

Identification of an Autophagy-Related lncRNA Prognostic Signature and Related Tumor Immunity Research in Lung Adenocarcinoma

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Abstract

Background: Autophagy is closely associated with the tumor immune microenvironment (TIME) and prognosis of patients with lung adenocarcinoma (LUAD). In the present study, we established a signature based on long noncoding RNAs (lncRNAs) related to autophagy (ARlncRNAs) to investigate the TIME and survival of LUAD patients.

Methods: We selected ARlncRNAs associated with prognosis to construct a model, and divided each sample into different groups based on risk score. Subsequently, Kaplan-Meier survival analysis was performed to investigate the survival outcomes of LUAD patients in different groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis explored the enriched pathways and functions. Several bioinformatics analyses were conducted to explore the TIME of LUAD patients in different groups.

Results: The ARlncRNAs signature could be recognized as an independent prognostic factor for LUAD patients, and patients in the low-risk group had a greater survival advantage. GO and KEGG enrichment analysis suggested that several immune functions and pathways were enriched in different groups. A high-risk score correlated significantly negatively with high abundance of immune cells and stromal cells around the tumor and high tumor mutational burden (TMB). Low-risk patients had a higher PD-1, CTLA-4 and HAVCR2 expression, and had a better efficacy of immune checkpoint inhibitor (ICIs), including PD-1/CTLA-4 inhibitor.

Conclusions: A reliable signature based on ARlncRNAs was constructed to explore the TIME and prognosis of LUAD patients, which could provide valuable information for individualized LUAD treatment.

1. Introduction

Lung cancer is one of the malignant tumors with the highest morbidity and mortality in the world¹. The incidence and mortality of lung cancer in the United States in 2021 are estimated to be 235,760 and 131,880, respectively². In China, there were estimated 733,000 new lung cancer cases and 610,000 deaths in 2015³. Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancers, of which adenocarcinoma accounts for about 50% of NSCLC⁴. Although the advent of radiotherapy and chemotherapy has revolutionized the NSCLC treatment, the 5-year survival rate of NSCLC with distant metastasis is only 7%⁵. Therefore, it is crucial to screen a reliable biomarker to guide individualized treatment of NSCLC.

As a crucial part of the recycling process in complicated tumor immune microenvironment (TIME), autophagy in tumor immunity has been increasingly appreciated⁶. For example, Jiang *et al.* illustrated the impact of autophagy on TIME from three perspectives, and proposed that autophagy-based therapy combined with immunotherapy may be promising⁷. Gerada *et al.*⁸ explained that TIME determines whether autophagy promotes or inhibits tumors. lncRNAs are a class of RNA that do not code proteins

with transcripts > 200 nucleotides⁹. They participated in the progression and metastasis of lung adenocarcinoma (LUAD) and were associated with immune pathways, and even served as a biomarker for prognosis of LUAD^{10 11}. Recently, the prognostic signatures based on coding or non-coding genes to predict the prognosis of LUAD patients has been a research hot spot^{12 13}. However, utilizing ARlncRNAs to construct models, and exploring the tumor immunity and the efficacy of immunotherapy in LUAD patients was still lacking. In this study, we established a novel ARlncRNAs signature to analyze the TIME and prognosis of LUAD patients, which represented a step toward individualized immunotherapy in LUAD.

2. Materials And Methods

2.1 Data acquisition

A signature based on ARlncRNAs was established by a multi-step approach (Fig. 1). We acquired the transcriptome profiles and the corresponding clinical information of LUAD patients and normal samples from The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>) database in December 2020. The gene transfer format (GTF) files was obtained from Ensembl (<http://asia.ensembl.org>) to distinguish lncRNAs and mRNAs. Furthermore, a list of autophagy-related genes (ARGs) from the Human Autophagy Database (HADb) (<http://www.autophagy.lu/>) was acquired. We identified ARlncRNAs by calculating the correlation coefficient between the ARGs and lncRNAs ($|cor| > 0.4$ and $p < 0.001$).

2.2 Construction of the ARlncRNAs signature

Kaplan-Meier survival analysis combined with univariate Cox analysis were performed to screen the ARlncRNAs expression levels that were significantly associated with the overall survival (OS) of the LUAD patients (log-rank $p < 0.05$, and $p < 0.05$). They were chosen for subsequent Cox proportional hazard regression analysis, and a heatmap was plotted for visualization. Subsequently, the multivariate Cox regression analysis was performed to construct the prognostic signature by running the R-x64-4.0.4 survival package, and the risk score of every LUAD patient was calculated based on the following formula:

n

$$\text{Risk score} = \sum_{i=1}^n \text{Coef}(i) \times E(i),$$

i = 1,

Coef (i) and E(i) represent the regression coefficient of the multivariate Cox analysis for each ARlncRNA and each ARlncRNA expression level, respectively. The median value of risk score was considered as the cut-off point for differentiating each patient into different groups.

2.3 Validation of the risk prognosis model

Several ROC curves were generated and AUC were calculated by R-x64-4.0.4 survival, survminer, timeROC packages to validate the predictive value of the prognostic signature. Kaplan–Meier log-rank test was conducted for comparing the OS between different risk groups to assess the predictive value of the prognosis model. A barplot and a boxplot was generated by R-x64-4.0.4 packages plyr, ggplot2, ggpubr to study whether there are statistical differences in OS between different risk groups. In addition, univariate and multivariate Cox regression analyses were performed to explore whether the prognostic signature was a potential independent prognostic indicator for patients with LUAD, and the results were visualized with two forest maps. To study the correlation between risk score and clinicopathological characteristics, several chi-square tests were performed to plot boxplots. A heatmap was generated to visualize the expression of ARlncRNAs included in the process of modeling.

2.4 Enrichment of functions and pathways in the ARlncRNA signature

Differentially expressed genes between different risk groups were identified by performing differential expression analysis. The R-x64-4.0.4 packages limma was utilized and the significance threshold for determining differentially expressed genes was log fold change [FC] > 1 and false discovery rate [FDR] < 0.05. To filter functional phenotypes in different risk groups, we performed GSEA 4.0.1 (<https://www.gseamsigdb.org/gsea/index.jsp>)¹⁴ by Kyoto Encyclopedia of Genes and Genomes (KEGG) ($p < 0.05$ and $q < 0.25$). GO function enrichment analysis was conducted to study the functional phenotypes that differentially expressed ARlncRNAs were enriched ($p < 0.05$ and $q < 0.05$).

