

PINK1 promotes the progression of lung squamous cell carcinoma through the FAK/AKT/RhoA/vinculin pathway

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Abstract. Lung squamous cell carcinoma (LUSC) is an aggressive malignancy with a poor prognosis. Given the association between mitochondrial dysregulation and LUSC, the aim of the present study was to identify mitochondrial unfolded protein response (UPR^{mt})-related candidate genes associated with LUSC and to explore their potential functional relevance. In the present study, differential expression, univariate Cox and survival analyses were combined to identify a UPR^{mt}-associated key gene in LUSC. Gene set enrichment analysis was then performed, and overexpression (OE) and knockdown cell lines were used to clarify its function using western blotting, Cell Counting Kit-8, colony formation and Transwell® invasion assays. Using bioinformatics tools and a review of the literature, PTEN-induced kinase 1 (PINK1) was singled out as a key gene in LUSC samples. Elevated PINK1 expression was associated with worse survival outcomes. A total of 117 pathways were enriched by PINK1, with the top 5 including ‘focal adhesion’ and ‘mitogen-activated protein kinase signaling pathway’. Using experimental analysis, it

was observed that PINK1 significantly enhanced the proliferative, migratory, invasive and colony forming abilities of tumor cells, thereby exacerbating LUSC. Furthermore, these enhancements were diminished by the application of focal adhesion kinase (FAK) inhibitor. It was also noted that the expression levels of protein kinase B (AKT), p-AKT, Ras homolog family member A (RhoA) and vinculin were all elevated in OE-PINK1 cells. Collectively, these findings indicated that PINK1 exerted its influence on LUSC through the FAK/AKT/RhoA/vinculin pathway. The present study identified PINK1 as a key gene in LUSC, exerting its influence through the FAK/AKT/RhoA/vinculin pathway, thereby presenting a potential novel therapeutic target for the treatment of LUSC in the future.

Introduction

Lung cancer is the most commonly diagnosed malignancy worldwide and the leading cause of cancer-related death, accounting for ~2.6 million new cases and ~1.9 million deaths, representing 12.8% of all new cancer cases and 19.1% of all cancer deaths globally in 2024 (1). Globally, non-small cell lung cancer (NSCLC) accounts for >85% of lung cancer cases, with lung squamous cell carcinoma (LUSC) representing the second most common subtype, accounting for 30% of NSCLC cases (2,3). Despite notable advancements in diagnostic techniques and therapeutic approaches for LUSC, patient prognoses continue to be unfavorable, due to the lack of identified driver targets and efficient targeted treatments (4). Furthermore, multiple cases are identified at advanced stages, and the restricted availability of systemic therapy alternatives exacerbates elevated mortality rates. Therefore, the identification and validation of novel key genes in LUSC are key to enhancing early diagnosis and long-term prognosis (5,6).

Mitochondria, which are organelles present in eukaryotic cells, produce adenosine triphosphate by oxidative phosphorylation, serving a key role in energy generation (7). Mitochondria are key to numerous biological processes, including redox equilibrium, calcium homeostasis, metabolism and apoptosis (8,9). The effective operation of these systems is maintained by various stress response mechanisms, including alterations in mitochondrial dynamics (10), selective autophagy (11) and the

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Abbreviations: NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; UPR^{mt}, mitochondrial unfolded protein response; UPR^{mt}-RGs, UPR^{mt}-related genes; DE-UPR^{mt}-RGs, differentially expressed UPR^{mt}-RGs; GEO, Gene Expression Omnibus; DEGs, differentially expressed genes; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; FAK, focal adhesion kinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WB, western blotting; CCK-8, Cell Counting Kit-8

Key words: FAK/AKT/RhoA/vinculin pathway, LUSC, UPR^{mt}, PINK1

mitochondrial unfolded protein response (UPR^{mt}) (12). UPR^{mt} denotes the transcriptional response initiated by the regulation of nuclear gene expression in the context of mitochondrial dysfunction, with the objective of restoring mitochondrial function and network integrity to facilitate cellular survival and adaptation (13). This reaction inhibits the buildup of misfolded proteins in mitochondria by facilitating proper protein folding and destruction, hence preserving cellular protein homeostasis (14). Previous studies have indicated that the UPR^{mt} is associated with multiple diseases, including cancer and neurological disorders (15,16). Bueno and Rojas (17) identified that in idiopathic pulmonary fibrosis, the compromised clearance of mitochondrial and endoplasmic reticulum stress by alveolar type II epithelial cells resulted in the simultaneous activation of both UPR^{mt} and endoplasmic reticulum UPR, subsequently triggering apoptosis, fibrosis and inflammation. Furthermore, Jiang *et al* (18) revealed that activating transcription factor 4 intensifies UPR^{mt} in the lungs, increasing pulmonary inflammation. These findings underscored the pivotal function of the UPR^{mt} in the genesis and progression of pulmonary illnesses. The aim of the present study was to identify key genes associated with the UPR^{mt} in LUSC to potentially identify novel therapeutic targets for LUSC treatment in the future.

The present study employed bioinformatics techniques to identify key genes associated with the UPR^{mt} in LUSC using public datasets. Functional enrichment analysis was performed on these genes to investigate their potential biological functions. The LUSC samples were subsequently categorized into high- and low-expression groups according to key gene expression levels. Analyses of the immunological microenvironment and mutations were conducted to compare the disparities between these two groups. Cell lines with knockdown (KD) or overexpression (OE) of the key genes were constructed. Proliferation, migration and invasion experiments were performed to assess the functions of key genes in LUSC and their impact on key pathways were subsequently examined using western blotting (WB). These analyses were performed to obtain theoretical insights for the identification of novel treatment targets for patients with LUSC.

Materials and methods

Data sources. A total of 496 LUSC (486 with survival information) and 51 control samples in The Cancer Genome Atlas (TCGA)-LUSC dataset (training set) were obtained from the University of California, Santa Cruz Xena Browser (<https://xena.ucsc.edu/>; dataset/project search terms: 'TCGA-LUSC' or 'Lung Squamous Cell Carcinoma'; Genomic Data Commons project ID: TCGA-LUSC). The validation sets, including GSE19188, GSE157010, GSE37745, GSE50081 and GSE29013 were all downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) and sequenced using the GPL570 platform ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array; version 2.0; Affymetrix, Inc.; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL570>). The GSE19188 dataset, including the 27 LUSC and 65 control samples (19), the GSE157010 dataset with 235 LUSC samples (20), the GSE37745 dataset with 66 LUSC samples (21), the GSE50081 dataset with 42 LUSC samples (22) and the GSE29013 with

25 LUSC samples (23). Furthermore, 35 UPR^{mt}-related genes (UPR^{mt}-RGs) were acquired from the published literature (Table SI) (24).

Identification of key genes in LUSC samples. Expression levels of UPR^{mt}-RGs were compared between LUSC and control samples, with the results visualized via boxplot generated by 'ggplot2' package (version 3.3.6) (25). Differentially expressed UPR^{mt}-RGs (DE-UPR^{mt}-RGs) were obtained based on $P < 0.05$. To identify genes associated with LUSC survival, univariate Cox regression analysis was conducted using 'survival' package (version 3.4-0) (<https://cran.r-project.org/package=survival>), selecting genes with hazard ratio (HR) $\neq 1$ and $P < 0.05$. For data integration and normalization in four validation sets (excluding GSE19188), the 'sva' package (26) was utilized, resulting in 368 LUSC samples for survival analysis. Using 'surv_cutpoint' function in the 'survminer' package (version 0.4.9) (<https://cran.r-project.org/package=survminer>), the optimal gene-specific expression cut-off value was determined using maximally selected rank statistics, with minprop set to 0.3 to ensure that each resulting expression group contained at least 30% of the total samples. Based on the gene-specific cut-off values, LUSC samples in both training and merged validation sets were categorized into high- and low-expression groups. Survival differences across two groups were assessed using log-rank test ($P < 0.05$) and HR was calculated. Genes exhibiting consistent HR direction and significant differences were designated as candidate key genes for subsequent analysis. In conjunction with a review of the existing literature (6,27), genes with strong associations to LUSC were ultimately identified as key genes.

