

CHCHD2 as a prognostic biomarker and therapeutic target in lung adenocarcinoma: Insights from pan-cancer multi-omics and functional studies

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Abstract. Lung adenocarcinoma (LUAD) remains a leading cause of cancer-related mortality, and mitochondrial proteins have been increasingly implicated in tumor progression. Coiled-coil-helix-coiled-coil-helix domain-containing 2 (CHCHD2) has been reported in several malignancies, yet its pan-cancer landscape and LUAD-specific functional relevance have not been comprehensively evaluated. In the present study, multi-omics analyses were integrated using The Cancer Genome Atlas-derived datasets and public resources [including Gene Expression Profiling Interactive Analysis (GEPIA) 2, CIBERSORT and CellMiner] to characterize CHCHD2 expression patterns, clinical associations, tumor microenvironment (TME) features, stemness-related indices

and drug-response correlations across cancers, followed by experimental validation in LUAD models. CHCHD2 was found to be upregulated in multiple cancers and was correlated with advanced disease stage and poor overall survival, especially in patients with LUAD. CHCHD2 promoted cell proliferation and migration while inhibiting apoptosis. CHCHD2 knockdown reduced AKT and ERK phosphorylation and suppressed xenograft tumor growth *in vivo*. High CHCHD2 expression was associated with increased stemness, an immunosuppressive TME, and differential drug-response patterns in CellMiner analyses, suggesting that CHCHD2 expression may be associated with compound-specific sensitivity profiles. Collectively, these findings support an oncogenic role of CHCHD2 in LUAD and highlight its potential utility as a prognostic biomarker and therapeutic target.

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Abbreviations: CHCHD, coiled-coil-helix-coiled-coil-helix domain; LUAD, lung adenocarcinoma; TME, tumor microenvironment; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; GTEx, Genotype-Tissue Expression; RNAss, RNA-based stemness score; DNAss, DNA methylation-based stemness score; OS, overall survival; IHC, immunohistochemistry; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NSCLC, non-small cell lung cancer; HPA, Human Protein Atlas; CPTAC, Clinical Proteomic Tumor Analysis Consortium; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; KM plotter, Kaplan-Meier plotter platform

Key words: CHCHD2, LUAD, pan-cancer analysis, TME, stemness, drug sensitivity

Introduction

Lung cancer is the most prevalent malignancy globally, with ~2.5 million newly diagnosed cases constituting ~12.4% of all cancers worldwide. It remains the leading cause of cancer-related mortality worldwide, accounting for ~18.7% of cancer deaths (1). Among its subtypes, lung adenocarcinoma (LUAD) represents the most prevalent histological form and is often diagnosed at advanced stages, limiting the effectiveness of conventional therapies (2). Despite recent advances in targeted therapy and immunotherapy, the overall survival (OS) of patients with LUAD remains unsatisfactory (3). This highlights the urgent need for the identification of new biomarkers and therapeutic targets capable of enabling earlier detection and enhancing treatment efficacy. In recent years, attention has increasingly shifted towards mitochondrial proteins, key regulators of cellular processes that are frequently disrupted in cancer. Within this category, the CHCHD protein family has emerged as a notable focus because of its structural conservation and functional relevance in tumor biology.

Mitochondria are essential for cellular energy metabolism, apoptosis regulation and redox homeostasis, functions that are frequently reprogrammed by cancer cells to sustain uncontrolled proliferation and survival (4,5). CHCHD proteins

are nuclear-encoded mitochondrial factors characterized by a CHCH domain stabilized by disulfide bonds (6). Among them, CHCHD2 has attracted particular attention because of its dual mitochondrial and nuclear functions, as well as its emerging associations in the field of cancer biology.

CHCHD2 regulates mitochondrial metabolism, respiratory chain assembly and cell survival signaling. In neurodegenerative diseases, it modulates integrated mitochondrial stress responses and apoptosis via the BCL2/BAX pathway (7). More recently, increasing evidence has linked CHCHD2 to malignant phenotypes across multiple tumor types, including enhanced proliferation and migration, therapeutic resistance and altered microenvironmental features (8).