2.5 Correlation analysis of tumor mutational burden

Spearman analysis was conducted to calculate the correlation coefficient between the tumor mutational burden (TMB) and the constructed model based on R-x64-4.0.4 ggplot2, ggpubr and ggExtra packages. The differences in TMB between different risk groups were explored by Wilcoxon signed-rank test, and a column diagram was plotted. To study the connection between TMB and the prognostic signature comprehensively, Kaplan–Meier survival analyses was performed for comparing OS between high-TMB and low-TMB.

2.6 Estimation of tumor immune microenvironment of the prognostic signature

To explore the abundance of immune cells and stromal cells between different groups, StromalScore, ImmuneScore, and ESTIMATEScore (StromalScore + ImmuneScore) of each patient were calculated by R-x64-4.0.4 estimate and limma packages. Then, Wilcoxon signed-rank tests were utilized to explore the differences in StromalScore, ImmuneScore, and ESTIMATEScore between different groups, and three column diagrams were plotted for visualization. Single-sample gene-set enrichment analysis (ssGSEA) was conducted for scoring LUAD-infiltrating immune cells to quantify their relative content. The scores of immune cells and pathways in different groups were shown on multi-boxplots, respectively.

2.7 Evaluation of clinical immunotherapy efficacy of ARlncRNAs signature

The emergence of immunotherapy has revolutionized the treatment of NSCLC. National Comprehensive Cancer Network (NCCN) guidelines¹⁵ recommend a series of immunotherapy drugs, including CTLA-4 and PD-1 blocking antibodies for NSCLC treatment. Expression analyses of common ICI-related immunosuppressive molecules (e.g., PD-1, CTLA-4, and HAVCR-2) were conducted by R-x64-4.0.3, ggpubr and limma packages to detect any statistical differences in expression of common ICI-related immunosuppressive molecules between different risk groups, and several violin plots were generated for visualization. Information of immunotherapy efficacy for LUAD patients was obtained from the The Cancer Immunome Atlas (TCIA, <https://tcia.at/>) database¹⁶. The difference in immunotherapy efficacy for LUAD patients between different groups was calculated by Wilcoxon signed-rank tests, and violin plots were labeled as follows for visualization: ***<0.001, **<0.01, and *<0.05.

3. Results

3.1. Identification of ARlncRNAs

Figure 1 shows, a multi-step approach to identify ARlncRNAs was conducted. A total of 551 LUAD transcriptome data, corresponding clinical data from TCGA-LUAD cohort (54 normal samples and 497 LUAD samples) and 232 ARGs from HADb were downloaded. Subsequently, we distinguished the mRNAs and lncRNAs by GTF files and identified 1071 ARlncRNAs based on Spearman correlation analysis.

3.2. Establishment of the prognostic signature based on ARlncRNAs

Using univariate Cox analysis combined with Kaplan-Meier survival analysis, we screened out 57 ARlncRNAs associated with survival of LUAD patients (Fig. 2a). Multivariate Cox regression analysis yielded 14 ARlncRNAs among ARlncRNAs related to prognosis for subsequent modeling. The risk score of every patient was calculated based on correlation coefficients calculated by multivariate Cox regression analysis, and each sample was differentiated into different groups by median value of risk score.

3.3. The prognostic signature is a powerful LUAD prognostic indicator

To quantify the predictive ability of the ARlncRNAs signature, we generated several ROC for validation. The curves demonstrated that 1-, 3-, and 5-year AUC values were 0.764, 0.742, and 0.713, respectively (Fig. 2b-c). Furthermore, compared with other clinicopathological characteristics (e.g., age, gender, and stage), the 1-year AUC value was the maximum (Fig. 2d). The survival curve, boxplot, and barplot indicated that patients with low-risk had a better prognosis and were statistically significant (Fig. 3a-c).

To explore the relationship between the ARlncRNA signature with clinicopathological characteristics, we performed univariate and multivariate Cox regression analysis, which suggested that two factors, i.e., the risk score (hazard ratio [HR] = 1.589, confidence interval [CI] = 1.406–1.797, $p < 0.001$) and stage (HR = 1.470, [CI] = 1.265–1.710, $p < 0.001$), correlated with the survival (Fig. 3d-e). The results above suggested that the ARlncRNA signature could act as a potential independent prognostic indicator for patients with LUAD. The heatmap (Fig. 4a) suggested that stage ($p < 0.001$) and ImmuneScore ($p < 0.001$), N ($p < 0.01$) were statistically different in different groups. In addition, most ARlncRNAs included in the process of modeling were enriched in the low-risk group, suggesting autophagy was more active in the low-risk group. The scatter diagrams indicated that T (Fig. 4b, $p < 0.01$), stage (Fig. 4c, $p < 0.001$), and N (Fig. 4d, $p < 0.001$) were associated with risk score.

3.4. Functional annotation of the prognostic signature

GSEA analysis (Fig. 5a) indicated that several immune pathways such as B cell receptor signaling, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, and VEGF signaling pathway were enriched in patients with low risk. Cell cycle, P53 signaling pathway, pathways in cancer, small cell lung cancer, and thyroid cancer were relatively more active in the high-risk group. GO function enrichment analysis indicated that several functions related to tumor immunity such as humoral immune response, antimicrobial humoral response, antigen processing and presentation, and T cell mediated cytotoxicity, were enriched in different groups (Fig. 5b).

3.5. Assessment of the correlation between the TMB and ARlncRNA signature

The correlation curve (Fig. 5c) and column diagram (Fig. 5d) indicated that TMB was significantly negatively correlated with risk score, and the TMB of patients with high-risk was significantly higher than those with low-risk ($p < 0.001$). According to survival curves (Fig. 5e-f), patients with high TMB had a better survival advantage ($p = 0.035$), and patients with a combination of low-risk and high TMB showed a great prognosis.