Differential expression and enrichment analysis between different expression groups of key genes. To identify differentially expressed genes (DEGs) across different key gene expression groups, the 'limma' package (version 3.52.4) (28) was used, setting the criteria as $\log_2$ fold changel > 0.5 and adjusted $P < 0.05$. Subsequently, a volcano plot depicting these DEGs was generated using the 'ggplot2' package. In order to further explore molecular functions and underlying mechanisms associated with these DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out using the 'clusterProfiler' package (version 4.7.1.001) (29), with a significance threshold set at adjusted $P < 0.05$.

Gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA). To identify key gene functions in LUSC, GSEA was conducted through the 'clusterProfiler' package. The correlation between key genes and all genes was evaluated using Spearman's rank correlation analysis. GSEA was performed using normalized enrichment score $|(NES)| > 1$ and $P < 0.05$. Furthermore, to identify the divergent pathways associated with distinct expression groups of key genes, GSVA was implemented to identify enriched pathways. Differential expression analysis was then conducted using the 'limma' package to identify differentially expressed pathways between different expression groups, using the criteria $|t| > 2$ and $P < 0.05$. Background gene set for both GSEA and GSVA was 'c2.cp.kegg.v2023.2.Hs.symbols.gmt', which was

obtained from Molecular Signatures Database (<https://www.gsea-msigdb.org/>).

Evaluation of immune microenvironment. To investigate the immune landscape of LUSC samples, the quantity of immune cell infiltration in both expression groups was evaluated using the 'CIBERSORT' package (version 1.03) (30). The differences in immune cell composition across these groups were assessed using the Wilcoxon rank-sum test, with $P < 0.05$. Furthermore, the tumor immune dysfunction and exclusion (TIDE) score was calculated using the TIDE website (<http://tide.dfci.harvard.edu/>) (31) and differences in TIDE score between two expression groups of key genes were compared using the Wilcoxon rank-sum test ($P < 0.05$). The correlation between key gene expression and TIDE score was evaluated using Spearman's rank correlation analysis. Furthermore, to analyze the differences in immune, stromal and Estimation of STromal and Immune cells in MAlignant Tumors using Expression data (ESTIMATE) scores between two expression groups, 'estimate' package (version 1.0.13) (<https://r-forge.r-project.org/projects/estimate/>) was used to calculate the corresponding scores. The Wilcoxon rank-sum test was used to compare these scores between the groups ($P < 0.05$). The 'ImmuneSubtypeClassifier' package (version 0.1.0) (<https://github.com/CRI-iAtlas/ImmuneSubtypeClassifier>) (32) was used to divide LUSC samples into different immune subtypes. The expression levels of key genes in these subtypes were further explored using the Wilcoxon rank-sum test ($P < 0.05$).

Mutation analysis. To investigate the mutation profiles across groups with varying expression levels of key genes, 'maftools' package (version 2.12.0) (33) was used. A waterfall plot was generated to highlight the top 20 most frequently mutated genes.

Drug sensitivity analysis. The 'oncoPredict' package (version 0.2) (34) was used to determine the IC_{50} of 198 chemotherapeutic/targeted therapy drugs. The IC_{50} value for these drugs was compared between groups with diverse expression levels of key genes using the Wilcoxon rank-sum test ($P < 0.05$).

Cell culture, lentiviral vector construction and transfection. Human LUSC cells (NCI-H520 and SK-MES-1) were cultured in a medium consisting of 90% RPMI-1640, 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. These cells were incubated in an atmosphere of 5% CO_2 at 37°C.

293T cells (cat. no. IM-H222; Xiamen Yimo Biotechnology Co., Ltd.) were cultured and seeded onto a 6-well plate at a density of 6×10^4 cells per well in a final volume of 2 μ l medium per well, and transfection was carried out when cell confluence reached 60–80%. The transfection was performed using the jetPRIME® reagent (Polyplus-transfection SA). Briefly, 2 μ g target plasmid (PINK1-targeting short hairpin (sh)RNA-expressing pLKO.1-puro plasmid for PINK1 KD or PINK1 OE plasmid based on the pLVX-IRES-Hyg backbone for PINK1 OE), 0.25 μ g pMD2.G (cat. no. HG-VMA0648; HonorGene) and 0.75 μ g psPAX2 (cat. no. HG-VMA0649; HonorGene) were diluted in 200 μ l jetPRIME buffer and vortexed thoroughly. Subsequently, 4 μ l jetPRIME was

centrifuged at 1,360 x g for 5 sec at room temperature before being added to the diluted DNA solution. The resulting transfection mixture was then centrifuged at 1,360 x g for 10 sec at room temperature. The transfection mixture was incubated at room temperature for 10 min and 200 μ l of the resulting transfection complexes was added dropwise to each well. Plates were gently rocked horizontally and incubated at 37°C, and the medium was replaced with fresh medium after 4–6 h of transfection. A total of 48 h post-transfection, the viral supernatant was collected, filtered through a 0.22- μ m filter, aliquoted (1 μ l per tube) and stored at -80°C. The filtered supernatant was then used to infect NCI-H520 and SK-MES-1 cells.

NCI-H520 cell lines with key gene OE and SK-MES-1 cell lines with key gene KD (sh-key gene) were established and validated in 293T cells. Empty plasmids pLVX-IRES-Hyg (cat. no. V010432; NovoPro Bioscience Inc.) and pLKO.1-puro (cat. no. V010450; NovoPro Bioscience Inc.) were used as the negative controls for OE (OE-NC) and KD (empty pLKO.1-puro), respectively. All sequences for OE-key gene and sh-key gene are provided in Table SII. The effects of focal adhesion kinase (FAK) inhibitor GSK-2256098 (cat. no. HY-100498A; MedChemExpress) on NCI-H520 cells were assessed at concentrations of 0.2, 1.0, 5.0, 7.5, 15.0, 20.0 and 50.0 μ M and an optimal concentration was determined (35).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using FastPure Complex Tissue/Cell Total RNA Isolation Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions. Isolated RNA was then reverse transcribed into complementary DNA (cDNA) using ABScript III RT Master Mix for RT-qPCR with genomic DNA (gDNA) Remover (ABclonal Biotech Co., Ltd.). The reverse transcription reaction mixture consisted of 4 μ l 5X ABScript III RT Mix, 1 μ l 20X gDNA Remover Mix, 500 ng total RNA and nuclease-free H_2O added to a final volume of 20 μ l. The reverse transcription conditions were 37°C for 2 min, 55°C for 15 min, 85°C for 5 min, followed by a 4°C hold. Subsequently, qPCR was performed using an Archimed R4 real-time qPCR system (Beijing Kunpeng Gene Technology Co., Ltd.) and Genius 2X SYBR® Green Fast RT-qPCR Mix containing SYBR Green as the fluorescent dye (ABclonal Biotech Co., Ltd.). The RT-qPCR reaction mixture consisted of 10 μ l Genius 2X SYBR® Green Fast RT-qPCR Mix (NoX), 2 μ l DNA template (cDNA), 0.4 μ l forward primer (10 μ M), 0.4 μ l reverse primer (10 μ M) and double-distilled H_2O added to a final volume of 20 μ l. The RT-qPCR cycling conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 40–45 cycles of 95°C for 5 sec and 60°C for 30 sec. Gene expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method, as described by Livak and Schmittgen (36), with mRNA levels normalized to GAPDH (sequences are provided in Table SIII).

