Significance of *NKX2-1* as a biomarker for clinical prognosis, immune infiltration, and drug therapy in Lung Squamous Cell Carcinoma

Huiyue Lin [Corresp., 1], **Juyong Wang** [1], **Qing Shi** [2], **Minmin Wu** [2]

[1] Oncology Department, Longhua Hospital Shanghai University of Traditional Chinese Medicine, Shanghai, China

[2] Key Laboratory of Diagnosis and Treatment of Severe Hepato-Pancreatic Diseases of Zhejiang Province, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China

Corresponding Author: Huiyue Lin
Email address: linhuiyue65@163.com

Background This study was performed to determine the biological processes in which *NKX2-1* is involved and thus its role in the development of lung squamous cell carcinoma (LUSC) toward improving the prognosis and treatment of LUSC. **Methods** Raw RNA sequencing (RNA-seq) data of LUSC from The Cancer Genome Atlas (TCGA) were used in bioinformatics analysis to characterize *NKX2-1* expression levels in tumor and normal tissues. Survival analysis of Kaplan-Meier curve, the time-dependent receiver operating characteristic (ROC) curve, and a nomogram were used to analyze the prognosis value of *NKX2-1* for LUSC in terms of overall survival (OS) and progression-free survival (PFS). Then, differentially expressed genes (DEGs) were identified, and Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and Gene Set Enrichment Analysis (GSEA) were used to clarify the biological mechanisms potentially involved in the development of LUSC. Moreover, the correlation between the *NKX2-1* expression level and tumor mutation burden (TMB), tumor microenvironment (TME), and immune cell infiltration revealed that *NKX2-1* participates in the development of LUSC. Finally, we studied the effects of *NKX2-1* on drug therapy. To validate the protein and gene expression levels of *NKX2-1* in LUSC, we employed immunohistochemistry(IHC) datasets, The Gene Expression Omnibus(GEO) database, and qRT-PCR analysis.**Results** *NKX2-1* expression levels were significantly lower in LUSC than in normal lung tissue. It significantly differed in gender, stage and N classification. The survival analysis revealed that high expression of *NKX2-1* had shorter OS and PFS in LUSC. The multivariate Cox regression hazard model showed the *NKX2-1* expression as an independent prognostic factor.Then nomogram predicted LUSC prognosis.There are 51 upregulated DEGs and 49 downregulated DEGs in the *NKX2-1* high-level groups. GO, KEGG and GSEA analysis revealed that DEGs were enriched in cell cycle and DNA replication.The TME results show that *NKX2-1* expression was positively

associated with mast cells resting, neutrophils, monocytes, T cells CD4 memory resting, and M2 macrophages but negatively associated with M1 macrophages. The TMB correlated negatively with *NKX2-1* expression.The pharmacotherapy had great sensitivity in *NKX2-1* low-level group, the immunotherapy is no significant difference in *NKX2-1* low-level and high-level groups. The analysis of GEO data demonstrated concurrence with TCGA results. IHC revealed *NKX2-1* protein expression in tumor tissues of both LUAD and LUSC. Meanwhile qRT-PCR analysis indicated a significantly lower *NKX2-1* expression level in LUSC compared to LUAD. These qRT-PCR findings were consistent with co-expression analysis of *NKX2-1*.**Conclusion** We conclude that *NKX2-1* is a potential biomarker for prognosis and treatment LUSC. A new insights of *NKX2-1* in LUSC is still needed further research.

Significance of *NKX2-1* as a biomarker for clinical prognosis, immune infiltration, and drug therapy in Lung Squamous Cell Carcinoma

Huiyue Lin[1]; Juyong Wang[1]; Qing Shi[2]; MinminWu[2];

[1]Oncology Department, Longhua Hospital Shanghai University of Traditional Chinese Medicine, Shanghai, China
[2]Key Laboratory of Diagnosis and Treatment of Severe Hepato-Pancreatic Diseases of Zhejiang Province, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China;

Corresponding Author *:
Huiyue Lin[1],
725 Wanping South Road, Fenglin Street, Xuhui District, Shanghai, 200030, China.
E-mail**:** linhuiyue65@163.com.

Abstract:

Background This study was performed to determine the biological processes in which *NKX2-1* is involved and thus its role in the development of lung squamous cell carcinoma (LUSC) toward improving the prognosis and treatment of LUSC.

Methods Raw RNA sequencing (RNA-seq) data of LUSC from The Cancer Genome Atlas (TCGA) were used in bioinformatics analysis to characterize *NKX2-1* expression levels in tumor and normal tissues. Survival analysis of Kaplan-Meier curve, the time-dependent receiver operating characteristic (ROC) curve, and a nomogram were used to analyze the prognosis value of *NKX2-1* for LUSC in terms of overall survival (OS) and progression-free survival (PFS). Then, differentially expressed genes (DEGs) were identified, and Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and Gene Set Enrichment Analysis (GSEA) were used to clarify the biological mechanisms potentially involved in the development of LUSC. Moreover, the correlation between the *NKX2-1* expression level and tumor mutation burden (TMB), tumor microenvironment (TME), and immune cell infiltration revealed that *NKX2-1* participates in the development of LUSC. Finally, we studied the effects of *NKX2-1* on drug therapy. To validate the protein and gene expression levels of *NKX2-1* in LUSC, we employed immunohistochemistry(IHC) datasets, The Gene Expression Omnibus (GEO) database, and qRT-PCR analysis.

Results *NKX2-1* expression levels were significantly lower in LUSC than in normal lung tissue. It significantly differed in gender, stage and N classification. The survival analysis revealed that high expression of *NKX2-1* had shorter OS and PFS in LUSC. The multivariate Cox regression hazard model showed the *NKX2-1* expression as an independent prognostic factor.Then nomogram predicted LUSC prognosis.There are 51 upregulated DEGs and 49 downregulated DEGs in the *NKX2-1* high-level groups. GO, KEGG and GSEA analysis revealed that DEGs were enriched in cell cycle and DNA replication.The TME results show that *NKX2-1* expression was positively associated with mast cells resting, neutrophils, monocytes, T cells CD4 memory resting, and M2 macrophages but negatively associated with M1 macrophages. The TMB correlated negatively with *NKX2-1* expression.The pharmacotherapy had great sensitivity in *NKX2-1* low-level group, the immunotherapy is no significant difference in *NKX2-1* low-level and high-level groups. The analysis of GEO data demonstrated concurrence with TCGA results. IHC revealed *NKX2-1* protein expression in tumor tissues of both LUAD and LUSC. Meanwhile qRT-PCR analysis indicated a significantly lower *NKX2-1* expression level in LUSC compared to LUAD. These qRT-PCR findings were consistent with co-expression analysis of *NKX2-1*.

Conclusion We conclude that *NKX2-1* is a potential biomarker for prognosis and treatment LUSC. A new insights of *NKX2-1* in LUSC is still needed further research.

Keywords: *NKX2-1/TTF-1*; lung squamous cell carcinoma; prognosis; immune infiltration; therapy

Introduction

Lung cancer is the world's most common and deadliest malignant respiratory tumor, with 2.2 million estimated cases based on the 2020 report of the International Agency for Research on Cancer (https://gco.iarc.fr/, accessed on 15 September 2022). Small cell lung cancer (SCLC, 15%) and non‐small cell lung cancer (NSCLC, 85%) are the two main types of lung cancer, with NSCLC patients demonstrating lower rates of overall survival and 5-year survival[1,2]. NSCLC is divided into LUAD and LUSC according to pathogenesis and histological morphology [1]. LUSC, which comprises 30% of cases of NSCLC, has a high rate of metastasis and recurrence [3]. The current clinical first-line therapy for LUSC involves use of immune checkpoint inhibitors in combination with carboplatin and paclitaxel[4]. Although several therapies are confirmed to be beneficial for LUSC in prolonging progression-free survival, the clinical benefits for LUSC patients remains limited [5,6]. Therefore, an investigation into novel biomarkers is required to improve diagnoses and treatment of LUSC patients. Many studies on LUSC-related genes and prognostic markers have reported that the molecular mechanisms underlying the pathogenesis and progression of LUSC are not clear[6]. Thus, clarification of these mechanisms is required for the development of new promising biomarkers or potential drug treatments, which are urgently needed.

NK2 homeobox 1 (*NKX2-1*), also known as thyroid transcription factor-1 (*TTF-1*), is a member of the *NKX2* family of homeodomain-containing transcription factors[7]. *NKX2-1* regulates normal lung development and morphogenesis, especially in lung epithelial cell differentiation, and was demonstrated to be important for the occurrence of lung cancer [7,8]. An independent study showed that *NKX2-1* possibly regulated the adeno-to-squamous transdifferentiation to shape the tumor microenvironment or affected immune cell types shaping the correspongding tumor microenvironment, then determined tumor phenotype[9]. *NKX2-1/TTF-1* has been used as a diagnostic marker for LUAD and SCLC and is approximately 70% positive for LUAD, in which it is an indicator of favorable prognosis [7,10]. Although the expression of *NKX2-1/TTF-1* in LUSC appears to very low or indetectable, a couple of studies have demonstrated a close connection exists between *NKX2-1* and LUSC [2,7]. As there are currently few studies on this association, we sought to examine this further by downloading RNAseq data on LUSC from TCGA, which contains the genetic profiles of more than 20 different types of tumors [11]. In the present study, the database was analyzed to characterize the expression levels of *NKX2-1* in LUSC compared with normal tissues. We then explored the relationship between *NKX2-1* expression and clinical characteristics, TMB, the infiltration of immune cells, immune checkpoint genes, the TME, and the pharmacotherapy response. Furthermore, we studied the co-expression of DEGs in *NKX2-1* high-level and low-level groups in conjunction with GO and KEGGanalyses of DEGs to identify significant biological functions and pathways. In summary, the results of this study may provide new clues to understand the underlying molecular mechanisms of *NKX2-1* in LUSC and its influence on immune landscapes, TME, and the

pharmacotherapy of LUSC.

Materials & Methods

TIMER Database

TIMER (http://timer.cistrome.org, accessed on 29 July 2022) is a comprehensive resource for the systematic analysis of immune infiltrates, which includes more than 10,000 samples across 32 cancer types from TCGA[12,13]. We used TIMER to explore the mRNA transcriptional level of *NXK2-1* in various cancer types.

Data Processing

RNA-seq data profiles and relevant clinical information were downloaded from the TCGA database (https://portal.gdc.cancer.gov, accessed on 29 July 2022), which included 502 LUSC and 49 normal lung tissue samples. In our study, RNA-seq data were processed and normalized using the 'limma' package in R. Expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM), which were then transformed to log2 fold-change (Log2FC) values for subsequent analysis[14]. The missing OS values of patients were excluded to reduce statistical bias. All downloaded files were calibrated, normalized, and log2-transformed by R software (version 4.1.3, R Core Team, Vienna, Austria, https://www.r-project.org/).

Identification and Validation of *NKX2-1* Gene Expression in TCGA Database

The identification of DEGs was performed between normal tissue and tumor tissue using the "limma" R package, with absolute Log2FC > 1 and false discovery rate (FDR) < 0.05. A heatmap plot was drawn to exhibit the expression difference of other genes between *NKX2-1* high-level and low-level groups via the pheatmap R package.

Functional and Pathway Enrichment Analysis

The R package "clusterProfiler" was used to performed function and pathway analyses in both *NKX2-1* groups according to GO and KEGG. GSEA was performed in R software with c2.cp.kegg.v7.0 symbols.gmt as the reference gene set and Top 5 enrichment analysis results were visualized, with $p < 0.05$ indicating the significant enrichment of functional annotations.

The Relationship between *NKX2-1* and Other Genes

The "limma", "ggplot2", "ggpubr", "ggplot2"and "ggExtra" packages in R were used to analyze the relationship between co-expressed genes and *NKX2-1* using the Pearson method. Circos was used to intuitively exhibit the correlation between co-expression genes, with red representing positive and green representing negative correlation.

Predictive Nomogram Design

A nomogram was constructed and predicted based on the age, gender, stage, and risk score using the "rms" package and Cox regression model to predict the OS of LUSC patients at 1, 3, and 5 years. A calibration plot was used to evaluate the nomogram, which was based on Harrell's concordance index (C-index). "Points" was the scoring scale for each factor, and "total points" was the scale for total score. Based on the total score of the patient, the 1-, 3- and 5-year survival rate was inferred.

Correlation Analysis of *NKX2-1* Expression in TME and TMB

The TME, contains tumor cells, surrounding immune, and stroma cells[15]. The R package

"ESTIMATE" was used to compute the StromalScore, ImmuneScore, and ESTIMATEScore[16]. CIBERSORT was applied to estimate the proportion of 22 immune cells for each sample in both *NKX2-1* groups[17]. The p-values were based on the Wilcoxon signed-rank test, and $p < 0.05$ was considered to indicate a statistically significant difference. The correlation of *NKX2-1* expression with immune cells was conducted by using Pearson correlation analysis in the R package. The Pearson correlation test was used to investigate the correlation between *NKX2-1* and 17 immune checkpoint-related genes (such as BTLA, TNFSF14, CD80, and CD244), with results visualized using the pheatmap R package. The tumor mutational burden (TMB) is defined as the total number of base mutations per million cells in the tumor, and represents the number of mutations per megabase (Mut/Mb) of DNA in cancer, that is assessed by whole exome sequencing (WES), the systematic sequencing of all exons[18]. The correlation of *NKX2-1* expression with TMB was analyzed by the Spearman correlation test in R software.