3.6. Tumor immune microenvironment of the ARlncRNA signature

We found that StromalScore (Fig. 6a, $p < 0.001$), ImmuneScore (Fig. 6b, $p < 0.001$), and ESTIMATEScore (Fig. 6c, $p < 0.001$) in low-risk patients were significantly higher than that of high-risk patients. The multi-boxplots (Fig. 6d-e) indicated that the abundance of activated dendritic cells (aDCs), B cells, dendritic cells (DCs), immature dendritic cells (iDCs), mast cells, neutrophils, plasmacytoid dendritic cells (pDCs), T helper cells, tumor infiltrating lymphocytes (TIL), and regulatory T cells (Tregs) associated significantly negatively with the risk score. Compared with the high-risk group, several immune pathways, e.g., clinical complete response (CCR), check-point, cytolytic activity, human leukocyte antigen (HLA), T cell co-inhibition, T cell co-stimulation, and type II interferons (type II IFN) response were more active in the low-risk group.

3.7. ARlncRNA signature in the role of immunotherapy

The column diagrams showed that the expression of common ICI-related immunosuppressive molecules, e.g., PD-1 (Fig. 6f, $p < 0.05$), CTLA-4 (Fig. 6g, $p < 0.001$), and HAVCR-2 (Fig. 6h, $p < 0.001$), in low-risk group were significantly higher than those in high-risk group, which indicated that the efficacy of the above-mentioned ICIs seem to be better for low-risk patients. The violin plots based on TCIA validated the above results, suggesting that whether it is PD-1 inhibitor alone (Fig. 6i, $p < 0.05$), CTLA-4 inhibitor alone (Fig. 6j, $p < 0.001$) or a combination of the two (Fig. 6k, $p < 0.001$), the efficacy of low-risk patients is better than that of high-risk patients.

4. Discussion

Although there are already lots of signatures utilizing ARGs or ARlncRNAs to predict LUAD patients' survival outcomes, we are the first to explore the tumor immunity of the ARlncRNA model in detail^{17 18}. We conducted a detailed study on the tumor immunity of the ARlncRNA signature, which renders the constructed signature applicable for guiding the clinical personalized treatment of LUAD patients.

First, the lncRNA and ARG transcriptome profiles were obtained, and ARlncRNAs related to prognosis of LUAD patients based on co-expression analysis and univariate Cox analysis were identified. Next, we calculated the AUC value of 1-, 3-, and 5-year to obtain an ideal signature and differentiated each patient into different groups based on median value. Then, the survival and clinicopathological characteristics was analyzed to assess the predictive value of the ARlncRNA signature. Subsequently, we conducted a comprehensive assessment of the tumor immunity of the ARlncRNA signature, including GO and KEGG function enrichment analyses, TMB, TME, infiltration of immune cells, expression of common ICI-related immunosuppressive molecules, and efficacy of ICIs.

Previous studies on LUAD have mostly focused on single genes or noncoding genes, which are unable to illustrate the complex tumorigenesis and development process^{19 20}. In recent years, a combination of several genes to improve the predictive value of OS in LUAD patients were gradually identified. For example, Duan *et al.*¹⁸ constructed a prognostic signature based on ARGs, which served as a novel biomarker in LUAD. Meanwhile, Zhu *et al.*²¹ identified a ferroptosis-related gene signature and explored the immune cells infiltration. In this study, several ARlncRNAs included in the modeling process have been already reported in various malignant tumors, such as CARD8-AS1^{22 23}, AC060780.1²⁴, AC123595.1^{25 26}, UGDH-AS1²⁷, LINC00996²⁸, LINC00861^{29 30 31}, AL606489.1¹⁷, HLA-DQB1-AS1³², LINC00654³³, LINC00847^{34 35}. While others have not been discovered yet and may be potential novel biomarkers for further study.

Researchers found that autophagy played a vital role in tumorigenesis and development. Gu *et al.* confirmed that bupivacaine induced autophagy through Akt/mTOR signaling, inhibiting the progression of NSCLC³⁶. Lin *et al.* proposed that high expression of miR-30a improved the prognosis of NSCLC after

neoadjuvant chemotherapy by reducing autophagy caused by chemotherapy drugs³⁷. In addition, Li *et al.* found that the dysfunction of autophagy mediated by c-myc/miR-150/EPG5 had a great impact on the progression of NSCLC³⁸. Collectively, autophagy was probably involved in the occurrence and development of LUAD through a certain signaling pathway, having a significant impact on the prognosis of LUAD patients.

Then, GSEA enrichment analysis suggested that patients with low-risk had more autophagy and were enriched in B cell receptor signaling, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, and the VEGF signaling pathway. Recent searches confirmed the strong correlation between autophagy and VEGF. For example, Chen *et al.* found that VEGF promoted the occurrence of autophagy and VEGF knockdown decreased the autophagy level³⁹. In addition, Spengler *et al.*⁴⁰ discovered that VEGF signaling pathway regulated autophagy in endothelial cells. Taken together, we speculated that autophagy probably contributed to the occurrence and development of LUAD through VEGF signaling pathway, and that the ARlncRNA signature was closely associated with tumor immunity.

Recently, the interaction between autophagy and tumor immunity was investigated comprehensively. For example, TMB and TIME were identified as important determinants of the efficacy of ICIs and in the prognosis of cancer patients⁴¹. Moreover, Jena *et al.*⁴² have demonstrated that autophagy was closely related to TIME and participated in tumor progression. To explore the relationship between the ARlncRNA signature and tumor immunity thoroughly, we conducted ssGSEA to investigate the immune status in different groups. The patients in low-risk group had a higher abundance of immune cells and were more active in immune pathways, most of which were validated closely associated with autophagy. For example, Di *et al.*⁴³ found that CALCOCO2, an autophagy receptor, was mainly expressed in B cells, which mediated autophagy. In addition, the function of DCs to secrete cytokines has been shown to be inhibited by autophagy⁴⁴. Turan *et al.*⁴⁵ proved through experiments that iDCs infected with TSV-1 could induce autophagy. Li *et al.*⁴⁶ demonstrated that autophagy of mast cells could serve as a therapeutic target for allergic reactions. Ding *et al.*⁴⁷ revealed that neutrophils were associated with autophagy. Autophagy has been validated to fuel pDCs⁴⁸. Schmid *et al.*⁴⁹ proposed that CD4(+) T helper cells could recognize MHCI molecules presented after autophagy. Lu *et al.*⁵⁰ demonstrated that autophagy could mediate the function of Tregs. Samuel *et al.*⁵¹ proved that cellular metabolism facilitated autophagy to mediate the cytolytic effect. Zhang *et al.*⁵² confirmed the firm correlation between autophagy and HLA. These studies above suggest that autophagy is closely linked to tumor immunity, and autophagy probably participates in LUAD progression by regulating tumor immunity.