WB analysis. Proteins were extracted from aforementioned cell samples using radioimmunoprecipitation assay buffer lysis buffer (cat. no. P0013B; Beyotime Biotechnology) and their concentrations were measured through the bicinchoninic acid assay (cat. no. P0010; Beyotime Biotechnology). Equal amounts of total protein (30 μ g/lane) were separated by

SDS-PAGE on 10% gels and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 30 min at room temperature. Primary antibodies were incubated overnight at 4°C, followed by incubation with secondary antibodies for 30 min at room temperature. Protein bands were visualized using Immobilon Western chemiluminescent HRP substrate (cat. no. WBULS0500; MilliporeSigma) and the images were analyzed with ImageJ software (version 1.42q; National Institutes of Health). The following primary antibodies were used in the present study: Anti-FAK (cat. no. A11131; 1:500) and anti-phosphorylated (p)-FAK (Y397) (cat. no. AP1447; 1:2,000) were obtained from ABclonal Biotech Co., Ltd., anti-protein kinase B (AKT; cat. no. CY5561; 1:1,000), anti-p-AKT1 (cat. no. CY6569; 1:1,000), anti-Ras homolog family member A (RhoA; cat. no. CY5640; 1:1,000) and anti-vinculin B (cat. no. CY5164; 1:1,000) were all purchased from Shanghai Abways Biotechnology Co., Ltd. GAPDH antibody (cat. no. 81640-5-RR; 1:5,000; Proteintech Group, Inc.) was used as the reference protein antibody. The secondary antibody [goat anti-rabbit IgG (H+L), HRP-conjugated; cat. no. 31460; 1:5,000] was obtained from Thermo Fisher Scientific, Inc.

Cell proliferation assays. To evaluate cell proliferation, Cell Counting Kit-8 (CCK-8) and colony formation assays were conducted. For the CCK-8 assay, a CCK-8 kit (cat. no. G1613-1ML; Wuhan Servicebio Technology Co., Ltd.) was used. NCI-H520 cells were seeded into a 96-well plate. At 0, 24, 48 and 72 h, 10 μ l CCK-8 solution was added into each well and incubated for 1 h. The optical density value of each well was measured at 450 nm. For colony formation assay, cells were precisely counted and plated into 6-well culture plates at a density of 2,000 cells/well. Where indicated, cells were treated with 20 μ M GSK-2256098. Cells were cultured continuously for 14 days or until the majority of individual colonies contained >50 cells. Following colony formation, 700 μ l of 4% paraformaldehyde was added to each well for fixation at room temperature for 30 min, followed by staining with 700 μ l of 0.1% crystal violet solution at 37°C for 20 min. Plates were air-dried at room temperature, and images were captured using a mobile phone and quantitatively analyzed using ImageJ software (version 1.42q) (35).

Transwell assay. To assess migratory and invasive capabilities of cells under various treatments, Transwell analysis was conducted. The steps of the Transwell invasion assay were as follows: In ice, Matrigel and serum-free medium were diluted at a ratio of 1:8. A 60 μ l mixture was applied to the membrane of the Transwell insert and incubated at 37°C for 3 h, following which 100 μ l of serum-free medium was added to the upper chamber. The residual Matrigel/serum-free medium mixture in the upper chamber of the Transwell insert was then removed, and 500 μ l complete medium consisting of basal medium containing 20% serum was added to the lower chamber. A total of 4×10^4 cells/well were seeded into the upper chamber. The 24-well plate was cultured at 37°C, 5% CO₂ and 90% humidity for 24–48 h. The Transwell insert was subsequently extracted, fixed with 4% polyformaldehyde at room temperature for 30 min and stained with 1% (600 μ l) crystal violet at room temperature for 20 min. After washing

3 times with PBS, images were captured using an optical microscope at x100 magnification (x10 objective and x10 eyepiece), and cell counts were determined using ImageJ software (version 1.42q) (35). The Transwell migration assay does not require Matrigel coating and the remaining steps aforementioned were followed.

Statistical analysis. Bioinformatics analysis was conducted using R software (version 4.2.2; R Development Core Team). Differences between two groups were compared using the Wilcoxon rank-sum test, while differences among multiple groups were assessed using the Kruskal-Wallis test followed by Dunn's post hoc test with Holm correction for multiple comparisons. Survival differences were assessed using the log-rank test. Experimental data were analyzed using GraphPad Prism software (version 10.1.2; Dotmatics). Experimental data were presented as the mean $\pm$ standard deviation. All experiments, including WB, CCK-8, colony formation and Transwell migration/invasion assays, were performed using three independent biological replicates. Comparisons between two groups were performed using a two-tailed unpaired Student's t-test. Comparisons among three or more groups were conducted using one-way ANOVA followed by a two-tailed Bonferroni post hoc test for multiple pairwise comparisons when the overall difference was statistically significant. For CCK-8 assays, because both group and time were included as variables, statistical analysis was performed using two-way ANOVA followed by Bonferroni post hoc test for pairwise comparisons. All statistical tests were two-tailed. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PINK1 as a key gene in LUSC. Using differential expression analysis, 31 DE-UPR^{mt}-RGs were identified between LUSC and control samples in TCGA-LUSC dataset (Fig. 1A). Among these, DE-UPR^{mt}-RGs, CCAAT/enhancer-binding protein β (CEBPB), caseinolytic mitochondrial matrix peptidase proteolytic subunit, high-temperature requirement A2 and PINK1 were identified to be associated with LUSC survival by univariate Cox analysis (Fig. 1B). Furthermore, LUSC samples were categorized into high- and low-expression cohorts based on the optimal cut-off value of 0.3, Kaplan-Meier (KM) survival analysis was then performed in these groups. In both training and merged validation sets, survival difference was observed among CEBPB and PINK1 diverse expression groups (Fig. 1C and D). Of note, the higher expression levels of these genes were closely associated with worse prognosis, indicating that CEBPB and PINK1 were risk factors for LUSC. Thus, CEBPB and PINK1 were identified as candidate key genes in LUSC. According to the published literature (6), PINK1 was associated with the poor prognosis of LUSC. Furthermore, KM survival analysis of the present study also demonstrated that PINK1 was the most relevant genes for LUSC. Therefore, PINK1 was selected as a key gene for further analysis.