Therapy in *NKX2-1* High-Level and Low-Level Groups

Pharmacotherapy sensitivity analysis was based on the half-maximal inhibitory concentration (IC50), an indicator of the rate of response of tumor cells to pharmacotherapy. The "pRRophetic" package was used to predict the drug sensitivity of the two *NKX2-1* groups[19,20]. Immunotherapy data were obtained from the TCIA website (https://tcia.at/) and visualized in a violin plot through R software.

Verification of *NKX2-1* in lung cancer

The GEO database (https://www.ncbi.nlm.nih.gov/geo/) served as the validation set. GEO datasets (GSE67061, GSE84784, GSE101420) were calibrated and normalized using R software. Mining analysis of the *NKX2-1* gene was performed. Verification of *NKX2-1* protein expression in LUSC and LUAD was conducted using The Human Protein Atlas (HPA, https://www.proteinatlas.org/). Additionally, human LUAD cell lines (PC-9) and human LUSC cell lines (H520) were procured from the Shanghai Institute of Biosciences and Cell Resources Center (Chinese Academy of Sciences, Shanghai, China). PC-9 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific), and H520 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific), both supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Cells were maintained in a humidified cell incubator at 37° C with 5% CO2. Total RNA was isolated from the cells using TRIzol Reagent (Invitrogen, USA), and RNA concentration was determined using a DS-11 Spectrophotometer (DeNovix, USA). Reverse transcription was performed using HiScript III RT SuperMix for qPCR (Vazyme, China), followed by qRT-PCR using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China) on the CFX96 Real-Time System (Bio-Rad, USA). Primers were obtained from Sangon Biotech (Shanghai, China), with their sequences shown in Table 1. GAPDH was used as the reference gene for normalization. The expression differences of genes were calculated using the $2^{-\Delta ct}$ method[21].

Statistical Analysis

The statistical analyses were performed in R (version 4.1.3, R Core Team, Vienna, Austria,

https://www.r-project.org/), which included the Wilcoxon, Kruskal–Wallis, and chi-square statistical tests. The relationship between *NKX2-1* expression and LUSC clinicopathological features was shown as box plots using the "limma" and "ggpubr" R packages. The data from the TCGA database were divided into *NKX2-1* high- level and low-level groups based on the median expression level. The "ComplexHeatmap" R package was used to show the differences in clinicopathological features between the groups. Kaplan–Meier survival analysis was performed using the R packages "survminer" and "survival" to assess the differences in OS and PFS between the groups. Univariate and multivariate analysis were performed using the Cox proportional hazards regression model to identify significant factors. The time-dependent ROC curve analysis and area under the curve (AUC) were plotted by using the "timeROC" package in R to evaluate the predictive accuracy of the *NKX2-1* expression at different endpoints (1, 3, or 5 years) of the prognostic risk score mode. Statistical analyses were conducted using SPSS 23.0 statistics software (SPSS, USA). Student's t-test was utilized to determine differences between two experimental groups, while one-way ANOVA was employed for multiple group comparisons. A p-value < 0.05 was considered statistically significant.

Results

NKX2-1 mRNA Expression Levels in Various Cancers

NKX2-1 expression levels in various cancers were explored using TIMER. The results reveal that *NKX2-1* expression levels were significantly lower in LUSC but significantly higher in thyroid carcinoma (THCA). Although *NKX2-1* expression levels were very low in these cancers, its expression levels were significantly different in bladder urothelial carcinoma (BLCA), colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), HPV-positive HNSC and HPV-negative HNSC, kidney renal papillary cell carcinoma (KIRP), prostate adenocarcinoma (PRAD), skin cutaneous melanoma (SKCM), SKCM-metastasis, and uterine corpus endometrial carcinoma (UCEC) (Figure. 1A). We analyzed the *NKX2-1* expression data from TCGA to further characterize *NKX2-1* expression in LUSC. According to the paired and unpaired results, its expression in LUSC tumor tissue was significantly lower than that in normal tissue (Figure 1B-C).

Evaluation of Clinical Parameters and Development of a Prognostic Prediction Model for *NKX2-1* in LUSC Patients

The correlations between *NKX2-1* gene expression and clinical characteristics, including age, gender, stage, and TNM stage, were explored. The results showed that *NKX2-1* expression did not significantly differ according to age, T stage, or M stage ($p>0.05$). *NKX2-1* expression significantly differed according to gender and for Stage I vs. Stage II, Stage II vs. Stage III, and N0 vs. N1 ($p<0.05$) (Figure.2A-F). In addition to stage ($p < 0.05$), there were no significant differences in the *NKX2-1* high-level and low-level groups due to age, gender, TNM stage, Race, smoking status, site of tumor and treatment($p>0.05$) (Table 2). We found that LUSC patients with higher *NKX2-1* expression had shorter OS ($p=0.015$) and PFS ($p=0.036$) (Figure 2G-H). Further, we performed univariate and multivariate Cox regression analyses. *NKX2-1* was significantly associated with OS

in univariate (HR=1.462, 95%CI =1.082-1.976, p=0.013) and multivariate (HR=1.495, 95%CI=1.104-2.025, p=0.009) Cox regression analysis (Table 3). This suggests that *NKX2-1* is an independent prognostic factor. ROC curves were constructed to evaluate the prognostic accuracy, and the 1-,3-, and 5-year AUC values of *NKX2-1* were 0.574, 0.564, and 0.542, respectively (Figure 2I). We constructed a nomogram to predict LUSC prognosis precisely (Figure 2J). The sum of four points could be obtained according to the *NKX2-1* expression level, gender, age, and stage, with each total point corresponding to the predicted 1-,3-, and 5-year OS. Good agreement was observed between the observed and predicted OS rates at 1 ,3 , and 5 years in plots (Figure 2K). These results demonstrate that *NKX2-1* expression has a certain reference value for LUSC prognosis.

Comparison Analysis in *NKX2-1* High-Level and Low-Level Groups and Co-Expression Analysis of *NKX2-1*

We used the R software (version 4.1.3, R Core Team, Vienna, Austria, https://www.r-project.org/)to perform a comparative study between *NKX2-1* high-level and low-level groups. A total of 51 upregulated DEGs and 49 downregulated DEGs in the *NKX2-1* high-level groups were plotted in a heatmap (Figure 3A). *NKX2-1-AS1*, *SLC22A31*, *NAPSA*, *SFTA2*, *C16orf89*, *SFTPD* correlated positively and *TRIM29*, *LINC01980*, *GJB5*, *KRT5*, and *IRF6* correlated negatively with *NKX2-1* in terms of expression (Figure 3B-L, $p < 0.05$). The top 11 co-expressed genes are shown in the Circos plot, light pink represents positive correlation, light blue represents negative correlation(Figure 3M).

Functional Enrichment Analyses of DEGs in *NKX2-1* High-Level and Low-Level Groups

GO function and KEGG pathway enrichment analysis were used to reveal the function and mechanisms of 729 DEGs in *NKX2-1* high-level and low-level groups. The GO terms were divided into biological process (BP), cellular component (CC) and molecular function (MF) ontologies. The GO analysis results indicate that the DEGs are mainly enriched in the following BP categories: humoral immune response, sodium ion transport, negative regulation of peptidase activity, antimicrobial humoral response, antibacterial humoral response, and bicarbonate transport. The analysis shows that the DEGs were significantly enriched in the CC categories of collagen-containing extracellular matrix, apical part of cell, apical plasma membrane, blood microparticle, multivesicular body, and lamellar body. DEGs enriched in MF were mainly enriched in the categories of metal ion transmembrane transporter activity, passive transmembrane transporter activity, ion channel activity, cation channel activity, gated channel activity, and potassium channel activity (Figure 4A-C). In addition, the results of the KEGG pathway analysis indicated that 729 DEGs were enriched in neuroactive ligand－receptor interaction, complement and coagulation cascades, cytokine－cytokine receptor interaction, bile secretion, and cAMP signaling pathway (Figure 4D-E).

GSEA Identifies DEG-Related Signaling Pathways in *NKX2-1* High-Level and Low-Level Groups

To explore the mechanisms in which DEGs are involved in LUSC, we identified pathways that showed significant differences between the *NKX2-1* high- and low-expression groups by conducting GSEA (Figure 5). KEGG_CELL_CYCLE, KEGG_PPAR_SIGNALING_PATHWAY, KEGG_DNA_REPLICATION, and KEGG_HOMOLOGOUS_RECOMBINATION were active in the low-level group, whereas KEGG_OLFACTORY_TRANSDUCTION was active in the high-level group.

Immune Infiltration Analysis and Tumor Mutational Burden of *NKX2-1* Expression

We explored the correlation of *NKX2-1* expression level in immunity, ESTIMATE, and CIBERSORT. The ESTIMATE results indicate that the Stromal, Immune, and Estimate scores were lower in the *NKX2-1* low-level group than the *NKX2-1* high-level group (Figure 6A). Moreover, we found that infiltration levels for mast cells resting, neutrophils, monocytes, T cells CD4 memory resting, and macrophages M2 were significantly higher in the *NKX2-1* high-level group than in the *NKX2-1* low-level group, and the macrophage M1 infiltration level was comparatively higher in the *NKX2-1* low-level group (Figure 6B). Further, we performed correlation analysis between *NKX2-1* expression and immune infiltration cells. The results show that *NKX2-1* expression was positively associated with mast cells resting, neutrophils, monocytes, T cells CD4 memory resting, and M2 macrophages but negatively associated with M1 macrophages (Figure 6C-I). Interestingly, we found that the tumor mutational burden correlated negatively with NKX2-1 expression (Figure 6J). We also analyzed the relationship between NKX2-1 and immune checkpoint genes, which correlated positively (Figure 6K).

Analysis of Differences in Immune Therapy and Pharmacotherapy Responsiveness in *NKX2-1* High-Level and Low-Level Groups

In *NKX2-1* high-level and low-level groups, we examined the therapeutic sensitivity to chemotherapy drugs and molecular targeting drugs using the pRRophetic package and then screened out data for common clinical pharmacotherapies of cancer. The results indicated that the IC50s of various chemotherapy drugs, including 5-fluorouracil, cisplatin, docetaxel, doxorubicin, etoposide, gemcitabine, paclitaxel, and vinorelbine, and drugs for molecular targeted therapy, including axitinib, BI-2536, and gefitinib sorafenib, were lower in the *NKX2-1* low-level group, indicating greater sensitivity to the above drugs (Figure 7A-L). We then obtained the immunotherapy score data from tumor-targeted immune cell agonist (TICA) and compared the differences in immunotherapy score between *NKX2-1* high-level and low-level groups; interestingly, there was no significant difference (Figure 7M-P).

Verification of *NKX2-1* in Lung Cancer

The GEO database (GSE67061, GSE84784, GSE101420) comprised 78 LUSC samples and 69 normal lung samples. *NKX2-1* expression was significantly lower in LUSC compared to normal tissue (Figure 8A). Co-expression analysis of *NKX2-1* genes and differential analysis were performed on LUSC mRNA data from TCGA and GEO databases using R software, followed by

the identification of shared genes from both analyses, resulting in 1014 genes (Figure 8B). Co-expression analysis of the GEO database revealed positive correlations of *NKX2-1* expression with *SLC22A31, NAPSA, SFTA2, C16orf89,* and *SFTPD*, while negative correlations were observed with *TRIM29, GJB5, KRT5,* and *IRF6* (Table 4). Functional annotation through GO and KEGG pathway enrichment analysis for the 1014 genes indicated enrichment in cell cycle processes (Figure 8C-F). IHC results showed *NKX2-1* protein expression in tumor tissues of both LUAD and LUSC, with LUAD displaying negative, moderate, and strong expression (Figure 9A-C), and LUSC showing negative, weak, and moderate expression (Figure 9D-F). Furthermore, qRT-PCR revealed significantly lower *NKX2-1* expression in LUSC compared to LUAD (Figure 9G), with no significant difference in *SFTPD* and *NAPSA* expression levels compared to *NKX2-1*. However, *IRF6* and *TRIM29* displayed significantly higher expression levels compared to *NKX2-1* (Figure 9H).

Discussion

NKX2-1, also known as *TTF-1*, is a lineage-specific transcription factor involved in the occurrence of lung cancer and regulate the adeno-to-squamous transdifferentiation to determined tumor phenotype[9,22]. Many studies have shown that *NKX2-1* has high sensitivity and specificity for diagnosing primary lung cancer and is expressed in most cases of LUAD [7]. Additionally, *NKX2-1* expression has been reported as a positive prognostic indicator for LUAD[23]. Due to the low or delection of NKX2-1 expression, the important role of NKX2-1 is neglected in LUSC[7]. With the developments in cancer research bioinformatics, we can use powerful tools to analyze and explore the underlying molecular mechanisms in cancer biology and development, providing further reference value for clinical research[24]. Currently, there is little research into the association between *NKX2-1* expression and LUSD, so we sought to explore this relationship further using the TCGA database.

