D) The distribution and annotation of cell types. UMAP, uniform manifold approximation and projection; NK, natural killer.

profile differences of different cells and identify biological issues such as cell development, differentiation, and function, which can aid in gaining a deeper understanding of single-cell data and discovering new biological knowledge and biomarkers (22,23). We selected two clusters for temporal analysis, and the trajectory showed the transcriptional states of cell development in the two

clusters (Figure 10A). We also visualized the trajectory of the state of cluster cells (Figure 10B) and determined the starting point of cell differentiation (Figure 10C), revealing changes related to tumor progression. Next, we studied the expression changes of four prognostic genes in the temporal analysis (Figure 10D). *JUN* expression was significantly decreased with cancer progression, while that of *AIFM3*,





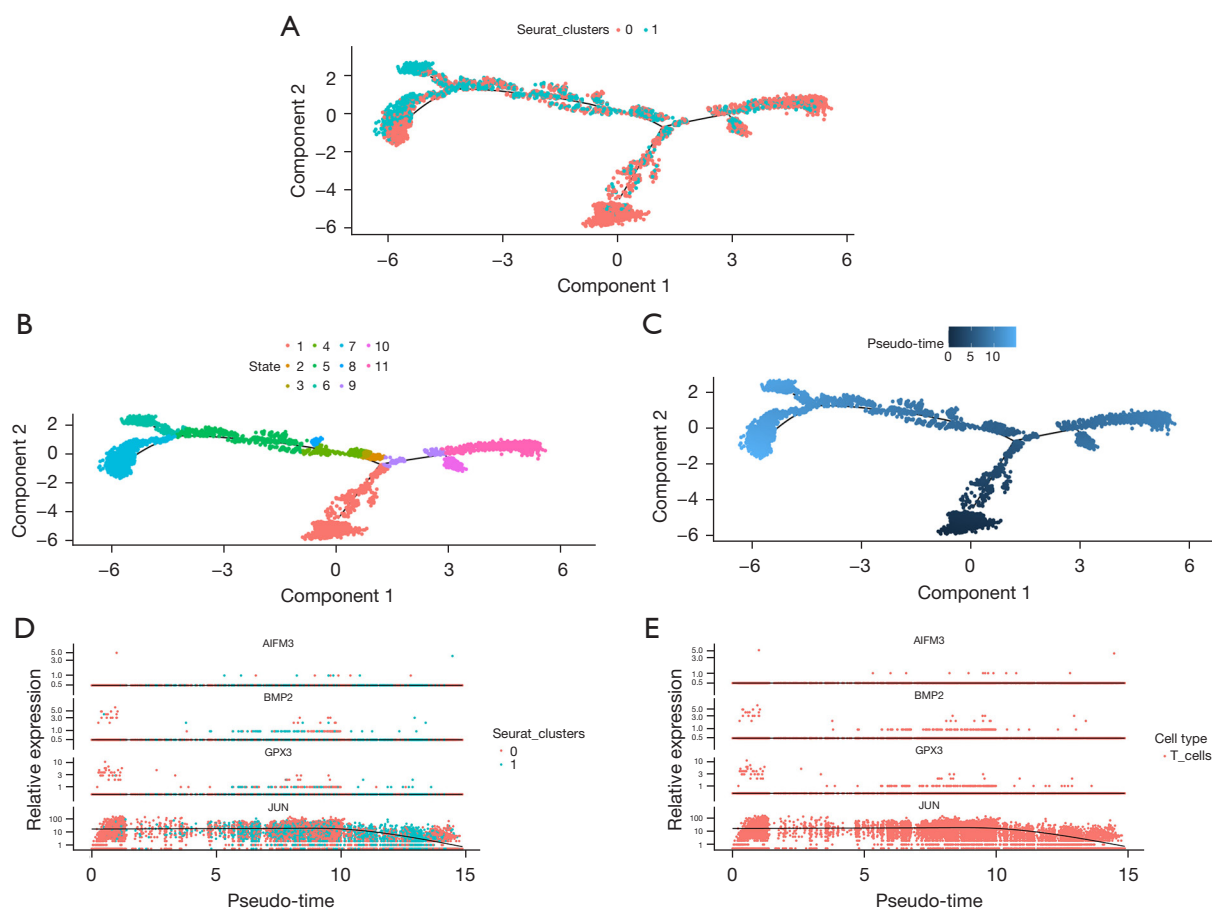
**Figure 9** The classification and expression of differential genes and prognostic genes in cell subpopulations. (A) Volcano plot showing significantly upregulated and downregulated genes in 16 clusters. (B) Heat map showing marker genes in 8 cell types. (C) The distribution of four prognostic genes (*AIFM3*, *BMP2*, *GPX3*, *JUN*) in 8 cell types. (D) Heat map showing the specific expression of *AIFM3*, *BMP2*, *GPX3*, and *JUN* in each cell type. FC, fold change; NK, natural killer; UMAP, uniform manifold approximation and projection.