Potential functions of PINK1 in LUSC. Differential expression analysis was conducted between the groups with high and low PINK1 expression in TCGA-LUSC dataset, identifying 1,336

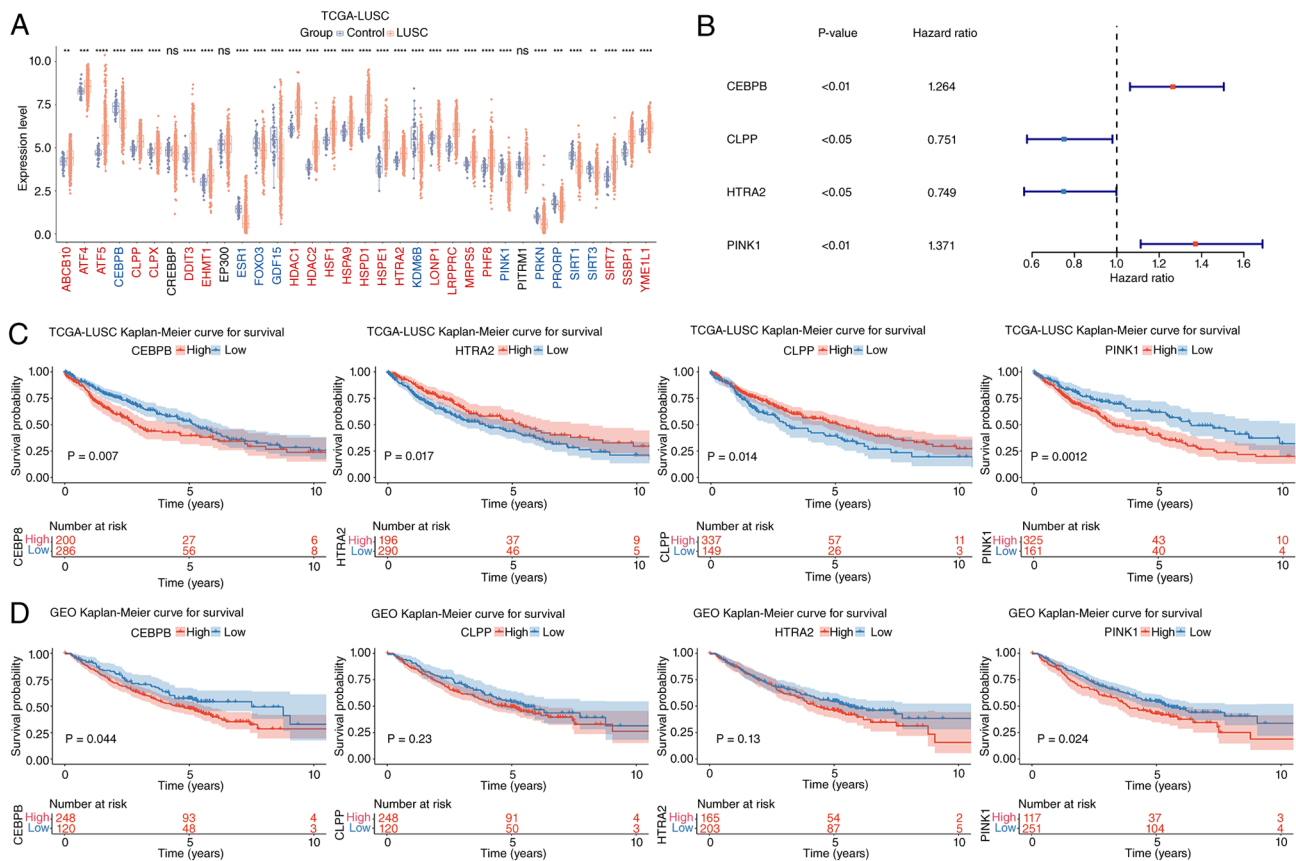


Figure 1. PINK1 was selected as a key gene in LUSC. (A) Differential expression levels of mitochondrial unfolded protein response-related genes between LUSC and control samples. (B) Analysis of LUSC survival-associated genes. Survival difference of genes in (C) training and (D) validation sets. **P<0.01, ***P<0.001 and ****P<0.0001. ns, no significance; PINK1, PTEN-induced kinase 1; LUSC, lung squamous cell carcinoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; CEBPB, CCAAT/enhancer-binding protein β ; CLPP, caseinolytic mitochondrial matrix peptidase proteolytic subunit; HTRA2, high-temperature requirement A2.

DEGs, comprising 1,300 upregulated and 36 downregulated genes (Fig. 2A; Table SIV). Functional enrichment analysis of these DEGs revealed 1,588 GO terms and 55 KEGG pathways. The GO terms encompassed processes such as 'extracellular matrix organization', 'extracellular matrix structural constituent' and 'collagen-containing extracellular matrix' (Fig. 2B), while KEGG pathways contained 'cytoskeleton in muscle cells', 'PI3K-AKT signaling pathway' and 'regulation of actin cytoskeleton' (Fig. 2C). To further investigate the functions of PINK1, GSEA was conducted. The results indicated that 117 pathways were enriched, with the top 5 pathways ranked by INES1 value being 'focal adhesion', 'regulation of actin cytoskeleton', 'endocytosis', 'ECM-receptor interaction' and 'MAPK signaling pathway' (Fig. 2D). These pathways were correlated with cellular processes, signaling molecules and interaction, suggesting that PINK1 possibly influenced LUSC through these mechanisms. Furthermore, to explore the differential pathways across PINK1 distinct expression groups, GSVA was carried out. The results revealed that 146 differential pathways between these groups. Among these, 26 were inhibited and 120 were activated in the LUSC samples (Fig. 2E). These inhibited pathways included 'oxidative phosphorylation', 'ribosome', 'metabolism of xenobiotics by cytochrome P450' and 'ascorbate and aldarate metabolism', while activated pathways contained 'focal adhesion', 'glycosaminoglycan biosynthesis chondroitin sulfate' and

'arrhythmogenic right ventricular cardiomyopathy arrhythmogenic right ventricular cardiomyopathy'.

Differential immune landscape of PINK1 distinct expression groups in LUSC. Immune infiltration abundance in two PINK1 expression groups is provided in Fig. 3A. Among the types of 22 immune cells, 9 exhibited significant differences between these groups, including resting memory CD4, activated memory CD4 and $\gamma\delta$ T cells and M0/M1/M2 macrophages (Fig. 3B). Furthermore, the TIDE score was also compared between different expression groups, revealing that the high-expression group had a significantly higher TIDE score (Fig. 3C). Correlation analysis revealed a significant positive correlation between the TIDE score and PINK1 expression ($\rho=0.16$; Fig. 3D), suggesting that higher PINK1 expression may be associated with greater immune escape potential. Furthermore, the stromal, immune and ESTIMATE scores were all significantly elevated in the high-expression group (Fig. 3E), highlighting the marked impact of PINK1 expression on the immune landscape of LUSC. Further immune subtypes revealed that LUSC samples could be categorized into six distinct subtypes, namely C1 (wound healing), C2 (IFN- γ dominant), C3 (inflammatory), C4 (lymphocyte depleted), C5 (immunologically quiet) and C6 (TGF- β dominant). Of note, a significant difference in PINK1 expression was detected between the C1 and C2 subtypes as compared with the C6