In present study, we examined and compared *NKX2-1* expression between the normal and tumor tissues of several pan-cancers using TIMER data. These results indicate that *NKX2-1* is mainly expressed in LUAD, LUSC, and THCA, consistent with current reports in the literature [8]. Interestingly, there was no significantly difference in *NKX2-1* expression between normal and tumor tissues in LUAD. We found that, in the diagnosis and treatment of LUAD, *NKX2-1* expression is often detected using immunohistochemical (IHC) methods, with fewer studies examining gene expression, which is a new finding[25,26]. Not surprisingly, the TIMER results are consistent with our results from R software, with *NKX2-1* expression found to be significantly lower in tumor than in normal tissue. This is consistent with the findings in several studies that *NKX2-1* expression is lower in LUSC[7,8].

Furthermore, we analyzed the relationship between *NKX2-1* expression and clinicopathological features, considering any differences between NKX2-1 high- and low-level groups, which revealed its prognostic value to a certain extent. The results of OS and PFS in K-M plotter indicated high expression of *NKX2-1* is clearly linked with poor prognosis in LUSC. Meanwhile, the univariate and multivariate Cox regression analysis revealed *NKX2-1* to be an independent prognostic factor in LUSC, which is consistent with the findings of Puglisi et al.[27].

The ROC curve, a graphical plot illustrating the diagnostic ability of a binary classifier system as its discrimination threshold is varied, juxtaposes sensitivity against 1-specificity. Its quantification, the AUC, is a widely recognized measure in clinical epidemiology to evaluate biomarkers' diagnostic capabilities [28]. However, our analysis revealed that *NKX2-1* expression's prognostic utility for LUSC over 1, 3, and 5-year intervals fell below expectations. Historically acknowledged for its scarcity in LUSC, recent advancements in gene detection technologies, such as those employed by TCGA, have identified *NKX2-1* amplification in LUSC, albeit at low levels in 2012 [29,30]. This underexpression likely contributes to the observed diminished sensitivity in ROC curve analysis. Given the inherent limitations of ROC curves for comprehensive analysis, we employed Nomograms to further delineate *NKX2-1*'s prognostic significance in LUSC. Nomograms offer a personalized risk assessment, integrating clinical or disease-specific characteristics, and have been instrumental in prognostication across various cancers for years [31,32]. Our study's nomogram, incorporating both clinical features and *NKX2-1* expression levels, indicates that higher *NKX2-1* expression correlates with reduced OS, consistently across predicted 1, 3, and 5-year outcomes.

NKX2-1 is recognized for its high specificity to LUAD and serves as a crucial biomarker for its diagnosis[33]. Nakraet al underscores the strong link between *NKX2-1* expression and EGFR mutation status, highlighting its association with favorable outcomes[26]. Independent of EGFR mutation presence, *NKX2-1* IHC positivity is correlated with improved PFS and OS[26,34-35]. Within cancer biology, *NKX2-1* plays a dual role, functioning as both an oncogenic driver and a tumor suppressor[36]. The beneficial prognostic implications of *NKX2-1* positivity in LUAD may be attributed to its anti-tumoral activities, suggesting a potential mechanism underlying its prognostic advantage[37]. Despite *NKX2-1*'s strong association with LUAD as opposed to LUSC, it is expressed in approximately three-quarters of LUSC cell lines, albeit not predominantly[22]. A retrospective analysis by Svaton et al. highlighted the presence of *NKX2-1*-positive cases in LUSC, revealing a longer PFS and OS in *NKX2-1*-negative scenarios, aligning with findings from this investigation[34]. Conversely, recent studies have identified a subset of LUSC cases with high *NKX2-1* cytoplasmic expression, identified using the ERP8190 antibody, exhibiting enhanced OS and disease-free survival[38]. This evidence suggests a prognostic and predictive significance of *NKX2-1* in LUSC. With ongoing advancements in genetic testing technologies, the observed expression levels of *NKX2-1* in LUSC and its impact warrant further exploration.

The occurrence and development of lung cancer is a complex and dynamic process that relies on the synergy between gene mutations and tumor microenvironment[39]. The immune microenvironment is involved in the development of LUSC and that *NKX2-1* is associated with lung inflammation, so we explored the relationship between the *NKX2-1* expression level and immunity in LUSC[40,41]. The immune infiltration algorithm was used to evaluate the level of *NKX2-1* expression with regard to immune infiltration and the distribution of immune cells. The results showed that the lower expression of *NKX2-1* had the less immune infiltration in LUSC. According to CIBERSORT algorithm analysis results, the expression of *NKX2-1* correlated positively with M2 macrophages, mast cells resting, neutrophils, monocytes and T cells CD4 memory resting, but negatively with M1 macrophages in LUSC. Researchers have found a link

between high macrophage M2 infiltration and worse prognosis in LUSC[42]. Mast cells was associated with the clinical stages of LUSC and implicated in metastasis of malignancies[43]. Monocytes has been proved the assoctiation with poor survial and metastasis in LUSC[44]. There are differences in immune microenvironment in LUSC and LUAD, particularly neutrophils[45].Compared with LUAD, LUSC had the more enrichment of neutrophils which foster squamous cell fate[9]. Loss of *NKX2-1* could led to tumor-associated neutrophils recruitment to shape the immune microenvironment suitable for the survival of squamous carcinoma, which in turn promotes the development of squamous carcinoma[9]. Lower infiltration of neutrophils had great prognosis[46]. Lower *NKX2-1* expression, lower neutrophils infiltration in our study. It may be the reason that *NKX2-1* low-level group has a better prognosis.

In recent years, LUSC treatment has evolved to encompass a variety of approaches. Chemotherapy has historically been the cornerstone of LUSC therapy due to the lack of identifiable driver mutations [47]. Emerging medical advancements have facilitated the approval and clinical integration of immunotherapeutic agents for treating LUSC, offering significant patient benefits [5]. Despite these developments, the prognosis for LUSC patients remains substantially suboptimal. TMB, defined as the aggregate number of mutations within the tumor genome, has demonstrated a robust correlation with responses to immunotherapy [18,48]. TMB is emerging as an evaluation method for immunotherapy and plays a vital role in immune response and as an indicator of favorable survival prognosis in LUSC patients[49]. Evidence indicates a correlation between TMB and tumor stage, with TMB median values in LUSC showing an upward trend from Stage I to Stage IV[43]. Furthermore, Devarakonda et al. observed a favorable prognosis in patients with high TMB who underwent resection for non-small cell lung cancer[50]. This is potentially because tumors with higher mutations are more likely to present neoantigens, rendering them susceptible to immune cell targeting, such targeting can enhance immune response activation, thereby augmenting the efficacy of immunotherapy[43]. In our investigation, a negative correlation was identified between the levels of TMB and *NKX2-1* expression. This suggests that lower *NKX2-1* expression may be indicative of a higher TMB, potentially correlating with a more favorable prognosis for those with diminished *NKX2-1* expression. Consequently, our findings propose that LUSC cases exhibiting low *NKX2-1* expression might derive more significant benefit from immunotherapeutic interventions. Above all, we believed that the expression level of *NKX2-1* has certain clinical guidance significance in LUSC.

To further understand the role of *NKX2-1* expression in therapy, our study was conducted to analyze the pharmacotherapy response by R package. The prediction of pharmacotherapy response through R package has been demonstrated in several clinical trials[17]. We used pRRophetic of R package to study the pharmacotherapy response, including chemotherapeutics and targeted drugs, will help physicians to select a suitable therapy for LUSC patients. The results indicated that the *NKX2-1* low-level group was significantly more sensitive to pharmacotherapy. Immune checkpoint inhibitors (ICIs) currently considered an effective anticancer therapy for lung cancer[46]. In this study, *NKX2-1* expression was shown to correlate positively with immune checkpoint genes. Interestingly, the responsiveness of immune checkpoint inhibitor therapy did not significantly differ between *NKX2-1* high-level and low-level groups. In the management of

LUSC, chemotherapy is the cornerstone of treatment [47]. Our findings corroborate this. In LUAD, positive *NKX2-1* status correlates with improved chemotherapy outcomes, and LUSC displays the opposite trend conversely [10]. Previous works have demonstrated that chemotherapeutic agents can enhance immune response by increasing tumor immunogenicity, similarly h-TMB elevates immunogenicity, potentially improving the efficacy of immune therapies, furthermore chemotherapy can augment TMB, suggesting a synergistic effect on tumor sensitivity to immunotherapy[47,51]. *NKX2-1*'s low expression is associated with higher TMB, indicating a heightened responsiveness to pharmacotherapy, inclusive of chemotherapy. Intriguingly, h-TMB might be amenable to immunotherapy[47]. However, our study did not observe this predicted outcome. Within the LUSC context, macrophages and neutrophils predominantly influence OS, and their interaction with ICIs is notable[52].Our analysis identified macrophages and neutrophils as the immune cells linked with *NKX2-1* expression, possibly elucidating the lack of association between *NKX2-1* expression and immunotherapeutic efficacy. A high macrophage density suggests a 'cool' tumor state, characterized by low TMB and reduced immunotherapy susceptibility[47, 52].Given that low *NKX2-1* expression corresponds to both lower macrophage density and higher h-TMB, it is plausible that increasing TMB, in response to chemotherapy, could render LUSC more receptive to immunotherapy.

Due to the complex oncogenic mechanisms involving *NKX2-1*, we explored the DEGs in both *NKX2-1* high-level and low-level groups and identified co-expression genes. *NKX2-1-AS1*, *SLC22A31*, *NAPSA*, *SFTA2*, *C16orf89*, *SFTPD*, *TRIM29*, *LINC01980*, *GJB5*, *KRT5*, and *IRF6* were found to be correlated with *NKX2-1*. *NKX2-1-AS1*, *NAPSA*, *SFTA2*, *SFTPD*, *TRIM29*, *KRT5*, and *IRF6* had previously been identified to be associated with lung cancer development[29,53-56]. To explore the biological mechanisms of DEGs in *NKX2-1* high-level and low-level groups, we conducted GO function and KEGG pathway enrichment analyses. Based on enrichment and GO function, the DEGs were mainly found to be involved in humoral immunity. KEGG pathway analysis further indicated the DEGs were mainly enriched in cytokine-cytokine receptor interaction, cAMP signaling pathway, viral protein interaction with cytokine − cytokine receptor, and PPAR signaling pathway categories. The cAMP and PPAR signaling pathway categories are known to be closely linked with LUSC carcinogenesis and development[57]. GSEA revealed that the downregulation of *NKX2-1* was involved in tumor progression of LUSC, suggesting that it may be important for the therapeutic benefits of LUSC.

To validate our findings, we analyzed the mRNA data of LUSC using the GEO database. The results from GEO were in agreement with those from TCGA, showing a significant down-regulation of *NKX2-1* expression in LUSC compared to normal lung tissue.The discrepancies in the number of DEGs and *NKX2-1* co-expressed genes in LUSC , obtained using R software, may be attributed to potential variations in the techniques employed for data collection between the GEO and TCGA databases. Then we performed a cross-analysis of differential genes and co-expressed genes obtained from both databases. The resulting common genes were subjected to GO and KEGG enrichment analyses, which further supported the findings of GSEA enrichment analysis in our study. The enrichment analyses indicated that the involvement of *NKX2-1* in LUSC

primarily revolves cell cycle regulation. Previous studies have demonstrated that *NKX2-1* directly regulates the cell cycle, by controlling over the expression of proliferation-related genes[58]. Several studies have demonstrated that the oncogenic mechanism of NKX2-1 involves numerous signaling pathways, such as the AKT, p38 signaling, PI3K, and WNT signaling pathways and uncovered direct transcriptional targets LMO3, EGFR, SOX2, and DUSP6[9,29,59-60]. Harada et al. demonstrated that *NKX2-1* binds to *cyclin D1* (*CCND1*) and plays a role in cell cycle progression, meanwhile over expression of *NKX2-1* leads to increased *cyclin D1* (*CCND1*) levels, potentially influencing metastasis incidence[61]. It is likely to contribute to the relatively poor prognosis associated with high *NKX2-1* expression in LUSC.

The Human Protein Atlas (HPA) provides valuable information on the protein levels of human gene expression profiles in both normal and tumor tissues[62]. Through immunohistochemistry data available in this database, we observed *NKX2-1* expression not only in LUAD but also in LUSC. These findings are consistent with some existing immunohistochemical studies [63]. Our analysis using TIMER data revealed a lower expression level of *NKX2-1* in LUSC compared to LUAD. These findings were further supported by qRT-PCR results, indicating a consistent trend. The observed disparity in expression levels between LUAD and LUSC raises the possibility that *NKX2-1* might exhibit strong expression in LUAD but only moderate expression in LUSC, warranting further investigation. Further exploration of co-expressed genes, such as *NAPSA, SFTPD, TRIM29,* and *IRF6* in LUSC cell lines (H520) through qRT-PCR correlated with our findings, providing additional insights into the mechanisms involving *NKX2-1* in LUSC development. Due to its low expression in LUSC and it is considered as a marker for identifying LUAD[7], research on *NKX2-1* in LUSC has been limited, and the related mechanisms have not been fully elucidated.