*BMP2*, and *GPX3* did not show significant changes. As revealed by previous dimensionality reduction clustering, T cells account for a large proportion in the sample, so we examined the expression changes of the four prognostic genes in the temporal analysis of T cells (Figure 10E).

## Discussion

LUSC is a histological subtype of NSCLC and is associated with poor prognosis and a high mortality rate mainly due to ineffective, highly challenging treatment (24). Despite a variety of therapies being available for LUSC, including

chemotherapy, radiotherapy, and targeted therapy, the prognosis for patients with LUSC remains unsatisfactory (25). Therefore, there is an urgent need to develop new molecular therapeutic targets and prognostic models for the diagnosis and treatment of LUSC. Previous study has shown that apoptosis plays a crucial role in maintaining the balance between cell death and division, and evading apoptosis can lead to uncontrolled cell proliferation, resulting in various diseases such as cancer (26). The mechanism of apoptosis is complex and involves many pathways. Any defect in any of these pathways can lead to malignant transformation of the affected cells, tumor metastasis, and resistance to anticancer



**Figure 10** Trajectory analysis of cell subpopulations and prognostic genes. (A–C) Trajectory analysis of clusters and cell state. Different colored dots represent corresponding clusters or cell states, arranged along the pseudotemporal branch, and their pseudo-temporal curve. Dark blue denotes an earlier time. (D) The pseudotemporal changes of *AIFM3*, *BMP2*, *GPX3*, and *JUN* in two clusters. (E) The pseudotemporal changes of *AIFM3*, *BMP2*, *GPX3*, and *JUN* in T cells.

drugs. Currently, many new therapeutic strategies targeting apoptosis may be applied (27,28). Although there is a connection between apoptosis and LUSC, no systematic study has been conducted that uses apoptosis-related features as prognostic indicators to predict the prognosis of patients with LUSC.

We thus conducted a systematic analysis based on TCGA-LUSC dataset and ARGs to identify differentially expressed ARGs in LUSC and nontumor tissues. Through multifactor and LASSO Cox regression analysis, we screened for prognostic-related genes and established a risk model to predict the prognosis of LUSC.

Bone morphogenetic proteins (BMPs) are multifunctional cytokines, belonging to members of the transforming growth factor- $\beta$  superfamily (29). BMP can trigger the

occurrence and progression of tumors through the signaling mediators of ligands and receptors. Meanwhile, BMPs can promote cell differentiation, including via inhibiting the process of epithelial-mesenchymal transition, which can prevent the malignant progression of cancer in the later stages (30). BMP can activate extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), protein kinase A (PKA), protein kinase C (PKC), and protein kinase D (PKD). Through the initiation of these pathways, BMP can exert its effects on cell survival, apoptosis, migration, and differentiation (31). Smad and Mad-related proteins (SMAD) is a classical pathway mediated by BMP2. A study has shown that BMP2 is highly overexpressed in human NSCLC and is associated with tumor grading and metastasis (32). Through mouse models, BMP2 has