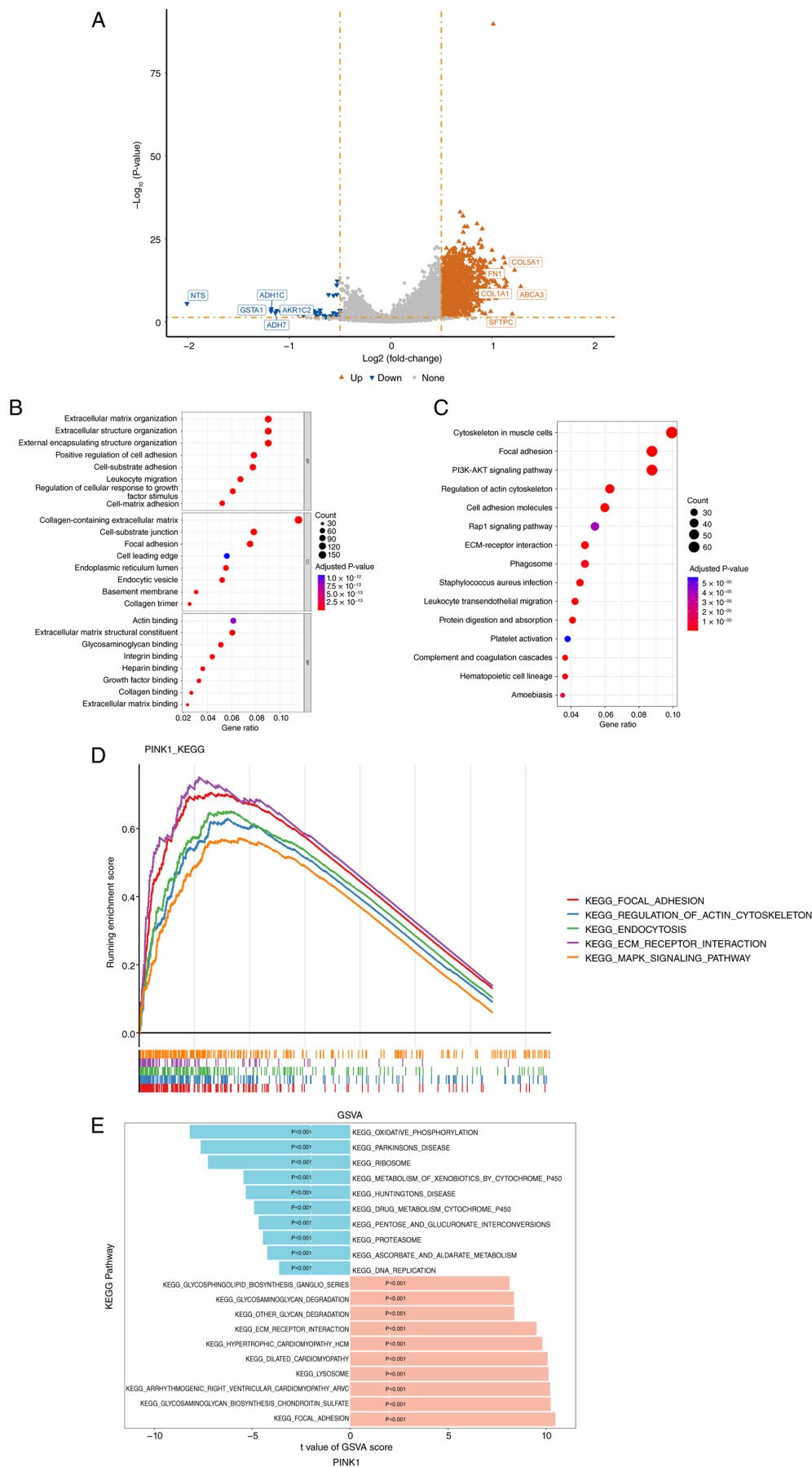


Figure 2. Potential functions of PINK1 expression in LUSC. (A) DEGs between PINK1 high- and low-expression groups. (B) Gene Ontology and (C) KEGG enrichment analysis of DEGs. (D) Gene set enrichment analysis of DEGs. (E) GSVA between high- and low-expression groups. PINK1, PTEN-induced kinase 1; LUSC, lung squamous cell carcinoma; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSVA, gene set variation analysis; ARVC, arrhythmogenic right ventricular cardiomyopathy; ECM, extracellular matrix.

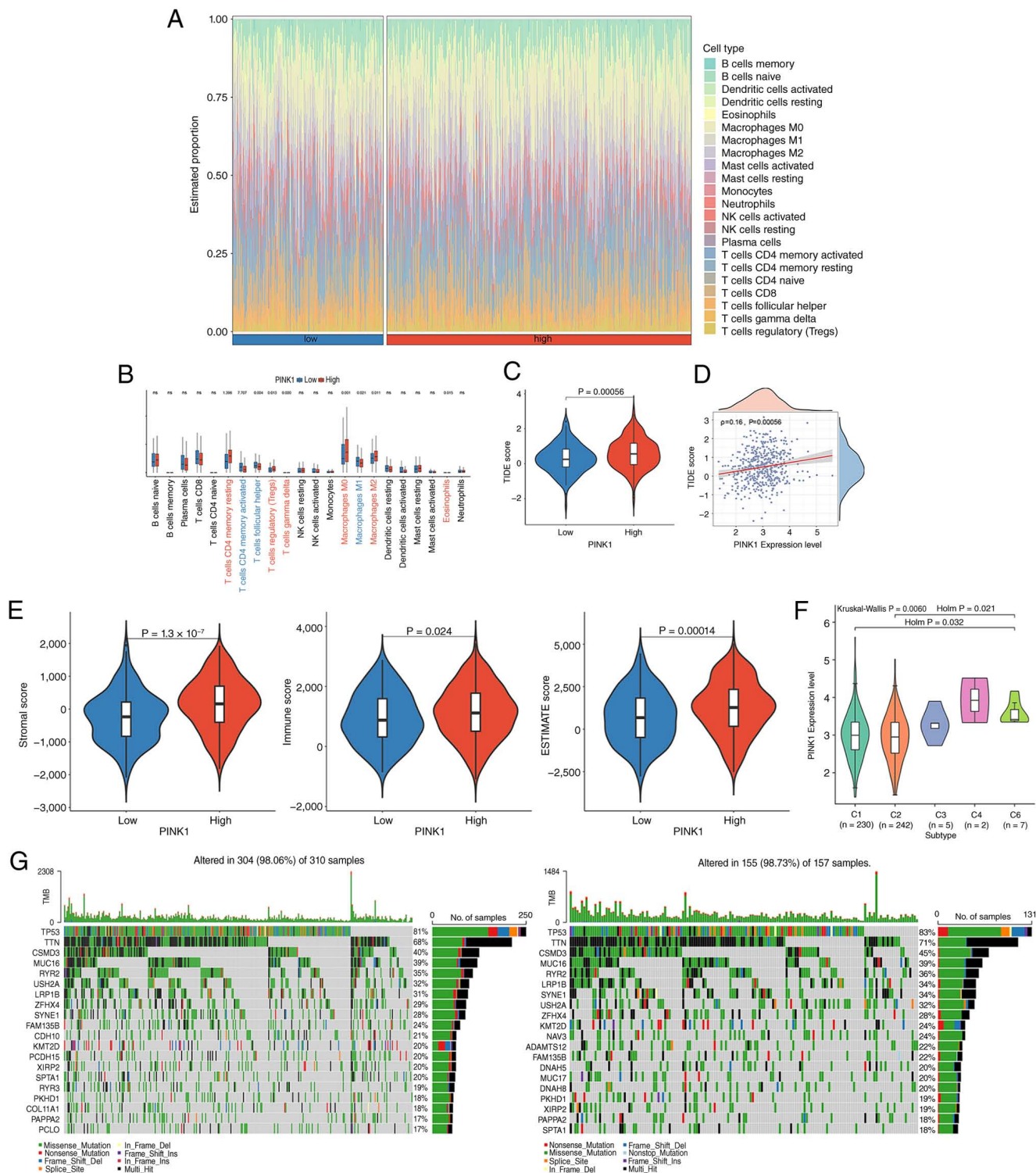


Figure 3. Immune and mutation landscape between high- and low-expression groups. (A) Immune landscape in distinct expression groups. (B) Difference in immune cell infiltration between high- and low-expression groups. (C) Difference and (D) correlation of the TIDE score between high- and low-expression groups. (E) Difference in immune, stromal and ESTIMATE scores between high- and low-expression groups. (F) Expression levels of PINK1 among different immune cell subtypes. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post hoc multiple comparisons test with Holm correction for multiple comparisons. (G) Mutation landscape of genes (left, high-expression group; right, low-expression group). ns, no significance; TIDE, tumor immune dysfunction and exclusion; PINK1, PTEN-induced kinase 1; TMB, tumor mutation burden; TIDE, Tumor Immune Dysfunction and Exclusion; NK, natural killer; ESTIMATE, Estimation of STromal and Immune cells in Malignant Tumor tissues using Expression data.

subtype (Fig. 3F). This finding suggested that PINK1 may exert distinct effects across different LUSC subtypes, thereby highlighting the necessity for personalized therapeutic approaches in the future.