In addition to CHCHD2, other members of the CHCHD family also play important roles in cancer. CHCHD4, for instance, enhances tumor proliferation and metabolic activity under both normoxic and hypoxic conditions by regulating mitochondrial respiratory chain subunits, particularly complex I, thereby creating vulnerabilities to complex I inhibition through increased reactive oxygen species (ROS) production (9). CHCHD3 promotes prostate cancer cell proliferation and migration via ROS signaling, the stability of which is regulated by ASB1-mediated K48-linked ubiquitination (10). Collectively, the intersection of mitochondrial biology with cancer hallmarks positions the CHCHD family as a promising interdisciplinary research focus that integrates oncology, immunology, and metabolism. However, despite increasing insights from other malignancies, the specific role and mechanisms of CHCHD2 in lung cancer, particularly LUAD, remain insufficiently understood.

Although individual studies have reported CHCHD2 upregulation in certain tumors, systematic, pan-cancer-level analyses that examine expression patterns, intrafamily correlations, and prognostic implications across diverse cancer types are lacking. Preliminary evidence suggests that CHCHD2 can be coamplified with EGFR in non-small cell lung cancer (NSCLC), enhancing cell migration, proliferation and mitochondrial respiration (11). Therefore, the present study was not designed to claim that CHCHD2 is a newly discovered oncogenic factor in NSCLC but rather to extend these previous observations through a LUAD-focused and pan-cancer multi-omics framework. In LUAD, the integrated picture linking CHCHD2 to clinical outcomes, tumor immune features, stemness-related phenotypes, drug-response patterns, and experimentally validated tumor behaviors remains insufficiently defined. Existing studies often examine isolated aspects of CHCHD2 function, whereas a comprehensive integration of pan-cancer expression, prognosis, tumor microenvironment (TME) features, single-cell localization, drug-sensitivity associations, and LUAD-specific functional validation remains limited. Moreover, the role of CHCHD2 in modulating the TME and immune subtypes remains insufficiently explored.

Considering these gaps, the present study was organized into three sequential parts. First, the pan-cancer expression and genomic alteration landscape of the CHCHD family were characterized and the clinical relevance of CHCHD2 in LUAD was evaluated. Second, CHCHD2-associated transcriptional pathways, TME features, stemness indices, immune infiltration patterns, single-cell expression distributions and

drug-response correlations were examined. Third, CHCHD2 expression and biological function were validated using LUAD clinical tissues, *in vitro* gain- and loss-of-function assays, and an *in vivo* xenograft model. This stepwise design links public multi-omics evidence with LUAD-specific experimental validation and provides an organized framework for evaluating the potential role of CHCHD2 in LUAD progression.

Materials and methods

Data collection and preprocessing. RNA sequencing (RNA-seq) expression matrices (HTSeq-FPKM) and corresponding clinicopathological annotations (including survival outcomes, immune subtype labels, and stemness indices) for 33 The Cancer Genome Atlas (TCGA) cancer types were downloaded from the UCSC Xena platform (<https://xena.ucsc.edu/>), which aggregates harmonized TCGA datasets (12,13). The expression values of CHCHD family members (CHCHD1-7 and CHCHD10) were extracted for downstream analyses, whereas CHCHD8 was excluded because of annotation and availability constraints in the downloaded expression matrix (14). For tumor types lacking matched normal samples in the TCGA, normal RNA-seq data from the Genotype-Tissue Expression (GTEx) project (<https://gtexportal.org/>) were incorporated to strengthen tumor-normal comparisons (15). Group comparisons between tumor and normal tissues were performed using the Wilcoxon rank-sum test in R (version 4.3.1; R Foundation for Statistical Computing; <https://www.r-project.org/>). The results were visualized as boxplots and heatmaps using the 'ggpubr' and 'pheatmap' packages.

Pan-cancer genomic, clinical and proteomic analyses. Genetic alteration profiles (including somatic mutations and copy number alterations) of the CHCHD gene family across TCGA pan-cancer cohorts were characterized using cBioPortal (16), which provides integrative visualization and summary statistics for TCGA multi-omics and clinical datasets. To examine clinicopathological stage-associated expression patterns, CHCHD2 mRNA levels across pathological stages (I-IV) were analyzed using the 'Stage Plot' module of GEPIA2 (<http://gepia2.cancer-pku.cn>). Log2(TPM+1) values were compared among stages by one-way ANOVA, and $P < 0.05$ was considered to indicate statistical significance (17).