been shown to promote lung cancer metastasis. Depletion of BMP2 or its receptor BMPR2 greatly reduces cell migration and invasiveness, and BMP2/BMPR2-mediated cell migration involves the activation of the SMAD1/5/8 signaling pathway. Depletion of SMAD1/5/8 significantly reduces cell migration by inhibiting SMAD1/5/8 (33). Epigenetic silencing of glutathione peroxidase 3 (GPX3), a member of the important antioxidant selenoprotein family (34), maintains genome integrity by inactivating reactive oxygen species (ROS) (35), which are believed to play various roles in cancer development. For example, when the gene expression of important molecules that control cell proliferation, apoptosis, or the cell cycle is abnormal, oxidative stress can lead to persistent DNA damage and may induce cancer (36). Research has shown that GPX3 is associated with ovarian cancer metastasis and cancer progression. In one study, stable OVCAR3 GPX3 knockdown cell line was constructed using lentiviral short hairpin RNA (shRNA), and it was found that reducing GPX3 expression inhibited colony formation and anchorage-independent cell survival (37). In a study on thyroid cancer, methylation-specific polymerase chain reaction (MSP), IHC staining, Transwell experiments, and small interfering RNA (siRNA) knockdown were used to investigate the role of GPX3. It was found that GPX3 inhibits thyroid cancer metastasis by suppressing the Wnt/ $\beta$ -catenin signaling pathway. Silencing GPX3 expression promotes human thyroid cancer metastasis (38). c-Jun has been found to be an oncogenic transcription factor in most cancers, and its overexpression plays an important role in various biological functions such as cell apoptosis, proliferation, invasion, and migration (39,40). One study reported that enforced expression of c-Jun increased anchorage-independent growth of human bronchial epithelial cell lines, and constitutive expression of a significant c-Jun-negative mutant suppressed anchorage-independent but not anchorage-dependent growth of lung cancer cell lines (41). The activity of c-Jun is regulated by post-translational modifications, which are mainly controlled by components of the mitogen-activated protein kinase (MAPK) family of kinases, including c-Jun N-terminal kinase (JNK), ERK, and p38 kinase (42). The significant and unique function of JNK is as an activator of c-Jun. Overexpression or activation of c-Jun has been shown to have antiapoptotic effects in various cancer cell lines, and its targeting may sensitize drug-resistant cancer cells to DNA-damaging agents (43). Therefore, these key apoptotic prognostic-related genes are closely associated

with LUSC and its prognosis, demonstrating the validity of our establishment of a prognostic model based on apoptotic prognostic-related genes.

After modeling, we conducted validation by dividing patients with LUSC into different risk groups based on the median risk score. Patients in the high-risk group had significantly worse prognosis. The results of ROC curve analysis showed that the prognostic model had good predictive performance. Furthermore, through analysis of the risk score of the model and other clinical features, the risk score of the model was found to be an independent prognostic indicator, and C-index analysis showed that the risk score had better predictive value than did other traditional clinical parameters. Therefore, the high predictive value of the model was again confirmed.

In addition, we conducted functional enrichment analysis on the apoptotic differentially expressed genes that we screened, identifying the key pathways through which they exert their function. According to GO and KEGG pathway analyses, we found that in cancer, cell apoptosis is mediated by changes in the mitochondrial membrane, which is a multifactorial process involving the BCL-2 family proteins, cysteine proteases, and large molecular complexes. A study has shown that in the preinitiation stage of mitochondria, different proapoptotic signal transduction or damage pathways are activated. When these signals or pathways converge on the mitochondria, the permeability of the inner and outer membranes increases, leading to the execution stage of the apoptotic process (44). The regulation of apoptotic mitochondrial events is achieved through the BCL-2 family of proteins. The BCL-2 family consists of more than 30 proteins and belongs to the BCL-2 superfamily, which includes antiapoptotic proteins, proapoptotic proteins, and homology domain 3 (BH3)-only proteins (45). In the presence of apoptotic stimuli, the expression of BH3 proteins increases, competitively binding to the antiapoptotic protein BCL-2 to release BAX/BAK from inhibition. Free BAX and BAK form oligomers, causing cytochrome C to form a channel through the outer membrane of the mitochondria, releasing it from the intermembrane space of the mitochondria into the cytoplasm. The released cytochrome C activates the caspase cascade reaction to induce cell apoptosis (46,47). Therefore, mitochondria can be considered to be the main integrator of the death pathway. Apoptosis depends on the activation of the above variety of signaling pathways, and these pathways are often dysregulated in cancer, providing a direction for further research on the treatment of apoptosis in LUSC.