At present, PD-1⁵³ and CTLA-4⁵⁴ inhibitors have been validated to benefit patients with advanced NSCLC in clinical trials. Furthermore, research indicated that the autophagy of tumor cells increased the expression of ICI-related immunosuppressive molecules (e.g., PD-1 and CTLA-4), and affected anti-tumor immune responses directly. In this study, expression analyses was conducted to study the correlation between the ARlncRNA signature and the expression of common ICI-related immunosuppressive molecules, which revealed low-risk patients always had a higher expression of them and a better efficacy

of ICIs. Subsequently, we analyzed the efficacy of ICIs and verified the results above; demonstrating that regardless of whether it is a PD-1 inhibitor alone, a CTLA-4 inhibitor alone or a combination of the two, the efficacy of patients in the low-risk group is better than that of the high-risk patients. Overall, the ARlncRNA signature could serve as a novel indicator for screening patients applicable for ICIs.

According to our data, we speculated that compared with high-risk patients, low-risk patients have more active autophagy, stronger tumor immunity, greater survival advantage and are more applicable for ICIs treatment. Autophagy could play an crucial part in the progression of LUAD by regulating the tumor immunity through VEGF signaling pathway, which had a great impact on prognosis of LUAD patients.

However, there are several limitations in our research. Firstly, bias of the information in the analysis process, as the profiles were obtained from public database. Secondly, it was difficult to find an ideal Gene Expression Omnibus (GEO) set including both 14 lncRNAs newly identified and detailed clinical information to validate the constructed ARlncRNA signature. Finally, external validation, such as quantitative real-time PCR and microarrays are necessary to increase credibility.

In conclusion, we constructed a novel ARlncRNAs signature and predicted the survival of LUAD patients, the state of TIME, and even the efficacy of ICIs accurately based on the expression of the 14 ARlncRNAs included in the modeling process, which may benefit patients with advanced NSCLC. Immunotherapy combined with TIME targeted therapy may improve individualized treatment of LUAD in the future.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

All data analysed during the current study are accessible from the TCGA database(<https://portal.gdc.cancer.gov/>).

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Hang Chen and Guodong Xu contributed to the conception of the study;

Menglu Sang and Saiqi Ni performed the R language;

Yao Lin, Chengfang Wu, and Yinyu Mu contributed significantly to analysis and manuscript preparation;

Hang Chen performed the data analyses and wrote the manuscript;

Kaitai Liu, Shibo Wu, and Ni Li helped perform the analysis with constructive discussions.

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Figures

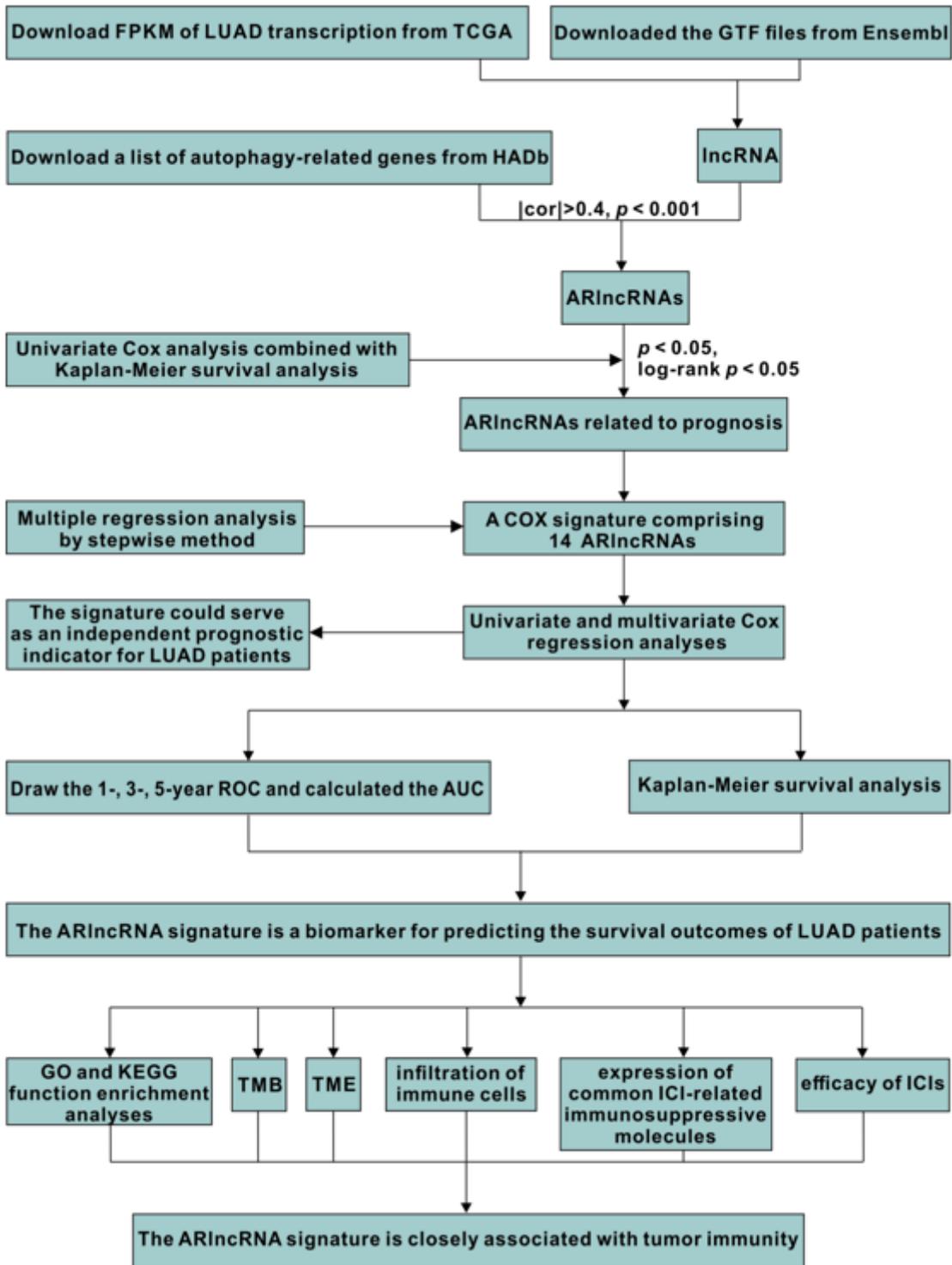


Figure 1

The flowchart for identifying a novel reliable ARlncRNAs signature and its implication.