Lineage plasticity contributes to the complexity of intratumoral heterogeneity, facilitates histological transitions among tumor subtypes, and may underlie the mechanisms of resistance to therapeutics observed in lung cancer[64]. *NKX2-1* is instrumental in regulating transcriptional programs within the pulmonary domain and is expressed not only in LUAD but also in LUSC[9]. The upregulation of USP13 during early lung tumorigenesis has been reported to suppress *NKX2-1* expression while enhancing *SOX2* expression, thus fostering LUSC development[65]. Interestingly, LUAD has the potential to undergo histological transformation to LUSC, which may confer resistance to targeted therapies[65]. Given the critical role of *NKX2-1* in LUSC, its study is of considerable importance. An analysis of *NKX2-1*-associated differentially expressed genes revealed an association with humoral immunity, aligning with the TME analysis. This association may explain why a significant subset of LUSC patients does not benefit from immunotherapy. Conversely, chemotherapy has been shown to elevate the TMB, and patients with a high TMB could be more responsive to immunotherapy. Therefore, a combination of chemotherapy and immunotherapy emerges as a pragmatic treatment strategy for LUSC. Studies support this approach, indicating improvements in median progression-free survival, overall survival, and response rates compared to chemotherapy alone[47, 52, 66]. In light of these findings, the intricate role of *NKX2-1* in LUSC and its implications for pharmacotherapy necessitate additional clinical and foundational research.

In our study, we comprehensively illustrate the importance of *NKX2-1* in LUSC, and validated by GEO database, HPA database and qRT-PCR. However, our study has some limitations. First, our study is based on public databases, although we conducted preliminary experiments to verify, the in-depth experimental verification is still thus lacking. Second, *NKX2-1* in LUSC are rarely reported in literature, lack of literature references for us. Finally, lack of further experiments in vitro and in vivo verified the biological mechanisms of *NKX2-1* in LUSC. Above all, the *NKX2-1* requires further in-depth study for LUSC.

Conclusion

In conclusion, this study demonstrated that low *NKX2-1* expression is closely associated with increased survival and favorable outcomes in terms of disease progression. The immune indicators of immune infiltration cells, TMB, and immune checkpoint genes were shown to be related to *NKX2-1* expression level, inferring that *NKX2-1* probably affects LUSC development via the TME. We also explored the responses to pharmacotherapy and immune checkpoint inhibitor therapy to offer robust new evidence for the development of potential LUSC therapies and diagnostic methods. Furthermore, validation was performed using the GEO databases, HPA databases, and qRT-PCR.As a result, we provide evidence demonstrating that *NKX2-1* is a potential target for the treatment of LUSC.

Acknowledgements

The manuscript has been reviewed and approved by all authors. We extend our gratitude to Xiaoying Huang's team from the Division of Pulmonary Medicine, the First Affiliated Hospital of Wenzhou Medical University for their valuable support and assistance. We also acknowledge The Cancer Genome Atlas (TCGA) and The Gene Expression Omnibus (GEO) database for providing their platforms, and all the contributors for sharing their meaningful datasets.

Funding

This research received no external funding.

Availability of data and materials

The R code is available at GitHub：
https://github.com/lannyRcode/LUSC-Rcode.git

Authors' contributions

HL, JW conceptualized and designed the present study.
HL performed the bioinformatics analysis and statistical analysis.
HL, QS, MW performed Methodology.
JW were responsible for study supervision.
HL wrote the manuscript.
JW critically revised the article.

All authors have read and approved the final manuscript.
JW confirm the authenticity of all the raw data.

Competing interests
The authors declare no conflicts of interest.

Reference
1. Gao L, Guo YN, Zeng JH, Ma FC, Luo J, Zhu HW, Xia S, Wei KL, Chen G. The expression, significance and function of cancer susceptibility candidate 9 in lung squamous cell carcinoma: A bioinformatics and in vitro investigation. Int. J. Oncol. 2019; 54:1651-1664.
2. Yu H, Pang Z, Li G, Gu T. Bioinformatics analysis of differentially expressed miRNAs in non-small cell lung cancer. J. Clin. Lab. Anal. 2021; 35:e23588.
3. Qian L, Lin L, Du Y, Hao X, Zhao Y, Liu X. MicroRNA-588 suppresses tumor cell migration and invasion by targeting GRN in lung squamous cell carcinoma. Mol. Med. Rep. 2016;14(4):3021-3028.
4. Fan FS, Yang CF, Chang CL. Nivolumab plus Carboplatin and Paclitaxel as the First-line Therapy for Advanced Squamous Cell Carcinoma of the Lung with Strong Programmed Death-ligand 1 Expression: A Case Report. Cureus 2019; 11:e5881.
5. Pan Y, Han H, Labbe KE, Zhang H, Wong KK. Recent advances in preclinical models for lung squamous cell carcinoma. Oncogene 2021;40:2817-2829.
6. Gao M, Kong W, Huang Z, Xie Z. Identification of Key Genes Related to Lung Squamous Cell Carcinoma Using Bioinformatics Analysis. Int. J. Mol. Sci. 2020; 21:2994.
7. Yang L, Lin M, Ruan WJ, Dong LL, Chen EG, Wu XH, Ying KJ. Nkx2-1, a novel tumor biomarker of lung cancer. J. Zhejiang Univ. Sci. B. 2012;13855-866.
8. Guan L, Zhao X, Tang L, Chen J, Zhao J, Guo M, Chen C, Zhou Y, Xu L. Thyroid Transcription Factor-1, Structure, Expression, Function and Its Relationship with Disease. BioMed Res. Int. 2021; 2021, 9957209.
9. Mollaoglu G, Jones A, Wait SJ, Mukhopadhyay A, Jeong S, Arya R, Camolotto SA, Mosbruger TL, Stubben CJ, Conley CJ, Bhutkar A, Vahrenkamp JM, Berrett KC, Cessna MH, Lane TE, Witt BL, Salama ME, Gertz J, Jones KB, Snyder EL, Oliver TG. The lineage-defining transcription factors SOX2 and NKX2-1 determine lung cancer cell fate and shape the tumor immune microenvironment. Immunity 2018;49:764-779.e9.
10. Park JY, Jang SH, Kim HI, Kim JH, Park S, Hwang YI, Jung KS, Seo J, Lee CY, Ko Y, Park YB. Thyroid transcription factor-1 as a prognostic indicator for stage IV lung adenocarcinoma with and without EGFR-sensitizing mutations. BMC Cancer 2019;19:574.
11. Chandran UR, Medvedeva OP, Barmada MM, Blood PD, Chakka A, Luthra S, Ferreira A, Wong KF, Lee AV, Zhang Z, Budden R, Scott JR, Berndt A, Berg JM, Jacobson RS. TCGA Expedition: A Data Acquisition and Management System for TCGA Data. PLoS ONE 2016;11:e0165395.
12. Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, Jiang P, Shen H, Aster JC, Rodig S,

Signoretti S, Liu JS, Liu XS. Comprehensive analyses of tumor immunity: Implications for cancer immunotherapy. Genome Biol. 2016;17:174.
13. He M, Han Y, Cai C, Liu P, Chen Y, Shen H, Xu X, Zeng S. CLEC10A is a prognostic biomarker and correlated with clinical pathologic features and immune infiltrates in lung adenocarcinoma. J. Cell Mol. Med. 2021;25:3391-3399.
14. Song K, Li L, Zhang G. Bias and Correction in RNA-seq Data for Marine Species. Mar. Biotechnol. 2017;19:541-550.
15. Arneth B. Tumor Microenvironment. Medicina 2019;56:15.
16. Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, Treviño V, Shen H, Laird PW, Levine DA, Carter SL, Getz G, Stemke-Hale K, Mills GB, Verhaak RG. Inferring tumour purity and stromal and immune cell admixture from expression data. Nat. Commun. 2013, 4, 2612.
17. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. J. Stat. Softw. 2010;33:1-22.
18. Addeo A, Friedlaender A, Banna GL, Weiss GJ. TMB or not TMB as a biomarker: That is the question. Crit Rev Oncol Hematol. 2021;163:103374.
19. Geeleher P, Cox NJ, Huang RS. pRRophetic: An R package for prediction of clinical chemotherapeutic response from tumor gene expression levels. PLoS ONE 2014;9:e107468.
20. Geeleher P, Cox NJ, Huang RS. Clinical drug response can be predicted using baseline gene expression levels and in vitro drug sensitivity in cell lines. Genome Biol. 2014;15:R47.
21. Zhang R, Deng Y, Zhang Y, Zhai GQ, He RQ, Hu XH, Wei DM, Feng ZB, Chen G. Upregulation of HOXA13 as a potential tumorigenesis and progression promoter of LUSC based on qRT-PCR and bioinformatics. Int J Clin Exp Pathol. 2017;10:10650-10665.
22. Boggaram V. Thyroid transcription factor-1 (TTF-1/Nkx2.1/TITF1) gene regulation in the lung. Clin. Sci. 2009;116:27-35.
23. Zhang Y, Wang R, Li Y, Pan Y, Hu H, Zhang Y, Li H, Shen L, Yu Y, Sun Y, Chen H. Negative Thyroid Transcription Factor 1 Expression Defines an Unfavorable Subgroup of Lung Adenocarcinomas. J. Thorac. Oncol. 2015;10:1444-1450.
24. Banwait JK, Bastola DR. Contribution of bioinformatics prediction in microRNA-based cancer therapeutics. Adv. Drug Deliv. Rev. 2015;81:94-103.
25. Doherty MK, O'Connor E, Hannon D, O'Reilly A, Yen D, Redmond M, Grogan LM, Hennessy BT, Breathnach OS, Morris PG. Absence of thyroid transcription factor-1 expression is associated with poor survival in patients with advanced pulmonary adenocarcinoma treated with pemetrexed-based chemotherapy. Ir. J. Med. Sci. 2019;188:69-74.
26. Nakra T, Singh V, Nambirajan A, Malik PS, Mohan A, Jain D. Correlation of TTF-1 immunoexpression and EGFR mutation spectrum in non-small cell lung carcinoma. J. Pathol. Transl. Med. 2021;55:279-288.
27. Puglisi F, Barbone F, Damante G, Bruckbauer M, Di Lauro V, Beltrami CA, Di Loreto C. Prognostic value of thyroid transcription factor-1 in primary, resected, non-small cell lung carcinoma. Mod. Pathol.1999; 12:318-324.

647 28. Hajian-Tilaki K. Receiver Operating Characteristic (ROC) Curve Analysis for Medical
648 Diagnostic Test Evaluation. Caspian J Intern Med. 2013;4(2):627-635.
649 29. Phelps CA, Lai SC, Mu D. Roles of Thyroid Transcription Factor 1 in Lung Cancer Biology.
650 Vitam. Horm. 2018;106:517-544.
651 30. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of
652 squamous cell lung cancers. Nature 2012;489:519-525.
653 31. Balachandran VP, Gonen M, Smith JJ, DeMatteo RP. Nomograms in oncology: More than
654 meets the eye. Lancet Oncol. 2015;16:e173-80.
655 32. Chen Q, Hu L, Chen K. Construction of a nomogram based on a hypoxia-related lncRNA
656 signature to improve the prediction of gastric cancer prognosis. Front. Genet. 2020;11:570325.
657 33. Yatabe Y, Dacic S, Borczuk AC, Warth A, Russell PA, Lantuejoul S, Beasley MB, Thunnissen
658 E, Pelosi G, Rekhtman N, Bubendorf L, Mino-Kenudson M, Yoshida A, Geisinger KR, Noguchi
659 M, Chirieac LR, Bolting J, Chung JH, Chou TY, Chen G, Poleri C, Lopez-Rios F, Papotti M, Sholl
660 LM, Roden AC, Travis WD, Hirsch FR, Kerr KM, Tsao MS, Nicholson AG, Wistuba I, Moreira
661 AL.Best Practices Recommendations for Diagnostic Immunohistochemistry in Lung Cancer. J
662 Thorac Oncol. 2019;14(3):377-407.
663 34. Svaton M, Fiala O, Krakorova G, Blazek J, Hurdalkova K, Barinova M, Mukensnabl P, Pesek
664 M. Thyroid transcription factor 1 and p63 expression is associated with survival outcome in
665 patients with non-small cell lung cancer treated with erlotinib. Oncol Lett. 2020;20(2):1376-1382.
666 35. Ma Y, Fan M, Dai L, Kang X, Liu Y, Sun Y, Yan W, Liang Z, Xiong H, Chen K. The
667 expression of TTF-1 and Napsin A in early-stage lung adenocarcinoma correlates with the results
668 of surgical treatment. Tumour Biol. 2015;36(10):8085-8092.
669 36. Schilsky JB, Ni A, Ahn L, Datta S, Travis WD, Kris MG, Chaft JE, Rekhtman N, Hellmann
670 MD. Prognostic impact of TTF-1 expression in patients with stage IV lung adenocarcinomas. Lung
671 Cancer. 2017;108:205-211.
672 37. Phelps CA, Lindsey-Boltz L, Sancar A, Mu D. Mechanistic Study of TTF-1 Modulation of
673 Cellular Sensitivity to Cisplatin. Sci Rep. 2019;9(1):7990.
674 38. Liao Y, Yang F, Li X, Chen K, Wang J. The Prognostic Value of TTF-1/NKX2-1 in Lung
675 Squamous Cell Carcinoma. Appl Immunohistochem Mol Morphol. 2023;31(6):414-420.
676 39. Wood SL, Pernemalm M, Crosbie PA, Whetton AD. The role of the tumor-microenvironment
677 in lung cancer-metastasis and its relationship to potential therapeutic targets. Cancer Treat Rev.
678 2014;40(4):558-566.
679 40. Wang L, Wang H, Xu K, Xu Y, Wang Y, Wei S, Zhang Z. Exploration of immune-related cells
680 and ceRNA in squamous cell lung cancer. Medicine 2021;100:e27058.
681 41. Maeda Y, Chen G, Xu Y, Haitchi HM, Du L, Keiser AR, Howarth PH, Davies DE, Holgate
682 ST, Whitsett JA. Airway epithelial transcription factor NK2 homeobox 1 inhibits mucous cell
683 metaplasia and Th2 inflammation. Am. J. Respir. Crit. Care Med. 2011;184:421-429.
684 42. Han Y, Li Y. Comprehensive exploration of M2 macrophages and its related genes for
685 predicting clinical outcomes and drug sensitivity in lung squamous cell carcinoma. J. Oncol.
686 2022;2022:1163924.
687 43. Zhang L, Chen J, Cheng T, Yang H, Li H, Pan C. Identification of the key genes and