Association between PINK1 expression and chemosensitivity in LUSC. The mutation profiles of high- and low-PINK1 expression groups were investigated. The present study results reported that the top 3 most frequently mutated genes in both

groups were tumor protein p53 (high, 81%; low, 83%), titin (high, 68%; low, 71%) and CUB and sushi multiple domains 3 (high, 40%; low, 45%) (Fig. 3G). The predominant type of mutation was missense mutation.

To assess drug sensitivity in PINK1 distinct expression groups, the IC_{50} value of these drugs was calculated and compared. A total of 100 drugs exhibited a marked difference between distinct expression groups (Table SV). Common chemotherapeutic drugs, such as cisplatin_1005, paclitaxel_1080 and gemcitabine_1190 (numbers indicate the corresponding unique drug IDs in the Genomics of Drug Sensitivity in Cancer database, <https://www.cancerrxgene.org/>), had a higher IC_{50} value in the high-expression groups, indicating that patients with a high PINK1 expression had a lower drug sensitivity.

PINK1 aggravates LUSC cell proliferation, migration, invasion and colony formation ability. To further elucidate the role of PINK1 in LUSC, lentivirus vectors for PINK1 OE and KD, together with their corresponding negative controls, were constructed. PINK1 OE was established in NCI-H520 cells, whereas PINK1 KD was established in SK-MES-1 cells. As illustrated in Fig. 4A and B, both mRNA and protein expression levels of PINK1 were significantly increased in NCI-H520 cells transfected with OE-PINK1-2, compared with the corresponding control group. Among the two OE constructs, OE-PINK1-2 was selected for the subsequent functional experiments. As displayed in Fig. 4C and D, PINK1 expression at both the mRNA and protein levels was significantly reduced in SK-MES-1 cells transfected with sh-PINK1-1 and -2, compared with the corresponding control group. Among the three KD constructs, KD-PINK1-2 was selected for subsequent functional experiments because it exhibited higher gene knockdown efficiency than KD-PINK1-1. Furthermore, CCK-8 assay results demonstrated that PINK1 OE significantly enhanced the proliferation of NCI-H520 cells compared with in the OE-NC group, with the effect beginning to appear at 24 h, becoming evident at 48 h and reaching the highest level at 72 h. By contrast, PINK1 KD significantly reduced the proliferation of SK-MES-1 cells compared with in the KD-NC group, with the inhibitory effect becoming marked from 48 h and being most notable at 72 h (Fig. 4E). In addition to the between-group comparisons, Bonferroni post hoc analysis also revealed significant time-dependent increases in cell viability within each group. In the OE-NC group, cell viability was significantly increased at 24, 48 and 72 h compared with 0 h and significant differences were also detected between 24, 48 and 72 h. In the OE-PINK1 group, cell viability increased significantly over time, with significant differences observed between 24, 48 and 72 h, indicating that PINK1 OE further enhanced the proliferative ability of cells over time. Similarly, in the KD-NC group, cell viability increased significantly at 24, 48 and 72 h compared with 0 h. The time-dependent increase in the KD-PINK1 group was significantly attenuated compared with that in the KD-NC group. Colony formation analysis revealed that PINK1 OE significantly promoted colony formation, whereas PINK1 KD exerted the opposite effect (Fig. 4F). Furthermore, the migratory and invasive capabilities of LUSC cells were significantly increased in NCI-H520 cells following PINK1 OE and significantly decreased in SK-MES-1 cells

following PINK1 KD (Fig. 4G). Collectively, these findings suggested that elevated PINK1 expression may exacerbate LUSC progression by promoting cell proliferation, migration, invasion and colony formation.

PINK1 promotes LUSC through the FAK/AKT/RhoA/vinculin pathway. Considering that PINK1 was significantly associated with focal adhesion by enrichment analyses and FAK has been recognized as a key regulator in NSCLC (37), the aim of the present study was to explore the association between FAK and PINK1 in LUSC. WB revealed that both FAK and p-FAK expression levels were significantly elevated in OE-PINK1 cells, as compared with OE-control cells (Fig. 5A). However, the p-FAK/FAK ratio was not significantly increased in OE-PINK1 cells compared with OE-control cells. A 20 μ M FAK inhibitor was then employed to investigate its effects on tumor cells in LUSC. PINK1 overexpression significantly increased the proliferation of NCI-H520 cells compared with the OE-NC group, whereas treatment with 20 μ M FAK inhibitor significantly attenuated this increase (Fig. 5B). Similarly, PINK1 overexpression significantly enhanced cell migration, invasion and colony formation compared with in the OE-NC group; by contrast, treatment with the FAK inhibitor significantly reduced the PINK1 overexpression-induced increases in cell migration, invasion and colony formation (Fig. 5C and D). These findings suggested that PINK1 may activate FAK to promote the aforementioned processes, thereby exacerbating LUSC progression. Further WB analysis revealed markedly increased expression levels of AKT, p-AKT, RhoA, vinculin and p-AKT/AKT in OE-PINK1 cells (Fig. 5E-G). Notably, the protein-level changes of FAK, AKT, RhoA and vinculin were consistent with the transcriptomic analysis results in TCGA-LUSC cohort, in which PTK2, AKT1, RHOA and VCL were significantly upregulated in the PINK1-high group (Table SIV). Collectively, these findings indicated that PINK1 aggravated LUSC progression by enhancing tumor cell proliferation, migration, invasion and colony formation through the FAK/AKT/RhoA/vinculin pathway.

Discussion

LUSC is a major subtype of NSCLC, characterized by aggressive growth and poor prognosis (38). Increasing evidence indicates that mitochondrial dysfunction contributes to tumor progression. The UPR^{mt} is a stress-adaptive signaling pathway that maintains mitochondrial proteostasis by upregulating molecular chaperones and proteases in response to mitochondrial damage (39-41). The aberrant activation of UPR^{mt} has been implicated in several cancer types, including lung cancer (40), breast cancer (42) and prostate cancer (43), suggesting that UPR^{mt}-RGs may serve key roles in LUSC development.