Furthermore, in our multifaceted study of the immune microenvironment, we found significant differences in TMB and *TP53* between the high- and low-risk groups. Previous research (48) has shown that TMB in blood can be used to evaluate the efficacy of camrelizumab in combination with chemotherapy in patients with advanced LUSC. During treatment, blood TMB levels are positively correlated with treatment efficacy, indicating that higher TMB leads to better treatment efficacy and longer OS and progression-free survival (PFS) for patients. These findings are consistent with the results of our study, in which we found differences in gene mutations between the high- and low-risk groups, with the most common type of gene mutation, that of *TP53*, showing significant differences in mutation type between the high- and low-risk groups. Previous study has shown that *TP53* is one of the most common mutated genes in lung cancer, and its mutation is closely related to the occurrence and development of lung cancer. The type of *TP53* mutation is related to prognosis, and patients with nonsense mutations have a poorer prognosis (49). This is consistent with our research, as patients in the high-risk group had a poorer prognosis.

Although a previous study has investigated the relationship between apoptosis and cancer, there is sparse research concerning its relationship with tumor immunity (50). Stromal cells and immune cells are the main elements of the tumor microenvironment, and an important aspect of our research lies in exploring the correlation between the risk model of patients with LUSC and tumor immunity. We used the ESTIMATE algorithm to calculate these scores and found that the high-risk group had higher immune and stromal scores. This indicates that changes in immune status can also affect the process of cell apoptosis. We found that natural killer (NK) cells and macrophages, which are inherently linked with cell apoptosis, were highly expressed in the high-risk group. A study has shown that the mitochondrial apoptosis (mtApoptosis) pathway is crucial for efficient NK killing, and NK cells can preactivate cancer cells for mtApoptosis. Preactivated NK cells bind BH3 mimetics to NK cells, synergistically killing cancer cells *in vitro* and inhibiting tumor growth *in vivo* (51). This study suggests that mtApoptosis can enhance NK-based immunotherapy. Interestingly, research has found that macrophages are a heterogeneous group of cells in the innate immune system that are crucial for the initiation, progression, and resolution of inflammation. They have significant functional plasticity and can respond to abnormalities and initiate programs to overcome

them and restore normalcy (52,53). The cytokines and intracellular components released by apoptotic cells can activate macrophages, causing them to shift from the M1 to M2 phenotype, thereby promoting tumor growth and metastasis. In addition, apoptotic cells can also induce macrophages to produce growth factors and extracellular matrix components, promoting tumor cell proliferation and invasion. If the interaction between apoptotic cells and macrophages is not disrupted, surviving tumor cells may receive substantial support from the reaction induced by local macrophages in apoptotic tumor cells, thereby enhancing tumor recurrence (54). The consideration of these findings indeed provides valuable ideas and possible strategies for researchers to further explore the antitumor potential of cell apoptosis and tumor immunity.

Tumor heterogeneity is a significant challenge in cancer treatment, as different subpopulations of cells may exhibit varying sensitivity and resistance to therapeutic drugs (55). Therefore, understanding tumor heterogeneity may be considerably valuable for developing personalized treatment plans and predicting treatment outcomes. Compared to traditional “bulk” RNA-seq methods, which average potential differences in cell-specific transcriptomes, scRNA-seq analyzes the gene expression patterns of each individual cell, providing a clear insight into the entire tumor ecosystem, such as the mechanisms of intra- and intertumoral heterogeneity (56). Through scRNA-seq analysis, this study ultimately divided cells into 8 subgroups. To further understand the expression patterns and functions of apoptosis-related prognostic genes in different cell subgroups, we compared the expression of prognostic genes in different cell subgroups to determine their functions in these subgroups. We found that *BMP2* was highly expressed in epithelial cells and may thus exert critical functions in these cells. In a study on breast cancer, elevated levels of *BMP2* led to excessive activation of the *BMPR1B* signaling pathway, which is the receptor for *BMP2*. When *BMP2* binds to *BMPR1B*, it activates the *BMPR1B* signaling pathway, promoting the transformation of epithelial cells. These transformed epithelial cells have increased proliferation and invasiveness, thereby promoting tumor development (57). Similarly, in a study on lung injury, *BMP2* was found to activate the BMP signaling pathway, leading to increased BMP activity and ultimately resulting in the transformation of epithelial cells and the disruption of epithelial barrier function. This study successfully inhibited the increase in BMP activity and the disruption of epithelial barrier function by suppressing the expression of