Protein-level validation was performed using CPTAC proteomic datasets accessed via the UALCAN portal (<http://ualcan.path.uab.edu/>), which provides normalized protein abundance values for tumor and normal tissues. Differences between the tumor and normal groups were assessed using Student's t-test ($P < 0.05$), and the results were visualized as boxplots (18). Immunohistochemistry (IHC) images of CHCHD2 in tumor and normal tissues were retrieved from the Human Protein Atlas (HPA; <https://www.proteinatlas.org/>), and staining intensity and distribution patterns were visually compared across cancer types (19).

The prognostic value of CHCHD2 expression across cancers was evaluated using the Kaplan-Meier plotter platform (<https://kmplot.com/analysis/>). OS was compared between the high- and low-expression groups using the platform's default settings, and hazard ratios (HRs) with log-rank P-values were reported (20).

Identification of genes, pathways and cellular functions related to CHCHD2 in LUAD. To elucidate the LUAD-specific transcriptomic features associated with CHCHD2, co-expression analysis was first performed using TCGA-LUAD RNA-seq data and genes significantly correlated with CHCHD2 expression were identified under stringent criteria ($|r| > 0.4$, FDR < 0.05); significant correlations were visualized using Circos plots. TCGA-LUAD samples were then stratified into high- and low-expression groups, and differentially expressed genes (DEGs) were identified using thresholds of $|\log_2FC| > 1$ and FDR < 0.05 . The functional annotation of the DEGs was subsequently conducted via Gene Ontology (GO) enrichment analysis (21) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (22) to characterize the biological processes (BP) and molecular pathways associated with CHCHD2 in LUAD.

TME and immune analyses. TME infiltration levels were quantified from TCGA-LUAD RNA-seq data using the ESTIMATE algorithm to calculate the stromal score, immune score and ESTIMATE score (and infer tumor purity where applicable) (23). Stemness indices [RNA-based stemness score (RNAss) and DNA methylation-based stemness score (DNAss)] were obtained from previously published OCLR-based stemness models and/or corresponding TCGA PanCanAtlas stemness resources, and their associations with CHCHD2 expression were evaluated by Spearman correlation (24).

TCGA immune subtype annotations (C1-C6) were used to compare CHCHD2 expression across immune subtypes, following the pan-cancer immune landscape framework (25). In addition, correlations between CHCHD2 and immune checkpoint genes were assessed using Spearman correlation, with multiple-testing correction applied when appropriate. Relative abundances of tumor-infiltrating immune cells were estimated using CIBERSORT (LM22 signature matrix), and differences in immune cell fractions between the CHCHD2-high and CHCHD2-low groups were compared using Wilcoxon rank-sum tests (26). Finally, single-cell RNA-seq-based validation of CHCHD2 expression across malignant and immune/stromal cell populations was performed using the Tumor Immune Single-Cell Hub (TISCH/TISCH2) database (26).

Analysis of the association between CHCHD2 expression and drug sensitivity across multiple cancer types. Drug sensitivity data were downloaded from the CellMiner™ database (27,28) (version: 2020.3; database: 2.4.2; <https://discover.nci.nih.gov/cellminer/home.do>).

Patient specimens and cell culture conditions. A total of 9 matched pairs of LUAD tissues and corresponding adjacent nontumorous tissues were obtained from the Department of Cardiothoracic Surgery at the Affiliated Hospital of Nantong University. All specimens were preserved at -80°C for subsequent western blotting and reverse transcription-quantitative PCR (RT-qPCR) analyses. The present study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (approval no. 2023-K010-01; Nantong, China). Written informed consent was obtained from each participant in compliance with the Declaration of Helsinki.

Human LUAD cell lines (A549, H1975, H1299, PC9 and H1650) were obtained from the Cell Bank of the Chinese Academy of Sciences. All the lines were authenticated and confirmed to be mycoplasma free. Cells were cultured in appropriate basal medium supplemented with 10% fetal bovine serum and 1% penicillin at 37°C in a humidified incubator with 5% CO_2 . Standard procedures for revival, subculture and cryopreservation were performed.