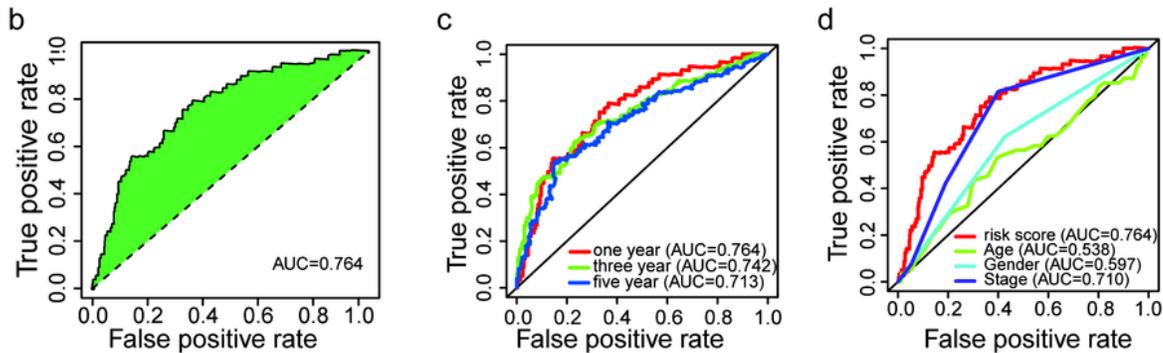
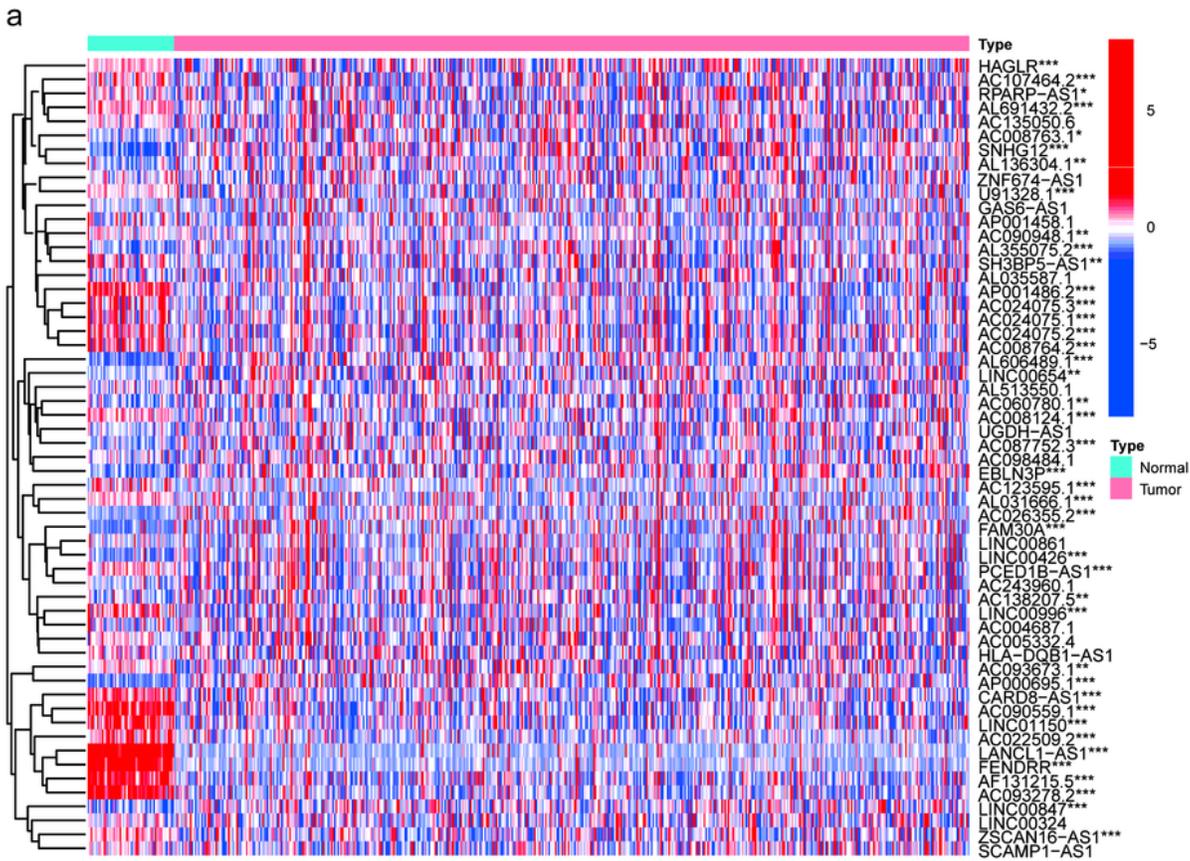


Figure 2

Identification of the ARlncRNA signature. (a) Heatmap shows the expression of ARlncRNAs related to survival in LUAD and normal samples. (b and c) The 1-, 3-, 5-year AUC values were >0.7. (d) Compared with AUC of age, sex, and stage, the 1-year AUC value was the maximum.