characterizations of Tumor Immune Microenvironment in Lung Adenocarcinoma (LUAD) and Lung Squamous Cell Carcinoma (LUSC). J Cancer. 2020;11:4965-4979.
44. Porrello A, Leslie PL, Harrison EB, Gorentla BK, Kattula S, Ghosh SK, Azam SH, Holtzhausen A, Chao YL, Hayward MC, Waugh TA, Bae S, Godfrey V, Randell SH, Oderup C, Makowski L, Weiss J, Wilkerson MD, Hayes DN, Earp HS, Baldwin AS, Wolberg AS, Pecot CV. Factor XIIIA-expressing inflammatory monocytes promote lung squamous cancer through fibrin cross-linking. Nat Commun. 2018;9:1988.
45. Kargl J, Busch SE, Yang GH, Kim KH, Hanke ML, Metz HE, Hubbard JJ, Lee SM, Madtes DK, McIntosh MW, Houghton AM. Neutrophils dominate the immune cell composition in non-small cell lung cancer. Nat Commun. 2017;8:14381.
46. Liu X, Wu S, Yang Y, Zhao M, Zhu G, Hou Z. The prognostic landscape of tumor-infiltrating immune cell and immunomodulators in lung cancer. Biomed Pharmacother. 2017;95:55-61.
47. Qiang H, Li J, Chang Q, Shen Y, Qian J, Chu T. Mining GEO and TCGA Database for Immune Microenvironment of Lung Squamous Cell Carcinoma Patients With or Without Chemotherapy. Front Oncol. 2022;12:835225.
48. Ku BM, Kim Y, Lee KY, Kim SY, Sun JM, Lee SH, Ahn JS, Park K, Ahn MJ. Tumor infiltrated immune cell types support distinct immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. Eur J Immunol. 2021;51(4):956-964.
49. Yan D, Chen Y. Tumor mutation burden (TMB)-associated signature constructed to predict survival of lung squamous cell carcinoma patients. Sci Rep. 2021; 11:9020.
50. Devarakonda S, Rotolo F, Tsao MS, Lanc I, Brambilla E, Masood A, Olaussen KA, Fulton R, Sakashita S, McLeer-Florin A, Ding K, Le Teuff G, Shepherd FA, Pignon JP, Graziano SL, Kratzke R, Soria JC, Seymour L, Govindan R, Michiels S. Tumor Mutation Burden as a Biomarker in Resected Non-Small-Cell Lung Cancer. J Clin Oncol. 2018;36(30):2995-3006.
51. Kersten K, Salvagno C, de Visser KE. Exploiting the Immunomodulatory Properties of Chemotherapeutic Drugs to Improve the Success of Cancer Immunotherapy. Front Immunol. 2015;6:516.
52. Lu J, Duan Y, Liu P, He X, Yang Y, Zhang R, Weng L. Identification of tumour-infiltrating myeloid subsets associated with overall survival in lung squamous cell carcinoma. J Pathol. 2023;259(1):21-34.
53. Xiao J, Lu X, Chen X, Zou Y, Liu A, Li W, He B, He S, Chen Q. Eight potential biomarkers for distinguishing between lung adenocarcinoma and squamous cell carcinoma. Oncotarget 2017;8:71759-71771..
54. Davé V, Childs T, Whitsett JA. Nuclear factor of activated T cells regulates transcription of the surfactant protein D gene (Sftpd) via direct interaction with thyroid transcription factor-1 in lung epithelial cells. J. Biol. Chem. 2004,;279:34578-34588.
55. Xu W, Chen B, Ke D, Chen X. TRIM29 mediates lung squamous cell carcinoma cell metastasis by regulating autophagic degradation of E-cadherin. Aging 2020;12:13488-13501.
56. Liu Y, Shao G, Yang Z, Lin X, Liu X, Qian B, Liu Z. Interferon regulatory factor 6 correlates with the progression of non-small cell lung cancer and can be regulated by miR-320. J. Pharm.

Pharmacol. 2021;73:682-691.
57. Ma X, Yang S, Jiang H, Wang Y, Xiang Z. Transcriptomic analysis of tumor tissues and organoids reveals the crucial genes regulating the proliferation of lung adenocarcinoma. J. Transl. Med. 2021;19:368.
58. Tagne JB, Gupta S, Gower AC, Shen SS, Varma S, Lakshminarayanan M, Cao Y, Spira A, Volkert TL, Ramirez MI. Genome-wide analyses of Nkx2-1 binding to transcriptional target genes uncover novel regulatory patterns conserved in lung development and tumors. PLoS One. 2012;7:e29907.
59. Ingram K, Samson SC, Zewdu R, Zitnay RG, Snyder EL, Mendoza MC. NKX2-1 controls lung cancer progression by inducing DUSP6 to dampen ERK activity. Oncogene 2022;41:293-300.
60. Zewdu R, Mehrabad EM, Ingram K, Fang P, Gillis KL, Camolotto SA, Orstad G, Jones A, Mendoza MC, Spike BT, Snyder EL. An NKX2-1/ERK/WNT feedback loop modulates gastric identity and response to targeted therapy in lung adenocarcinoma. eLif 2021;10:e66788.
61. Harada M, Sakai S, Ohhata T, Kitagawa K, Mikamo M, Nishimoto K, Uchida C, Niida H, Kotake Y, Sugimura H, Suda T, Kitagawa M. Homeobox Transcription Factor NKX2-1 Promotes Cyclin D1 Transcription in Lung Adenocarcinomas. Mol Cancer Res. 2017;15:1388-1397.
62. Navani S. Manual evaluation of tissue microarrays in a high-throughput research project: The contribution of Indian surgical pathology to the Human Protein Atlas (HPA) project. Proteomics. 2016;16:1266-70.
63. Ordóñez NG. Value of thyroid transcription factor-1 immunostaining in tumor diagnosis: a review and update. Appl Immunohistochem Mol Morphol. 2012;20:429-444.
64. Quintanal-Villalonga Á, Chan JM, Yu HA, Pe'er D, Sawyers CL, Sen T, Rudin CM. Lineage plasticity in cancer: a shared pathway of therapeutic resistance. Nat Rev Clin Oncol. 2020;17(6):360-371.
65. Kwon J, Zhang J, Mok B, Allsup S, Kim C, Toretsky J, Han C. USP13 drives lung squamous cell carcinoma by switching lung club cell lineage plasticity. Mol Cancer. 2023;22(1):204.
66. Yu J, Wu X, Ma J, Chen X, Li L. Clinical Observation of Immunotherapy Efficacy and Adverse Effects in Chinese Patients with Lung Squamous Cell Carcinoma. Zhongguo Fei Ai Za Zhi. 2022 Jul 20;25(7):546-554.

Table 1(on next page)

Table1 The primer sequences of genes

Table 1. The primer sequences of genes

Gene	Forward primer	Reverse primer
NKX2-1	AGCACACGACTCCGTTCTC	GCCCACTTTCTTGTAGCTTTCC
NAPSA	TCTTCGTACCTCTCTCGAACTAC	GGCAACAGTGAAGTTTTGTGG
SFTPD	CCTTACAGGGACAAGTACAGCA	CTGTGCCTCCGTAAATGGTTT
TRIM29	CTGTTCGCGGGCAATGAGT	TGCCTTCCATAGAGTCCATGC
IRF6	CCCCAGGCACCTATACAGC	TCCTTCCCACGGTACTGAAAC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Table 2(on next page)

Table 2. Relationship of Clinical Parameters for LUSC patients in *NKX2-1* High-Level and Low-Level Groups

Table 2. Relationship of Clinical Parameters for LUSC patients in *NKX2-1* High-Level and Low-Level Groups

Characteristics	Low expression of NKX2-1	High expression of NKX2-1	*P*-value
Number	251	250	
Age, N(%)			0.329
<65	93(37.1)	77(30.8)	
≥65	154(61.4)	168(67.2)	
NA	4(1.6)	5(2.0)	
Gender, N(%)			0.097
Male	194(77.3)	177(70.8)	
Female	57(22.7）	73(29.2)	
Race, N(%)			0.569
White	181(72.1)	168(67.2)	
Asia	4(1.6)	5(2.0)	
Black of African American	12(4.8)	18(7.2)	
NA	54(21.5)	59(23.6)	
Smoking status, N(%)			0.112
Smoker	206(82.1)	218(87.2)	
Non-smoker	45(17.9)	32(12.8)	
Site of tumor, N(%)			0.193
Upper lobe	122(48.6)	138(55.2)	
Middle lobe	5(2.0)	11(4.4)	
Lower lobe	92(36.7)	81(32.4)	
Main bronchus	4(1.6)	3(1.2)	
Overlopping lesion of lung	6(2.4)	2(0.8)	
Lung NOS	22(8.8)	15(6.0)	
Stage, N(%)			0.070
I	109(43.4)	135(54.0)	
II	96(38.8)	66(26.4)	
III	40(15.9)	44(17.6)	
IV	4(1.6)	3(1.2)	
NA	2(0.8)	2(0.8)	
T classification, N(%)			0.649
T1	53(21.1)	61(24.4)	
T2	146(58.2)	147(58.8)	
T3	39(15.5)	32(12.8)	
T4	13(5.2)	10(4.0)	
M classification, N(%)			0.984
M0	205(81.7)	206(82.4)	
M1	4(1.6)	3(1.2)	

MX	40(15.9)	39(15.6)	
NA	2(0.8)	2(0.8)	
N classification, N(%)			0.116
N0	151(60.2)	168(67.2)	
N1	77(30.7)	54(21.6)	
N2	20(8.0)	20(8.0)	
N3	1(0.4)	4(1.6)	
NX	2(0.8)	4(1.6)	
Treatment, N(%)			0.073
No treatment	125(49.8)	144(57.6)	
Pharmaceutical therapy	59(23.5)	38(15.2)	
Radiation therapy	9(3.6)	14(5.6)	
Pharmaceutical and Radiation therapy	28(11.2)	20(8.0)	
NA	30(12.0)	34(13.6)	

Table 3(on next page)

Table 3. Univariate and multivariate Cox regression hazard analyses of *NKX2-1* expression.

Chracteristics	Univariate analysis		Multivariate analysis	
	HR(95% CI)	p value	HR(95% CI)	p value
Age(<65 vs. ≥65)	1.440(1.030-2.014)	0.033	1.484(1.058-2.081)	0.22
Gender(Male vs. Female)	0.736(0.511-1.059)	0.099		
Smoking status(No-smoker vs. Smoker)	0.935(0.586-1.491)	0.778		
Site of tumor(Upper lobe vs. Other sites)	0.932(0.846-1.027)	0.155		
Stage(Stage I vs. Stage II-IV)	1.230(1.026-1.474)	0.025	1.295(1.082-1.551)	0.005
T classification(T1 vs. T2-4)	1.218(0.998-1.487)	0.053		
M classification(M0 vs. M1)	2.431(0.897-6.586)	0.081		
N classification(N0 vs. N1-3)	1.118(0.901-1.387)	0.311		
NKX2-1(Low vs. High)	1.462(1.082-1.976)	0.013	1.495(1.104-2.025)	0.009

Table 3. Univariate and multivariate Cox regression hazard analyses of *NKX2-1* expression.