Reagents and antibodies. CHCHD2 knockdown and overexpression plasmids were obtained from Corues Biotechnology (<https://www.corues.cn/>). Lipofectamine 2000 was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). TRIzol™ Reagent was obtained from Invitrogen; Thermo Fisher Scientific, Inc., and HiScript III RT SuperMix and ChamQ SYBR qPCR Master Mix were obtained from Vazyme Biotech Co., Ltd. Primary antibodies against CHCHD2 (1:1,000; cat. no. 19424-1-AP; Proteintech Group, Inc.) and GAPDH (1:10,000; cat. no. AB0037; Abways Technology) were used for western blotting. For pathway validation, primary antibodies against phospho-AKT (Ser473) (1:3,000; cat. no. 28731-1-AP; Proteintech Group, Inc.), total AKT (1:10,000; cat. no. 60203-2-Ig; Proteintech Group, Inc.), phospho-ERK1/2 (Thr202/Tyr204) (1:3,000; cat. no. 28733-1-AP; Proteintech Group, Inc.) and total ERK1/2 (1:8,000; cat. no. 11257-1-AP; Proteintech Group, Inc.) were used for western blotting. HRP-conjugated anti-rabbit IgG (1:3,000; cat. no. 7074) and anti-mouse IgG (1:3,000; cat. no. 7076) secondary antibodies were obtained from Cell Signaling Technology, Inc. Western blotting consumables, including RIPA buffer, PMSF, PVDF membranes, and ECL reagents, were obtained from Beyotime Institute of Biotechnology, MilliporeSigma and Thermo Fisher Scientific, Inc. Reagents used for functional assays, including Cell Counting Kit-8, Annexin V-FITC, and Transwell chambers, were obtained from the aforementioned suppliers.

Cell transfection. For CHCHD2 knockdown, PC9 and H1975 cells were transfected with $2.5 \mu\text{g}/\text{well}$ short hairpin RNA (shRNA) plasmids targeting CHCHD2 (Corues Biotechnology; <https://www.corues.cn/>) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in 6-well plates according to the manufacturer's instructions. The shRNA oligonucleotides were subsequently cloned and inserted into the pRNA3-SV40-EGFP(2A)Puro-U6 vector, which contains a U6 promoter, an EGFP reporter, and a puromycin resistance cassette. A total of 3 CHCHD2-targeting shRNAs and a non-targeting negative-control shRNA (shNC) were used. The target sequences were as follows: shCHCHD2-1, 5'-CAGTGG AGGAAGTAATGCTGA-3'; shCHCHD2-2, 5'-CCTCTATGA GATCAACAGTT-3'; shCHCHD2-3, 5'-GAAACAGTGCCG ACTTGCAAA-3'; and shNC, 5'-GGAGTACTTTATCCGTGT ATT-3'. Knockdown efficiency was quantitatively evaluated at both the mRNA and protein levels. Among the three shRNAs tested, shCHCHD2-1 produced the strongest and most consistent reduction in CHCHD2 expression across PC9 and H1975 cells and was therefore selected for subsequent functional experiments. For CHCHD2 overexpression, H1299 and H1650 cells were transfected with $2.5 \mu\text{g}/\text{well}$ CHCHD2 expression plasmids (Corues Biotechnology) using Lipofectamine 2000. The knockdown and overexpression efficiencies were

confirmed by RT-qPCR and western blotting, respectively, at 48 h post-transfection, and the cells were collected at 48-72 h post-transfection for functional experiments.

RNA extraction, reverse transcription and RT-qPCR. Total RNA was extracted with TRIzol and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) by measuring absorbance at 260 nm; RNA purity was assessed using the A260/A280 ratio. Reverse transcription was performed with HiScript III RT SuperMix (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. RT-qPCR was conducted on an ABI 7500 system with ChamQ SYBR Master Mix (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 30 sec. GAPDH served as the internal reference gene, and relative CHCHD2 expression was calculated using the $2^{-\Delta\Delta C_q}$ method (29). The sequences of the primers used were as follows: CHCHD2 forward, 5'-ACCA GTCGCTCAGCCACCAG-3' and reverse, 5'-CTCGCAGGC TCAGCATTACTTCC-3'; and GAPDH forward, 5'-CAGGAG GCATTGCTGATGAT-3' and reverse, 5'-GAAGGCTGG GGCTCATTT-3'.