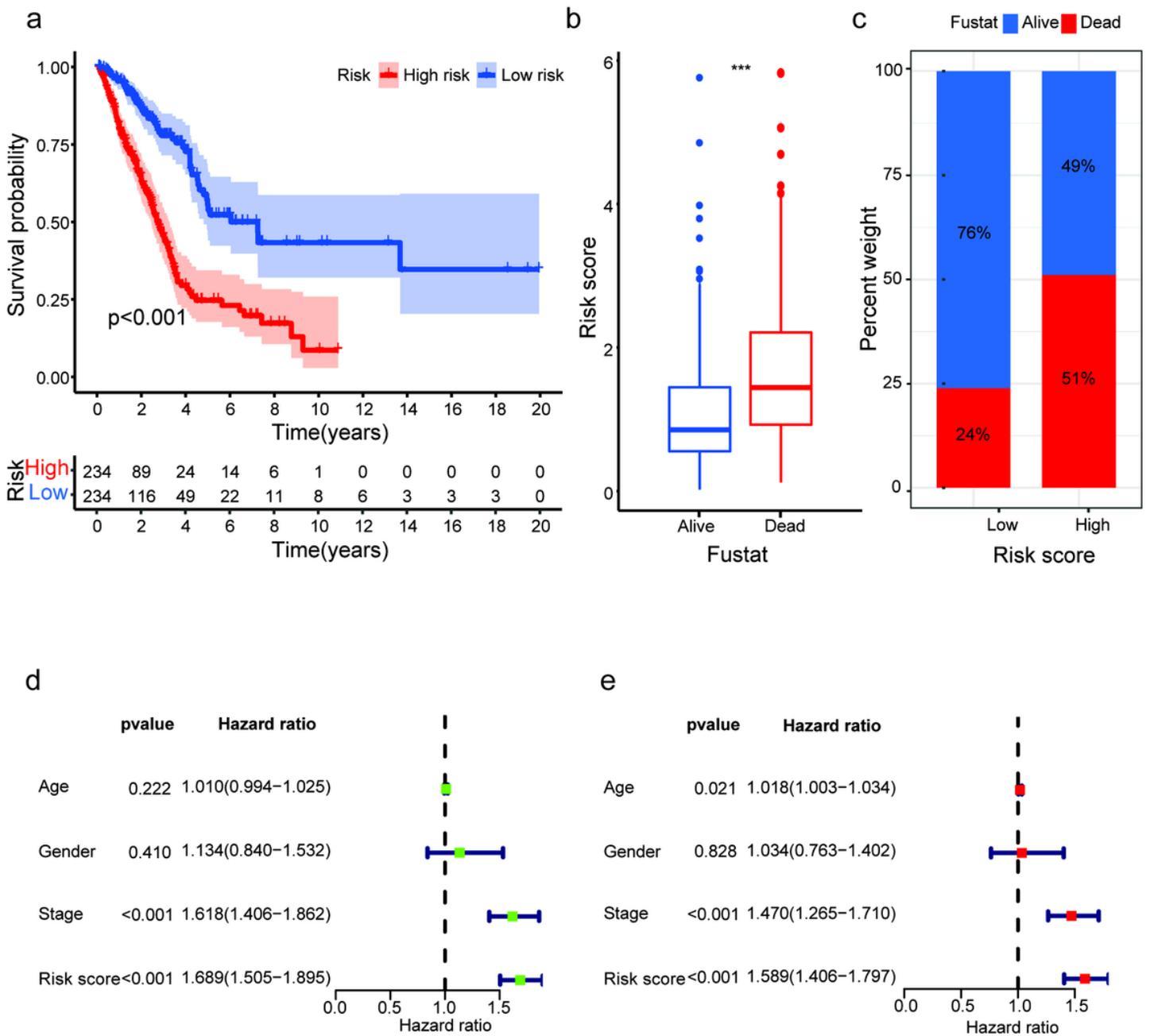


Figure 3

Validation of the predictive value of the prognostic signature. (a-c) The survival curve (a), boxplot (b), and barplot (c) indicate that the ARlncRNA signature was associated with survival outcomes of LUAD patients and patients with low risk have a significant survival advantage. (d and e) Univariate (d) and multivariate (e) Cox regression analyses including age, gender, stage, risk score suggests that risk score (HR = 1.589, CI = 1.406–1.797, $p < 0.001$) and stage (HR = 1.470, CI = 1.265–1.710, $p < 0.001$) could act as an independent prognostic factor for LUAD.

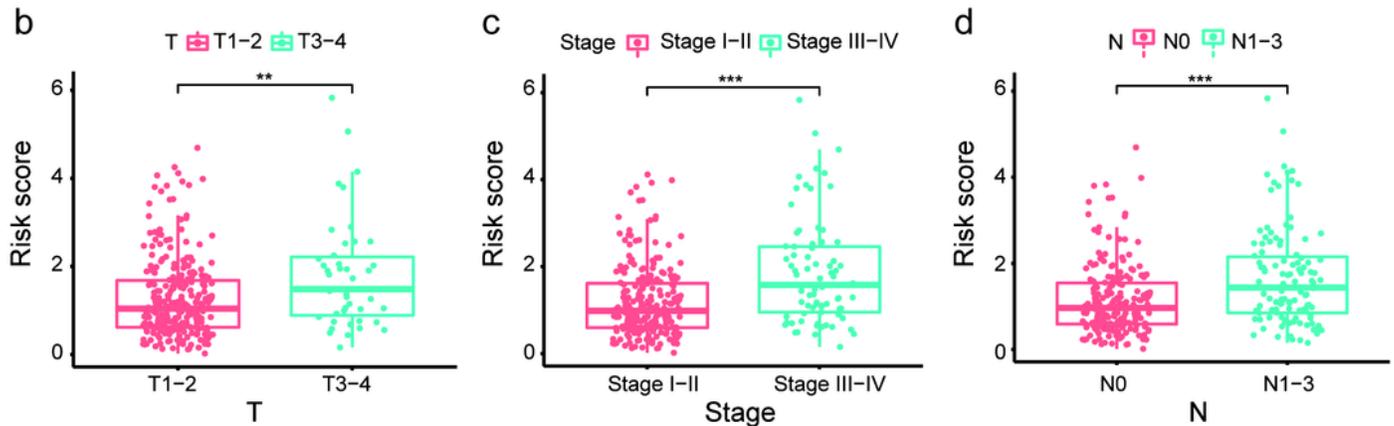
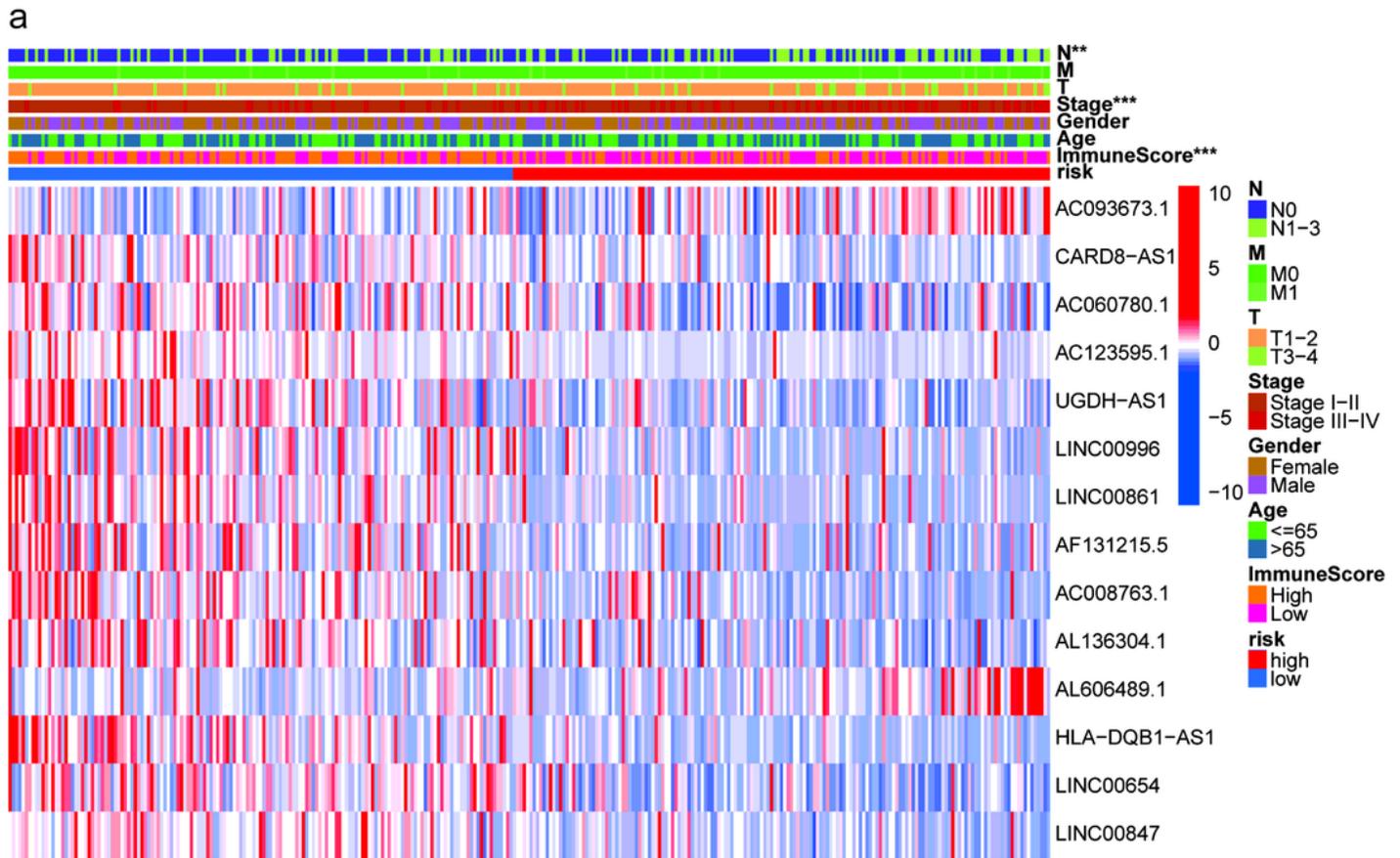