Table 4(on next page)

Table 4. The co-expression genes of *NKX2-1* in GEO and TCGA

Table 4. The co-expression genes of *NKX2-1* in GEO and TCGA

	GEO		TCGA	
Gene	Pearson's correlation	p-value	Pearson's correlation	p-value
NAPSA	0.876646645	<0.0001	0.845383966	<0.0001
SFTPD	0.806601106	<0.0001	0.813702366	<0.0001
SLC22A31	0.833582363	<0.0001	0.873599353	<0.0001
SFTA2	0.867622911	<0.0001	0.852154614	<0.0001
C16orf89	0.882838078	<0.0001	0.815555528	<0.0001
GJB5	-0.451335552	<0.0001	-0.378450636	<0.0001
KRT5	-0.320750235	<0.0001	-0.370243082	<0.0001
TRIM29	-0.582457298	<0.0001	-0.397324635	<0.0001
IRF6	-0.404342555	<0.0001	-0.370549304	<0.0001

Figure 1

NKX2-1 mRNA Expression Levels in Various Cancers

NKX2-1 expression levels in (**A**) various cancer types, (**B**) LUSC vs. normal tissue, and (**C**) in LUSC vs. normal tissue with *NKX2-1* paired expression analysis. Characterization based on the tumor immune estimation resource (TIMER) database. * $p < 0.05$, *** $p < 0.001$.

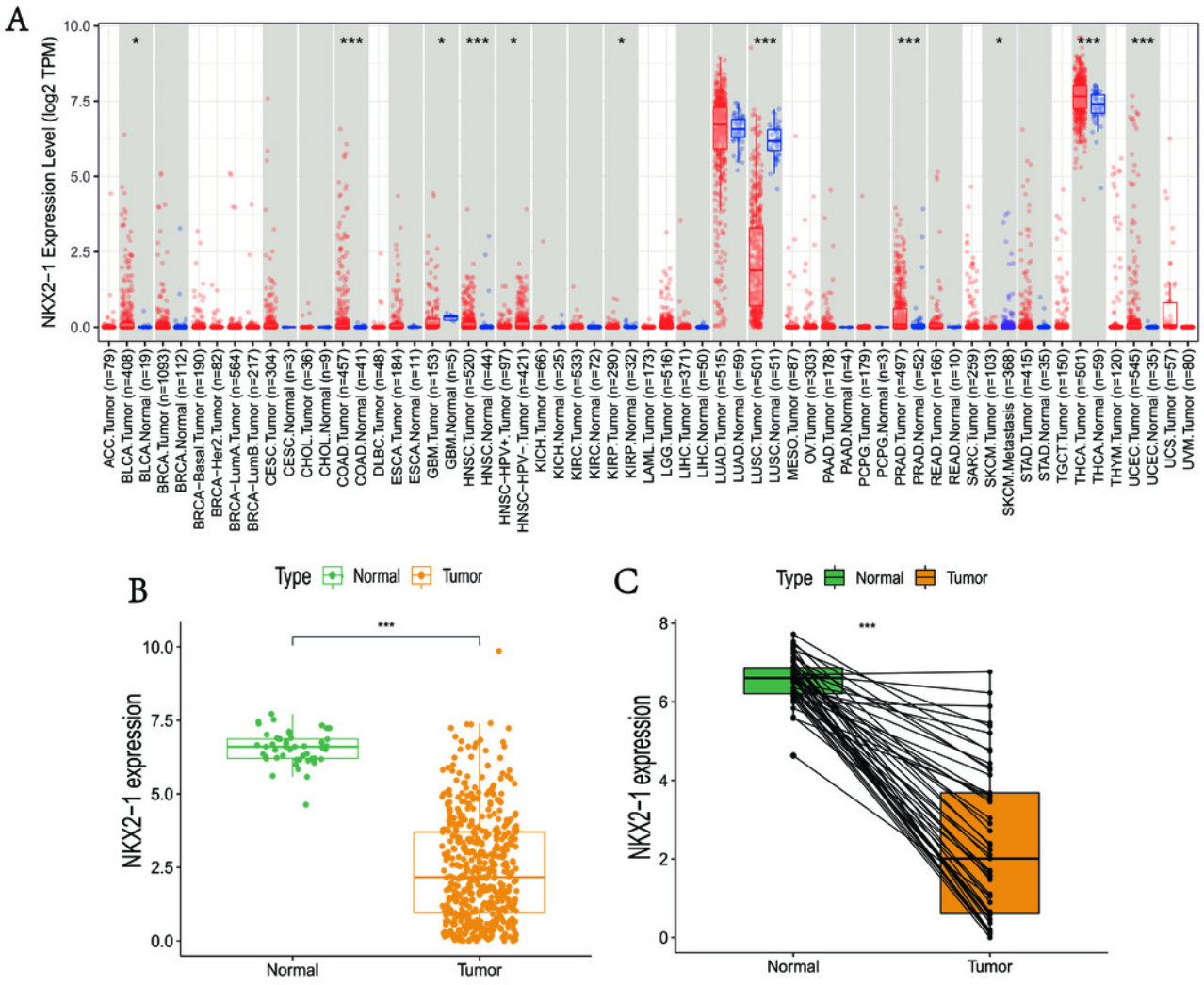

Figure 2

Evaluation of Clinical Parameters and Development of a Prognostic Prediction Model for *NKX2-1* in LUSC Patients

Association between *NXK2-1* expression and (**A**) age, (**B**) gender, (**C**) stage, (**D**) tumor, (**E**) metastasis, and (**F**) node in LUSC patients. (**G**) Kaplan–Meier curves for OS and (**H**) PFS according to *NKX2-1* mRNA expression levels, stratified into high and low levels based on the median ($p < 0.05$). (I) ROC curves for 1, 3, and 5-year OS. (**J**) Nomogram predicting the probability of OS at 1, 3, and 5 years. (K) Calibration plot predicting the agreement between observed and predicted rates of OS at 1, 3, and 5 years. * $p < 0.05$, *** $p < 0.001$.

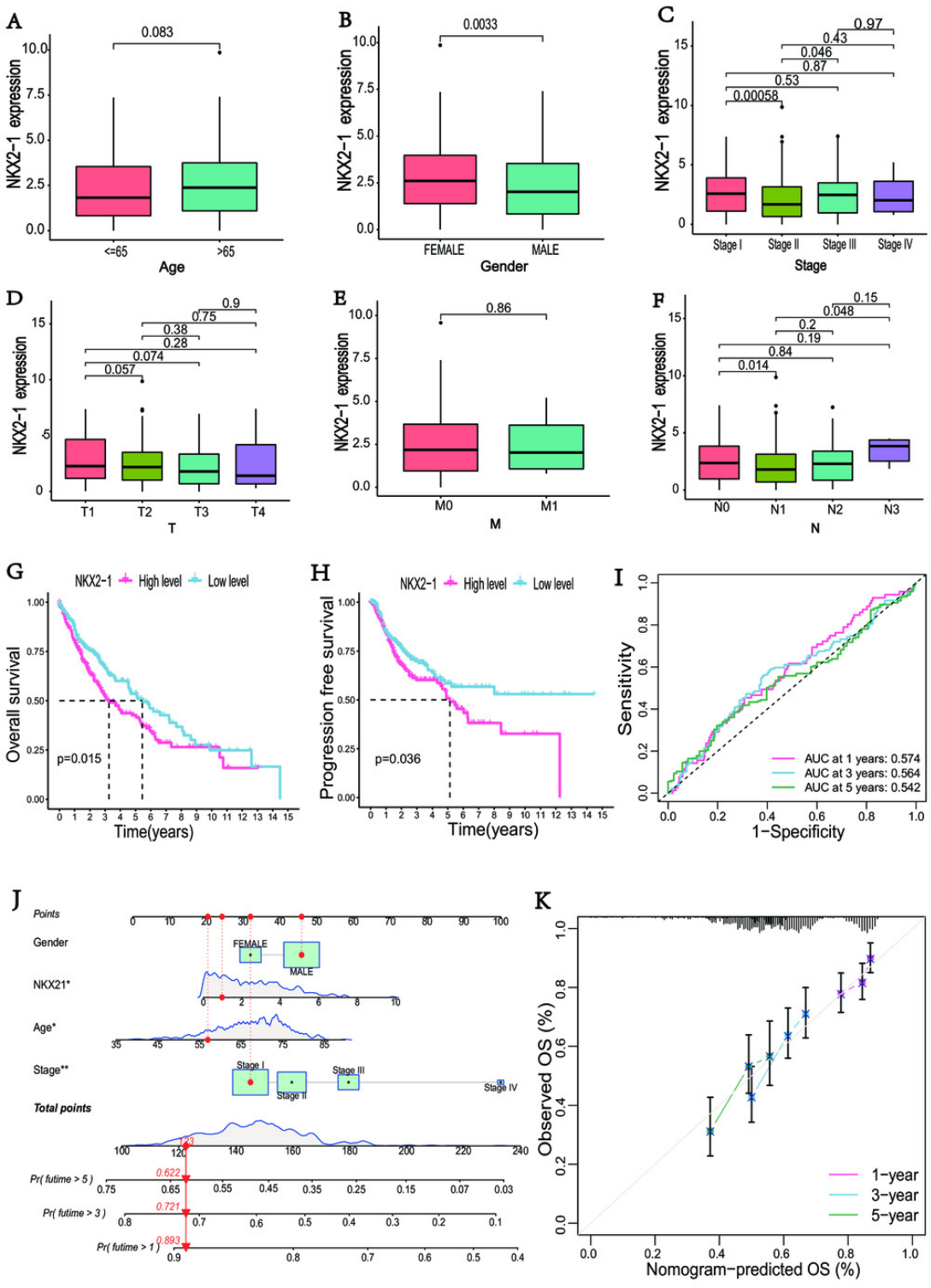
A
NKX2-1 expression
0.083
<=65
>65
Age
B
NKX2-1 expression
0.0033
FEMALE
MALE
Gender
C
NKX2-1 expression
0.97
0.43
0.046
0.87
0.53
0.00058
Stage I
Stage II
Stage III
Stage IV
Stage
D
NKX2-1 expression
0.9
0.75
0.38
0.28
0.074
0.057
T1
T2
T3
T4
T
E
NKX2-1 expression
0.86
M0
M1
M
F
NKX2-1 expression
0.15
0.048
0.2
0.19
0.84
0.014
N0
N1
N2
N3
N
G
NKX2-1
High level
Low level
Overall survival
p=0.015
Time(years)
H
NKX2-1
High level
Low level
Progression free survival
p=0.036
Time(years)
I
Sensitivity
1−Specificity
AUC at 1 years: 0.574
AUC at 3 years: 0.564
AUC at 5 years: 0.542
J
Points
Gender
FEMALE
MALE
NKX21*
Age*
Stage**
Stage I
Stage II
Stage III
Stage IV
Total points
Pr(futime > 5)
Pr(futime > 3)
Pr(futime > 1)
0.622
0.721
0.893
K
Observed OS (%)
Nomogram−predicted OS (%)
1−year
3−year
5−year

Figure 3

Comparison Analysis in *NKX2-1* High-Level and Low-Level Groups and Co-Expression Analysis of *NKX2-1*

(**A**) Heatmap showing the top 50 genes with the highest expression variation of DEGs in the *NKX2-1* high-level and low-level groups; graded color scale of blue to red represents levels of gene expression. *NKX2-1* expression correlated positively with (**B**) *NKX2-1-AS1*, (**C**) *SLC22A31*, (**D**) *NAPSA*, (**E**) *SFTA2*, (**F**) *C16orf89*, and (**G**) *SFTPD* and correlated negatively with (**H**) *TRIM29*, (**I**) *LINC01980*, (**J**) *GJB5*, (**K**) *KRT5*, and (**L**) *IRF6* expression. (**M**) The top 11 significant genes that were either positively or negatively correlated with *NKX2-1* shown in a Circos plot.