PINK1 is a mitochondrial serine/threonine kinase that serves a key role in mitochondrial quality control and mitophagy (44). Dysregulated PINK1 expression has been reported in multiple malignancies, including glioblastoma (45) and lung cancer (46), and has been reported to be closely associated with tumor progression, apoptosis resistance and chemoresistance, particularly in lung cancer (45-47). Previous studies have suggested that

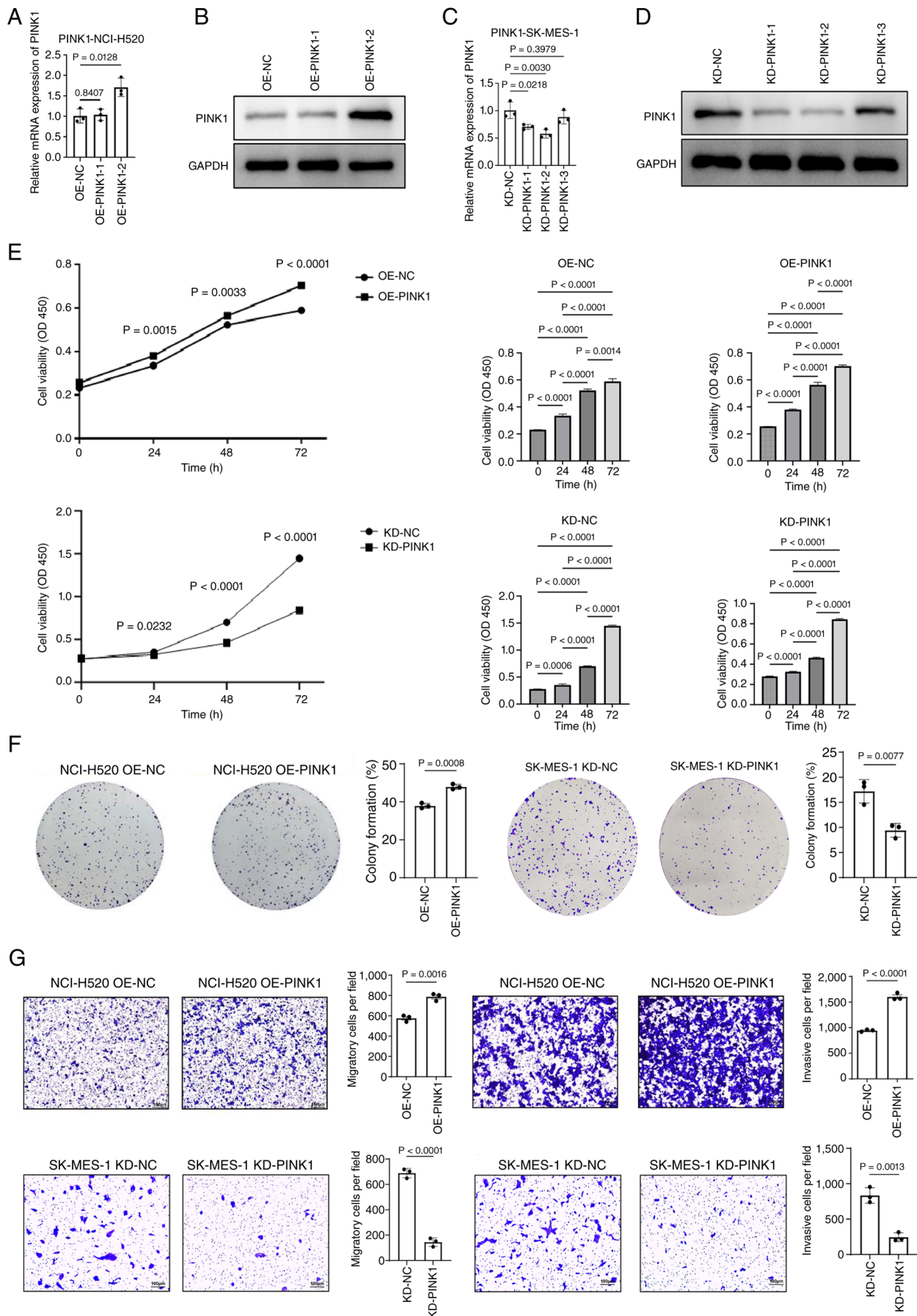


Figure 4. PINK1 expression is associated with mutation landscape and chemotherapy resistance in lung squamous cell carcinoma. Expression levels of OE-PINK1 at the (A) mRNA and (B) protein levels. Expression levels of sh-PINK1 at the (C) mRNA and (D) protein levels. (E) Cell activity of OE- and sh-PINK1. (F) Colony formation of OE- and sh-PINK1. (G) Migration and invasion of OE- and sh-PINK1. PINK1, PTEN-induced kinase 1; shRNA/sh, short hairpin RNA; OE, overexpression; NC, negative control; KD, knockdown.