*BMP2*, thereby avoiding lung injury (58). This provides a good explanation of the role of prognostic genes in tumor development at the cellular level.

Different subpopulations of cells exist within tumors and have different gene expression profiles, which results in heterogeneity within the tumor. Sequencing multiple tumor regions can reveal the evolutionary pattern of the tumor, with tumor cells having different gene mutations and expression profiles at different time points and locations, forming a branched evolutionary tree structure (59). Pseudotime analysis is a method for inferring trajectories from scRNA-seq data. It sorts cells along a trajectory based on the similarity of their expression patterns and determines the lineage structure by identifying branching events (60). In this study, we determined trajectories with different differentiation states based on the scRNA-seq data of NSCLC and located the selected prognostic genes on the cell differentiation trajectories. We observed the changes of prognostic genes in different clusters and the same cell types. Combining pseudotemporal analysis and the localization of cell types in different clusters over time, we were able to intuitively witness the evolution of cells in NSCLC. This provides new insights into the mechanisms and driving factors of tumor evolution.

Our study represents the inaugural attempt to construct a prognostic model based on differentially expressed ARGs to predict the survival rates of LUSC patients. This model holds the potential to introduce novel molecular markers for personalized treatment in LUSC, thereby enhancing treatment efficacy. Our research employs a variety of statistical methods to ensure the stability and reliability of the model. Furthermore, through correlation analysis of prognostic ARGs, we have augmented the biological interpretability of the model. The integrated application of these methods enhances the potential value of our prognostic model in clinical applications.

Although the prognostic model established in this study demonstrated good predictive performance, was proven to be a positive prognostic indicator for patients with LUSC, and provided more accurate information for the treatment and prognostic evaluation of patients with LUSC by incorporating scRNA-seq data, there are still some limitations that need to be considered. The analysis conducted in this study was based on retrospective data from the GEO and TCGA databases, and due to the scarcity of scRNA-seq data in the GEO database, we could not obtain complete single-cell sequencing results for LUSC samples. Our results need to be further functionally

validated. Despite these limitations, our findings can serve as a valuable concept-validation study, providing biomarkers and targets for future research and serving as a meaningful reference for the personalized treatment of patients with LUSC.

## Conclusions

In summary, based on bioinformatics analysis, this study identified apoptosis-related genes that are associated with prognosis in LUSC, and constructed a risk model based on these prognostic genes that can accurately predict the prognosis of LUSC. Through functional enrichment analysis, we explored the role of mitochondrial membrane changes in mediating apoptosis in LUSC, involving various factors such as BCL-2 family proteins, cysteine proteases, and macromolecular complexes. Apoptosis depends on the activation of different signaling pathways, but these pathways are often dysregulated in LUSC. We also studied the differences in the immune microenvironment between high- and low-risk groups, and found that patients with high TMB had better prognosis than those with low TMB, consistent with previous study showing a positive correlation between TMB and the efficacy and survival of immunotherapy. Finally, single-cell sequencing analysis provided insights into the cellular mechanisms of tumor development, revealing the important biological function of *BMP2* in epithelial cells, which, when overexpressed, can lead to the excessive activation of the BMPRII signaling pathway, promoting the transformation of epithelial cells and increasing the risk of tumor development. Our study provides guidance for the diagnosis and treatment of LUSC patients.

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**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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