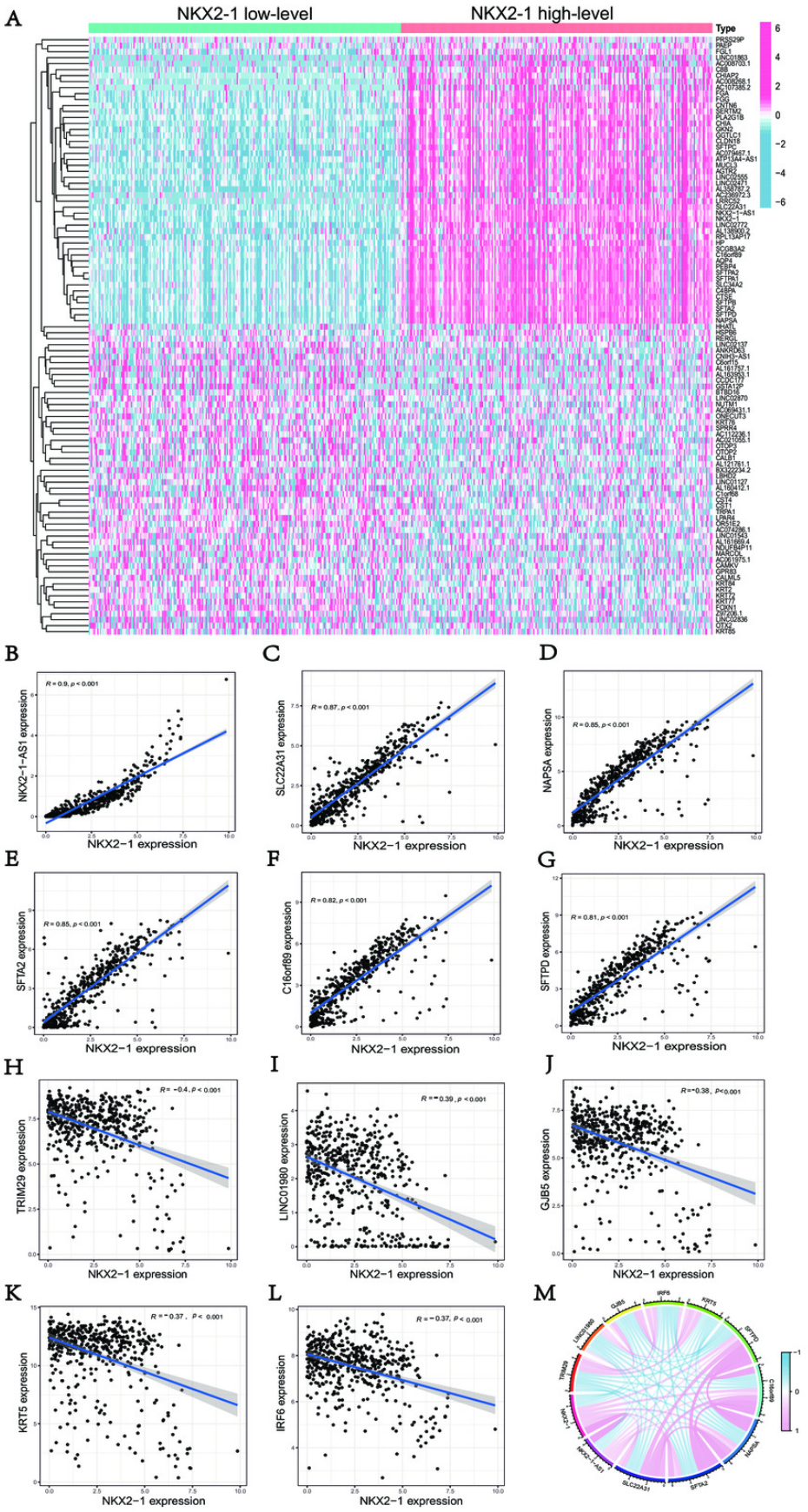
A
NKX2-1 low-level
NKX2-1 high-level
Type
B
NKX2−1−AS1 expression
NKX2−1 expression
C
SLC22A31 expression
NKX2−1 expression
D
NAPSA expression
NKX2−1 expression
E
SFTA2 expression
NKX2−1 expression
F
C16orf89 expression
NKX2−1 expression
G
SFTPD expression
NKX2−1 expression
H
TRIM29 expression
NKX2−1 expression
I
LINC01980 expression
NKX2−1 expression
J
GJB5 expression
NKX2−1 expression
K
KRT5 expression
NKX2−1 expression
L
IRF6 expression
NKX2−1 expression
M

Figure 4

Functional Enrichment Analyses of DEGs in *NKX2-1* High-Level and Low-Level Groups

(**A**) Circle plot of enriched biological process. The outer ring represents GO terms, with different colors distinguishing categories of biological process (BP), cellular component (CC), and molecular function (MF). The second ring within the outer ring shows the number of enriched genes. The third ring represents the number of enriched DEGs. The fourth ring represent the gene ratio. (**B-E**) Bar and bubble plots showing KEGG and GO enrichment analysis, respectively. Circle sizes represent the number of genes in each functional class. The graded color scale of blue to red represents the alterations of *p* values.

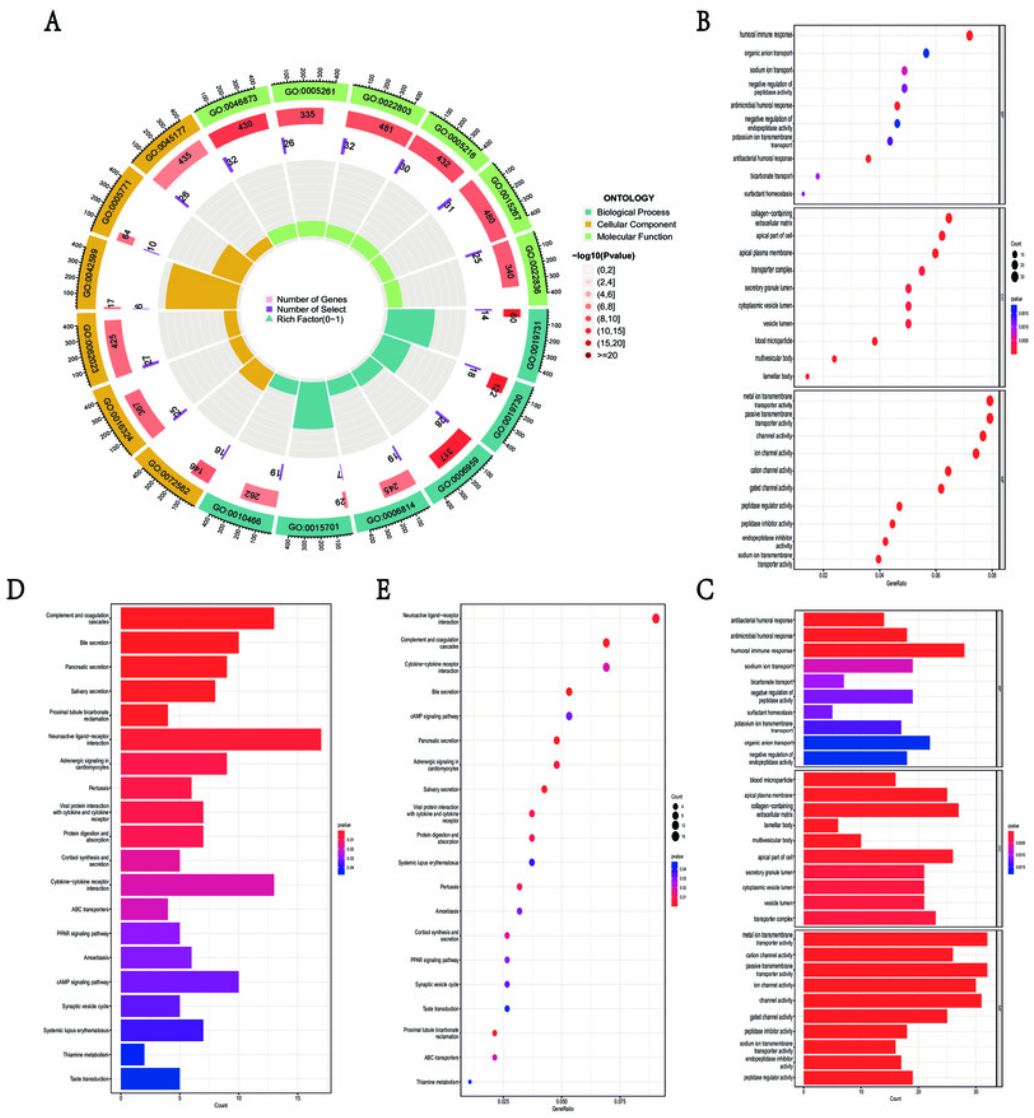
A
B
C
D
E
ONTOLOGY
Biological Process
Cellular Component
Molecular Function
−log10(Pvalue)
Number of Genes
Number of Select
Rich Factor(0~1)

Figure 5

GSEA Identifies DEG-Related Signaling Pathways in *NKX2-1* High-Level and Low-Level Groups

GSEA enrichment analysis of DEGs in *NKX2-1* high-level and low-level groups.

Figure 6

Immune Infiltration Analysis and Tumor Mutational Burden of *NKX2-1* Expression

(**A**) Violin plot of the immune score, stromal score, ESTIMATE score in *NKX2-1* high-level and low-level groups. (**B**) Box plot showing the fractions of the 22 immune cells in *NKX2-1* high-level and low-level groups. (**C**) Correlation between *NKX2-1* expression and the 22 immune cells. Dot size indicates the correlation coefficient, with negative correlation on the left and positive correlation on the right. (**D**) macrophages M1 and (**E**) macrophages M2, (**F**) monocytes, (**G**) neutrophils, (**H**) mast cells resting, (**I**) T cells CD4 memory resting. (**J**) The correlation between *NKX2-1* expression and tumor mutational burden. (**K**) Heatmap of the correlation between *NKX2-1* and immune checkpoints; Pearson coefficient was used to test significance. The darker the red, the stronger the positive correlation; and the darker the blue, the stronger the negative correlation. Pearson correlation between *NKX2-1* expression. *p < 0.05, **p < 0.01, ***p < 0.001.

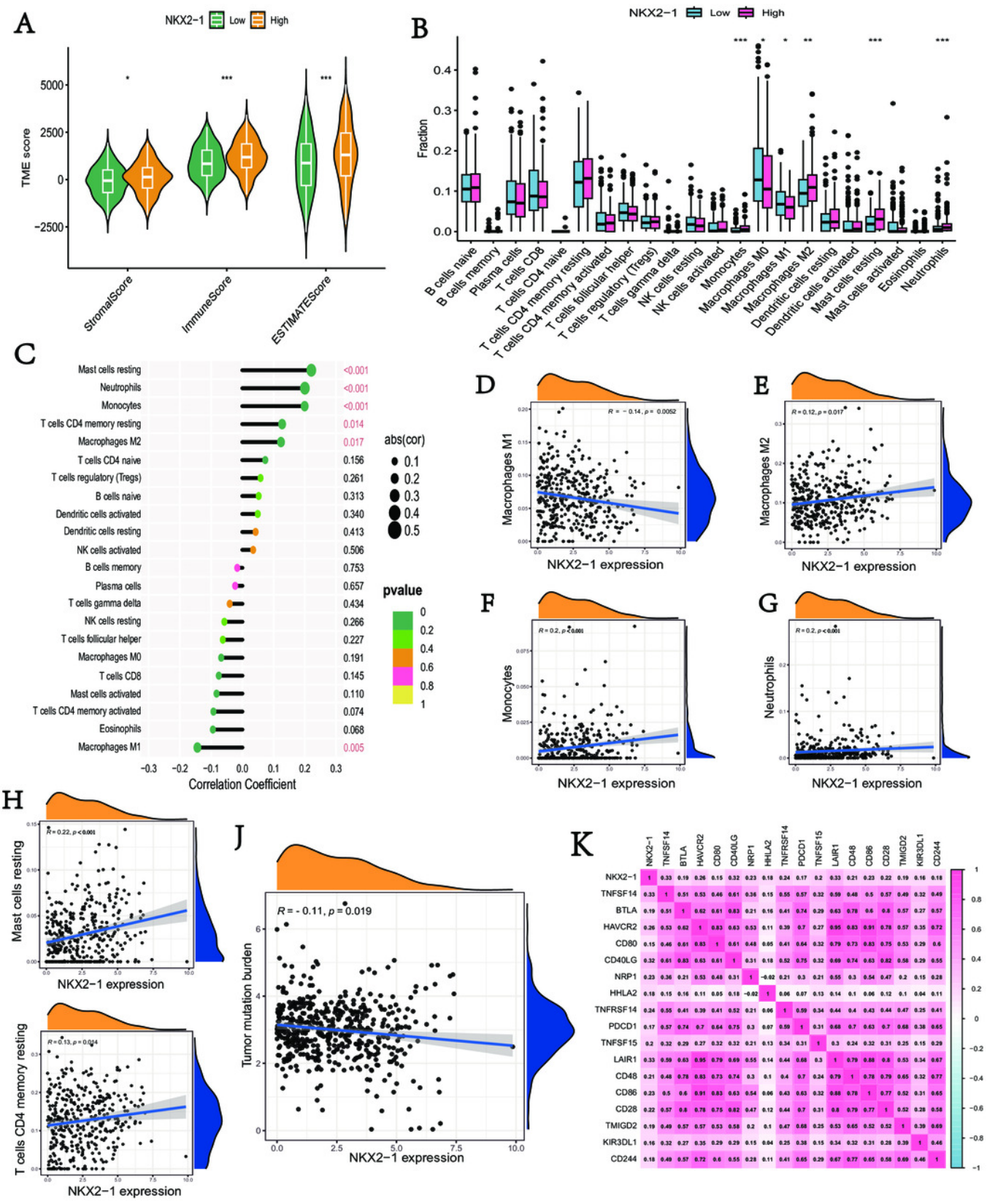
A
NKX2-1 Low High
TME score
StromalScore
ImmuneScore
ESTIMATEScore
B
NKX2-1 Low High
Fraction
B cells naive
B cells memory
Plasma cells
T cells CD8
T cells CD4 naive
T cells CD4 memory resting
T cells CD4 memory activated
T cells follicular helper
T cells regulatory (Tregs)
T cells gamma delta
NK cells resting
NK cells activated
Monocytes
Macrophages M0
Macrophages M1
Macrophages M2
Dendritic cells resting
Dendritic cells activated
Mast cells resting
Mast cells activated
Eosinophils
Neutrophils
C
Mast cells resting <0.001
Neutrophils <0.001
Monocytes <0.001
T cells CD4 memory resting 0.014
Macrophages M2 0.017
T cells CD4 naive 0.156
T cells regulatory (Tregs) 0.261
B cells naive 0.313
Dendritic cells activated 0.340
Dendritic cells resting 0.413
NK cells activated 0.506
B cells memory 0.753
Plasma cells 0.657
T cells gamma delta 0.434
NK cells resting 0.266
T cells follicular helper 0.227
Macrophages M0 0.191
T cells CD8 0.145
Mast cells activated 0.110
T cells CD4 memory activated 0.074
Eosinophils 0.068
Macrophages M1 0.005
abs(cor)
pvalue
Correlation Coefficient
D
Macrophages M1
NKX2-1 expression
E
Macrophages M2
NKX2-1 expression
F
Monocytes
NKX2-1 expression
G
Neutrophils
NKX2-1 expression
H
Mast cells resting
NKX2-1 expression
T cells CD4 memory resting
NKX2-1 expression
J
Tumor mutation burden
R = - 0.11, p = 0.019
NKX2-1 expression
K
NKX2-1
TNFSF14
BTLA
HAVCR2
CD80
CD40LG
NRP1
HHLA2
TNFRSF14
PDCD1
TNFSF15
LAIR1
CD48
CD86
CD28
TMIGD2
KIR3DL1
CD244

Figure 7

Analysis of Differences in Immune Therapy and Pharmacotherapy Responsiveness in *NKX2-1* High-Level and Low-Level Groups

IC50 was calculated for (**A**) 5-fluorouracil, (**B**) axitinib, (**C**) BI-2536, (**D**) cisplatin, (**E**) docetaxel, (**F**) doxorubicin, (**G**) etoposide, (**H**) gefitinib, (**I**) gemcitabine, (**J**) paclitaxel, (**K**) sorafenib, (**L**) vinorelbine, (**M**) The responsiveness in combination therapy of anti-CTLA4 and anti-PD-1. (**N**) The responsiveness in anti-PD-1 therapy. (**O**) The responsiveness in anti-CTLA4 therapy. (**P**) The responsiveness in other immune checkpoint inhibitor therapy. ***$p < 0.001$.