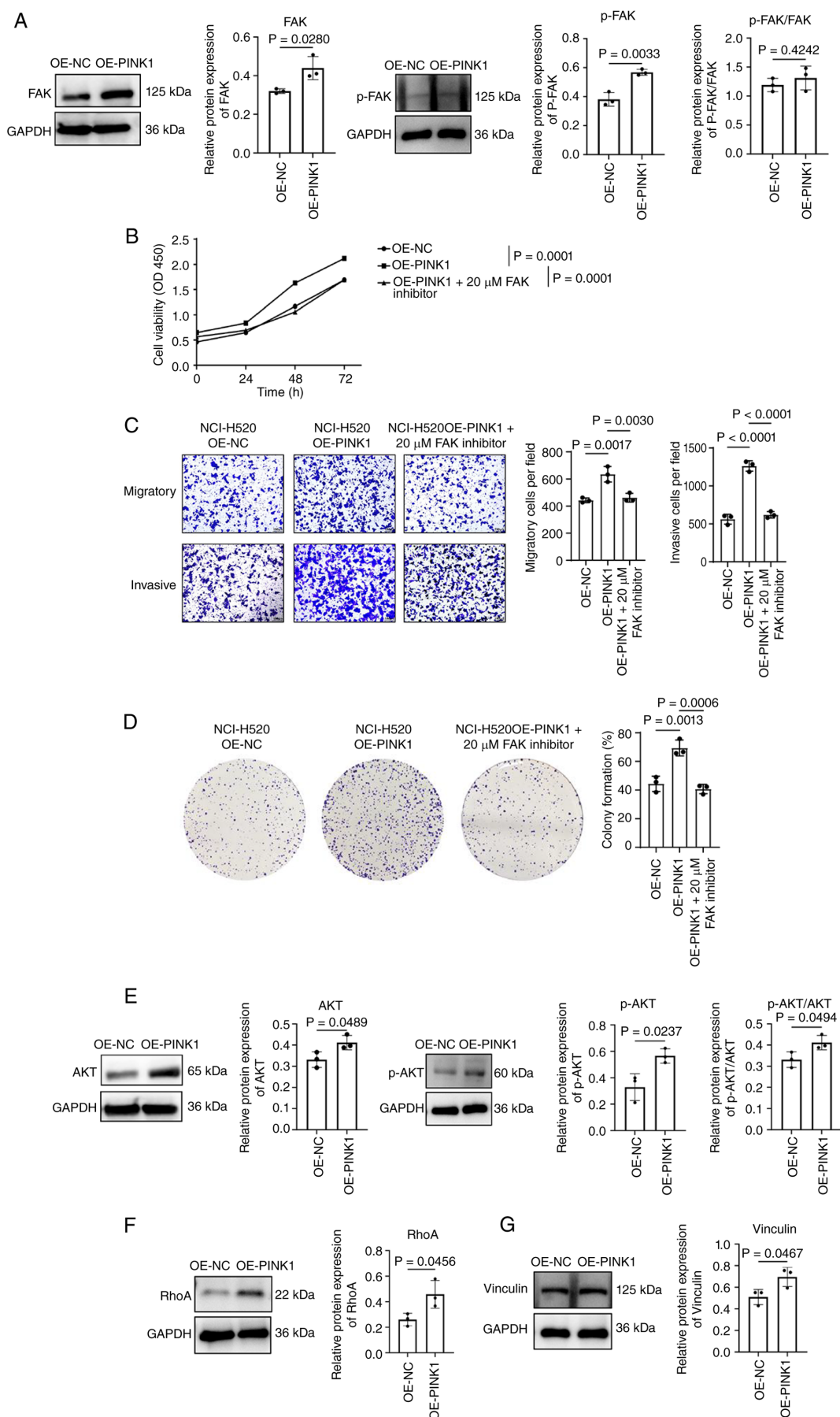


Figure 5. PINK1 promotes lung squamous cell carcinoma through the FAK/AKT/RhoA/vinculin pathway. (A) Protein expression levels of FAK and p-FAK, as well as the p-FAK/FAK ratio. (B) Cell activity with FAK inhibitor. (C) Migration and invasion with FAK inhibitor. (D) Colony formation with FAK inhibitor. Protein expression levels of (E) AKT and p-AKT (and the p-AKT/AKT ratio), (F) RhoA and (G) vinculin. PINK1, PTEN-induced kinase 1; FAK/AKT/RhoA, focal adhesion kinase/protein kinase B/Ras homolog family member A; p, phosphorylated; OE, overexpression; NC, negative control.

PINK1 exerts pro-tumorigenic effects mainly by regulating mitophagy and inhibiting apoptosis, and elevated PINK1 levels have been associated with poor prognosis and chemotherapy resistance in NSCLC (6,48,49).

However, to the best of our knowledge, majority of these studies focused on general NSCLC populations or primarily emphasized its role in mitochondrial autophagy, leaving the specific function and downstream signaling mechanisms of PINK1 in LUSC insufficiently characterized. Consistent with and extending these findings, the present study results demonstrated that PINK1 is not only associated with poor survival in LUSC but also directly promotes the proliferation, migration and invasion of LUSC cells, in part through the activation of focal adhesion-associated signaling pathways, highlighting its potential role as a key regulator of LUSC progression.

GSEA analysis demonstrated a significant enrichment of the 'focal adhesion' pathway in the PINK1 high-expression group. Consistently, PINK1 OE markedly increased FAK and p-FAK levels in LUSC cells. FAK, a non-receptor tyrosine kinase, has been reported to promote tumor growth by enhancing cell proliferation, survival, signal transduction and angiogenesis (50), and its OE is associated with unfavorable patient prognosis in multiple malignancies, including ovarian, breast, pancreatic, lung, prostate and colorectal cancer (51). Furthermore, FAK serves a key role in carcinogenesis, metastasis and drug resistance (52). The selective FAK inhibitor GSK2256098 has been reported to obstruct cancer cell adhesion, proliferation and migration (53), and to inhibit migration and metastasis by blocking FAK signaling in gastric cancer cells (54). In the present study, treatment with GSK2256098 significantly attenuated the enhanced proliferation, migration and invasion of LUSC cells induced by PINK1 OE, indicating that FAK activation is functionally involved in PINK1-mediated LUSC progression.

Furthermore, PINK1 OE increased the expression levels of AKT, p-AKT, RhoA and vinculin. The PI3K/AKT pathway, a principal downstream target of FAK (55,56), is known to regulate tumor cell proliferation, migration and invasion (57,58). AKT activation has also been reported to directly regulate RhoA activity through phosphorylation (59). RhoA, a member of the Rho-GTPase family, modulates cytoskeletal architecture by regulating actin stress fiber formation and focal adhesion complex assembly (60). Vinculin, a key protein linking integrin-mediated adhesion complexes to the actin cytoskeleton (61), has been associated with reduced overall survival in patients with lung cancer (62), and its KD suppresses cell proliferation and invasion (14). In combination, the present study findings suggested that PINK1 promotes LUSC progression through the activation of the FAK/AKT/RhoA/vinculin signaling axis.

To the best of our knowledge, the present study was the first to systematically screen UPR^{mt}-RGs in LUSC and identify PINK1 as a key prognostic factor using multi-cohort validation. Of note, experimental evidence associating PINK1 with the FAK/AKT/RhoA/vinculin signaling axis were provided, thereby establishing a novel mechanistic connection between mitochondrial stress responses and focal adhesion signaling in

LUSC. These findings potentially enhanced the current understanding of how mitochondrial quality control mechanisms interact with cytoskeletal remodeling pathways in LUSC. By integrating bioinformatics analysis with functional validation, the present study highlighted PINK1 as a potential biomarker for prognosis and therapeutic stratification. Targeting PINK1 or its downstream focal adhesion signaling components may represent a promising strategy in improving treatment outcomes in patients with LUSC.

Nonetheless, several limitations should be acknowledged. First, these conclusions were primarily based on bioinformatics analyses and *in vitro* experiments, without *in vivo* validation or clinical specimen confirmation, which may limit the translational strength of these findings. Although PINK1 promoted proliferation, migration and invasion of LUSC cells, its role in tumor progression and metastasis has not yet been verified in animal models. Future studies incorporating metastasis-associated *in vivo* experiments and clinical cohort validation are warranted to further evaluate its biological and clinical significance.

Secondly, although the present study was conducted within a UPR^{mt}-associated gene framework, the activation of canonical ER-UPR^{mt} signaling components or established UPR^{mt} markers were not directly assessed. Therefore, it remains to be elucidated whether UPR pathways are functionally involved in the observed phenotypes and the UPR^{mt}-associated mechanistic implications should be cautiously interpreted.

Thirdly, while PINK1 OE increased FAK, AKT, RhoA and vinculin expression and FAK inhibition attenuated PINK1-induced phenotypes, the present study findings primarily demonstrated association rather than a clearly defined hierarchical signaling cascade. Additional genetic, rescue or epistasis analyses are warranted to establish causal pathway dependence in future research.

Lastly, the mitochondrial localization or activation status of overexpressed PINK1 was not directly verified. Without confirmation of its mitochondrial engagement, the association between PINK1-driven effects and mitochondrial mechanisms remains to be elucidated. Furthermore, the present study mainly focused on proliferation, migration and focal adhesion-associated signaling, and other potential mechanisms regulated by PINK1, including autophagy or metabolic regulation, were not explored.

In conclusion, integrative analyses of TCGA and multiple GEO cohorts identified PINK1 as a UPR^{mt}-associated gene significantly associated with poor overall survival in LUSC. High PINK1 expression was reported to be enriched in 'focal adhesion' and 'PI3K/AKT-associated pathways' and associated with altered immune infiltration, elevated TIDE scores and reduced predicted chemosensitivity. Functional experiments demonstrated that PINK1 OE significantly promoted the proliferation, migration, invasion and colony formation of LUSC cells, whereas FAK inhibition significantly attenuated these effects. Mechanistically, PINK1 enhanced FAK phosphorylation and activated the downstream AKT/RhoA/vinculin signaling axis. Collectively, these findings indicated that PINK1 contributes to LUSC progression and may serve as a potential prognostic biomarker and therapeutic target; however, further *in vivo* validation is warranted in future research.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XL conceptualized the present study, obtained resources, curated the data, conducted the formal analysis, provided supervision, validated and visualized the data, wrote the original draft. YM acquired funding for the present study, validated and visualized the data, devised the methodology, participated in project administration, reviewed and edited the manuscript. XL and YM confirm the authenticity of all the raw data. Both authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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