Figure 4

Exploration of clinicopathological characteristics of the 14-ARlncRNA signature. (a) The heatmap suggested that stage ($p < 0.001$), ImmuneScore ($p < 0.001$), and N ($p < 0.01$) were statistically different in different groups. In addition, most ARlncRNAs included in the modeling process were enriched in the low-risk group, suggesting autophagy was more active in the low-risk group. (b-d) The scatter diagrams indicate that higher levels of T (b), N (c), and stage (d) tend to have a higher risk score.

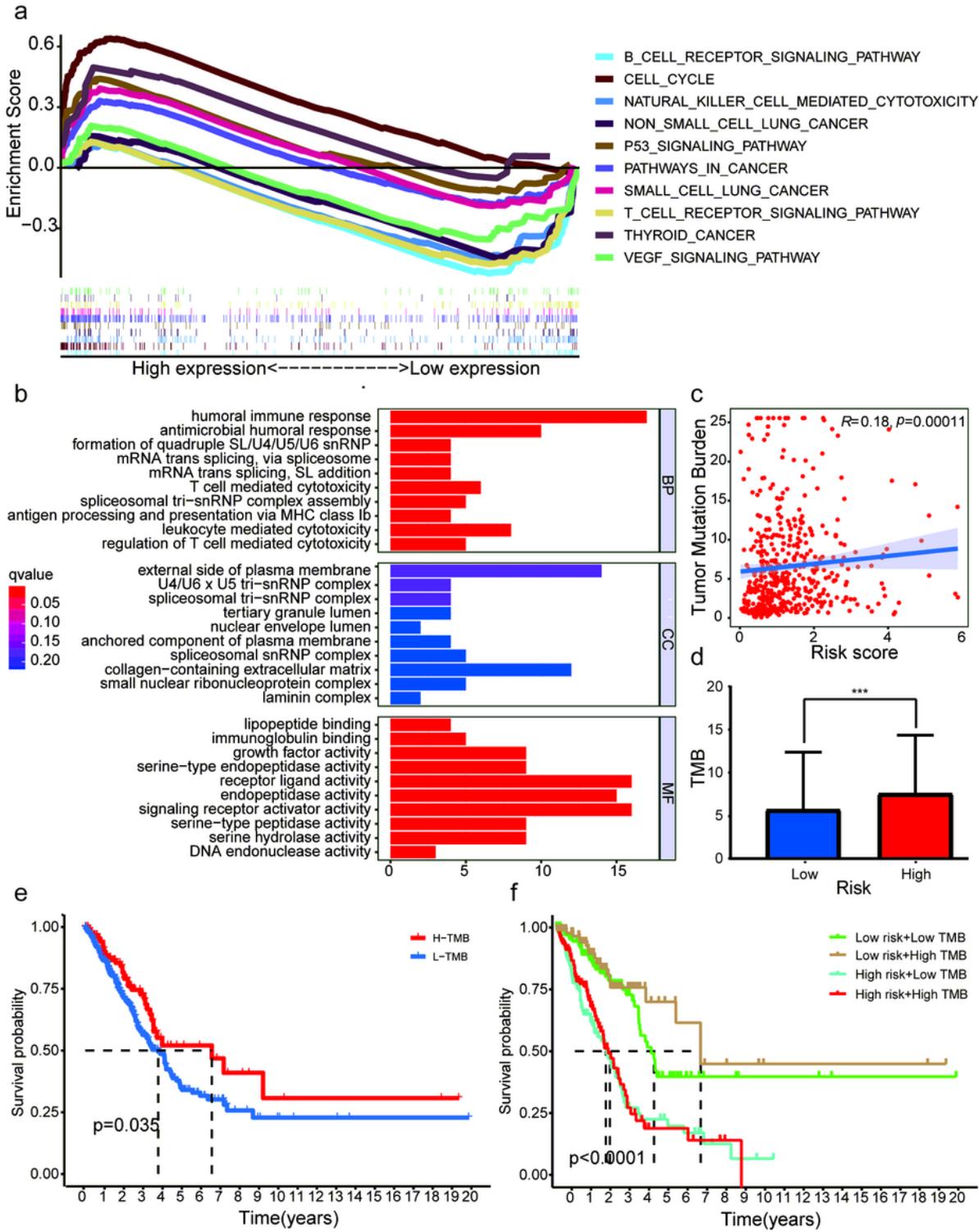


Figure 5

Research on tumor immunity of the 14-ARlncRNA signature. (a and b) KEGG (a) and GO (b) function enrichment analyses reveal that several immune pathways and immune function were enriched in different groups, suggesting the ARlncRNA signature may be associated with tumor immunity. (c and d) The scatter diagram (c) and column diagram (d) show that high-risk patients exhibit significantly higher

TMB. (e and f) The survival curves indicate that patients with a combination of low-risk and high TMB showed a greater prognosis.

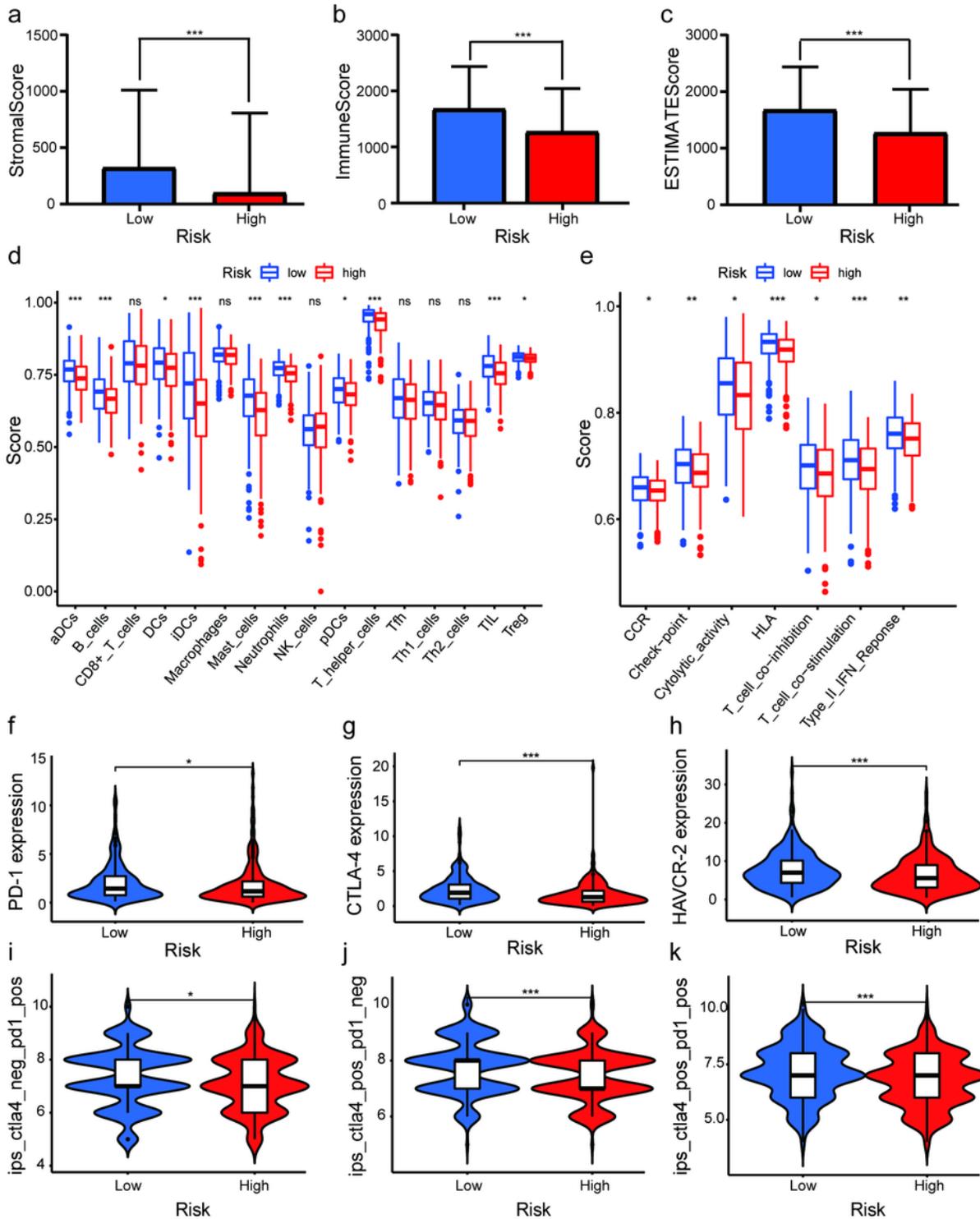


Figure 6

Exploration of the tumor immunity and ICIs efficacy of the ARlncRNA signature. (a-c) The column diagrams reveal that low-risk group patients have a higher StromalScore (a), ImmuneScore (b), and ESTIMATEScore (c). (d and e) The multi-boxplots show that patients in low-risk group have a higher

abundance of aDCs, B cells, DCs, iDCs, mast cells, neutrophils, pDCs, T helper cells, TIL, and Treg (d). Several immune pathways, including CCR, check-point, cytolytic activity, HLA, T cell co-inhibition, T cell co-stimulation, and type II IFN response were enriched in low-risk patients (e). (f-h) The violin plots indicate that high-risk score correlated significantly negatively with high PD-1 (f), CTLA-4 (g), and HAVCR-2 (h) expression, revealing low-risk patients are more applicable for ICIs treatment. (i-k) The violin plots validate the results above that regardless of whether it is a PD-1 blocker alone (i), a CTLA-4 blocker alone (j) or a combination of the two (k), the efficacy of patients with low-risk is better than that of high-risk patients.