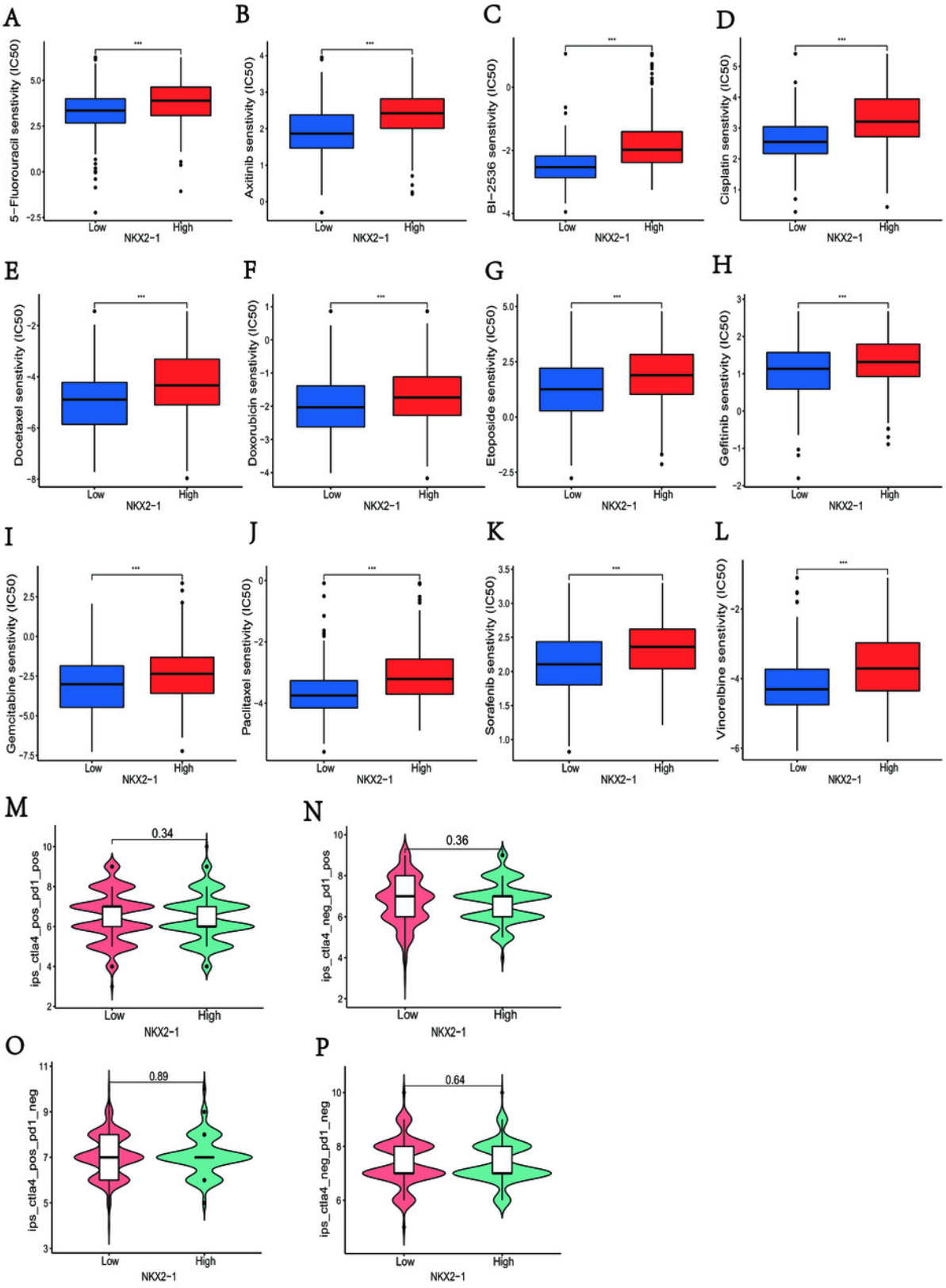
A
5−Fluorouracil senstivity (IC50)
B
Axitinib senstivity (IC50)
C
BI−2536 senstivity (IC50)
D
Cisplatin senstivity (IC50)
E
Docetaxel senstivity (IC50)
F
Doxorubicin senstivity (IC50)
G
Etoposide senstivity (IC50)
H
Gefitinib senstivity (IC50)
I
Gemcitabine senstivity (IC50)
J
Paclitaxel senstivity (IC50)
K
Sorafenib senstivity (IC50)
L
Vinorelbine senstivity (IC50)
Low
High
NKX2−1
***
M
ips_ctla4_pos_pd1_pos
0.34
N
ips_ctla4_neg_pd1_pos
0.36
O
ips_ctla4_pos_pd1_neg
0.89
P
ips_ctla4_neg_pd1_neg
0.64

Figure 8

Verification analysis of *NKX2-1* gene in LUSC.

(**A**) Comparison of *NKX2-1* expression level between LUSC and normal tissue. (**B**) Venn diagrams showing the intersection of co-expression genes of *NKX2-1* and DEGs in LUSC based on GEO and TCGA databases. (**C-D**) GO analysis of shared genes in co-expression and DEGs. (**E-F**) KEGG pathway analysis of shared genes in co-expression and DEGs. *** $p < 0.001$.

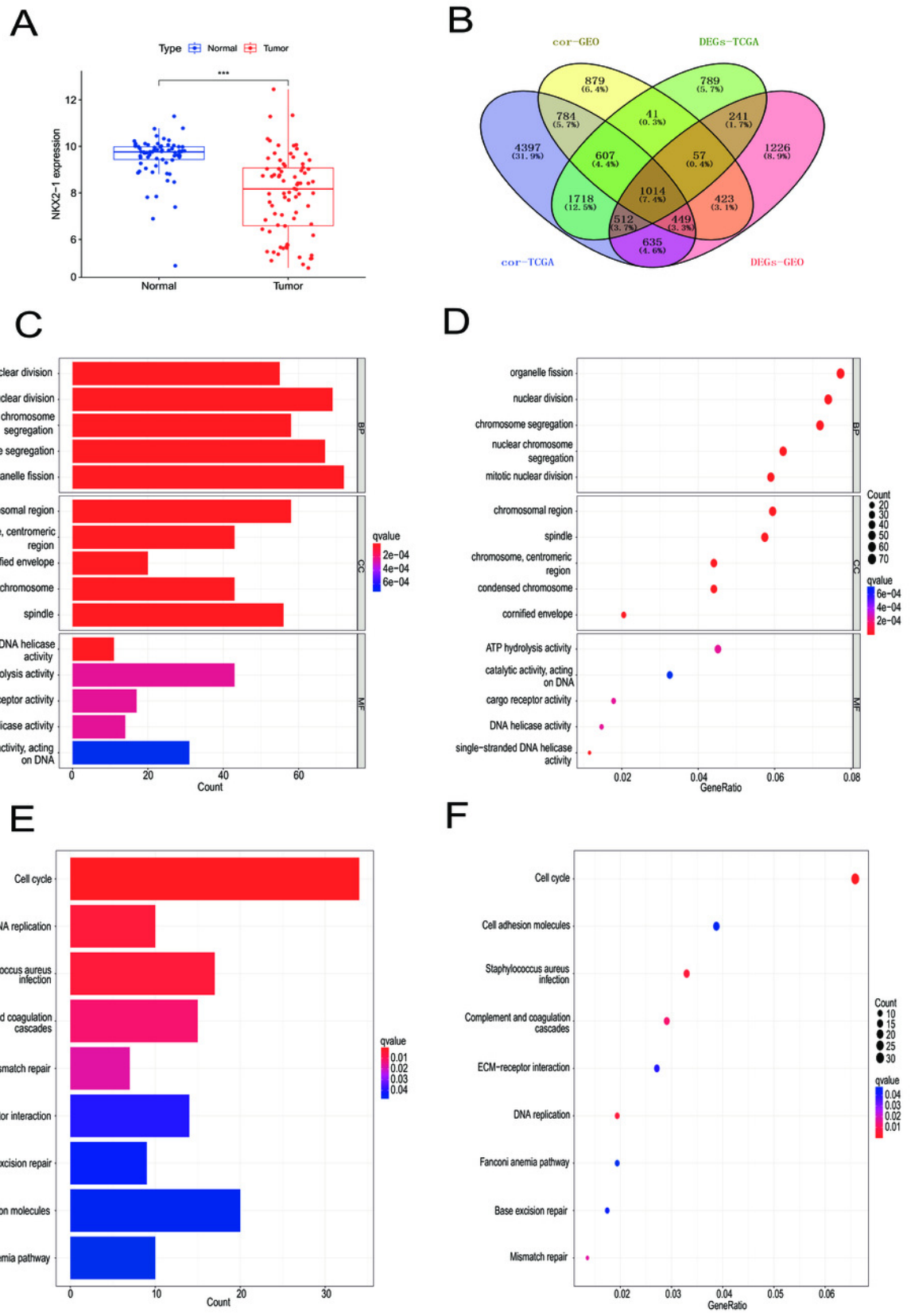
A
Type Normal Tumor
***
NKX2-1 expression
Normal
Tumor
B
cor-GEO
DEGs-TCGA
879 (6.4%)
789 (5.7%)
784 (5.7%)
41 (0.3%)
241 (1.7%)
4397 (31.9%)
607 (4.4%)
57 (0.4%)
1226 (8.9%)
1718 (12.5%)
1014 (7.4%)
423 (3.1%)
512 (3.7%)
449 (3.3%)
635 (4.6%)
cor-TCGA
DEGs-GEO
C
mitotic nuclear division
nuclear division
nuclear chromosome segregation
chromosome segregation
organelle fission
chromosomal region
chromosome, centromeric region
cornified envelope
condensed chromosome
spindle
single-stranded DNA helicase activity
ATP hydrolysis activity
cargo receptor activity
DNA helicase activity
catalytic activity, acting on DNA
BP
CC
MF
qvalue
2e-04
4e-04
6e-04
Count
D
organelle fission
nuclear division
chromosome segregation
nuclear chromosome segregation
mitotic nuclear division
chromosomal region
spindle
chromosome, centromeric region
condensed chromosome
cornified envelope
ATP hydrolysis activity
catalytic activity, acting on DNA
cargo receptor activity
DNA helicase activity
single-stranded DNA helicase activity
Count
qvalue
GeneRatio
E
Cell cycle
DNA replication
Staphylococcus aureus infection
Complement and coagulation cascades
Mismatch repair
ECM-receptor interaction
Base excision repair
Cell adhesion molecules
Fanconi anemia pathway
qvalue
Count
F
Cell cycle
Cell adhesion molecules
Staphylococcus aureus infection
Complement and coagulation cascades
ECM-receptor interaction
DNA replication
Fanconi anemia pathway
Base excision repair
Mismatch repair
Count
qvalue
GeneRatio

Figure 9

Expression of *NKX2-1* protein and RNA in lung cancer.

IHC results displaying *NKX2-1* protein levels in LUAD (**A-C**) and LUSC (**D-F**) based on data from The Human Protein Atlas. Expression levels are categorized as Negative (**A, D**), Moderate (**B, F**), Strong (**C**), and Weak (**E**). Relative expression level of *NKX2-1* in LUAD and LUSC (**G**), along with the expression levels of its co-expression genes in LUSC (**H**).