Western blotting. Proteins were extracted using RIPA lysis buffer supplemented with PMSF and phosphatase inhibitors and quantified using a BCA assay. Equal amounts of protein (20 μ g per lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat milk in TBST for 1 h at room temperature, the membranes were incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using ECL reagents (Thermo Fisher Scientific, Inc.) and quantified using ImageJ software (version 1.53; National Institutes of Health). GAPDH was used as the loading control for CHCHD2 expression. AKT and ERK pathway activation was evaluated using the p-AKT/total AKT and p-ERK1/2/total ERK1/2 ratios, respectively.

Cell proliferation assay. Cells were plated on 96-well culture plates at a density of 2,000 cells per well in 100- μ l complete medium with three replicate wells per condition. After a 12-h incubation period to ensure cell attachment, 10 μ l of Cell Counting Kit-8 (CCK-8) solution (Corning, Inc.) was added to each well at the indicated time points (24, 48, 72 and 96 h). The plates were then incubated at 37°C for an additional 2 h, after which the absorbance at 450 nm was recorded using a Bio-Rad microplate reader. Cell viability was assessed by constructing growth curves based on OD450 measurements over time.

Wound healing assay. After 24 h, the cells were seeded in 6-well plates to 90-100% confluence. Sterile 200- μ l pipette tips were used to create linear scratches (0.5-1 cm intervals). The wells were then washed three times with PBS, serum-free medium was replenished, and the wells were incubated at 37°C with 5% CO₂. Images of the scratches were captured at 0 and 24 h (Olympus inverted microscope), and closure was quantified via ImageJ software (National Institutes of Health).

Transwell migration assay. Trypsinized cells were centrifuged at 138 x g for 5 min and resuspended at 2-5x10⁴ cells/well. The upper chambers received 100 μ l of the suspension, and the lower chambers received 600 μ l of the complete medium. After 1-2 days, the contents of the upper chambers were removed, non-migratory cells were swabbed, and the membranes were fixed in 4% paraformaldehyde for 30 min, air-dried, stained with 0.1% crystal violet for 20 min, washed three times, and air-dried. The migratory cells were microscopically imaged and counted using ImageJ software (version 1.53; National Institutes of Health).

Flow cytometry for apoptosis. Cells (5x10⁶/sample) were trypsinized, incubated with Annexin V-FITC (20 μ g/ml) at 4°C in the dark for 20 min, resuspended in 400 μ l of PBS, stained with PI (50 μ g/ml, 1 μ l) for 2 min, and analyzed on a CytoFLEX S cytometer (Beckman Coulter, Inc.; 488 nm excitation, FITC at 515 nm, PI >560 nm). Flow cytometry data were analysed using CytExpert software (version 2.4; Beckman Coulter, Inc.). Apoptotic rates were determined by summing the early and late populations.

Xenograft mouse model. Male BALB/c nude mice (4-6 weeks old; 18-22 g) were randomly assigned to the shNC and shCHCHD2-1 groups (n=4 per group; 8 mice in total). The mice were housed under specific pathogen-free conditions at 22±2°C and 50-60% relative humidity under a 12-h light/dark cycle, with *ad libitum* access to food and water. PC9 cells transfected with shCHCHD2-1 or shNC were subcutaneously injected into the mice. Tumor dimensions were measured every 3 days using callipers, and tumor volume was calculated as $V=(\text{length} \times \text{width}^2)/2$. Animal health and behavior were monitored daily, including general condition, activity, food and water intake, and tumor-associated abnormalities. Humane endpoints included marked deterioration in body condition, inability to access food or water, severe impairment of activity, tumor ulceration or necrosis, or excessive tumor burden according to the institutional animal-welfare protocol. Animals meeting these criteria would have been euthanized immediately.

The experimental duration was 30 days. No animals succumbed or required early euthanasia. All mice were euthanized on Day 30 by CO₂ inhalation. CO₂ was gradually introduced from a compressed-gas cylinder into a non-pre-charged chamber at a displacement rate of 30% of the chamber volume per min and maintained for 3 min after respiratory arrest. Death was confirmed by irreversible cessation of respiration and cardiac activity. No anesthetic agent was used for cell inoculation or tumor measurements.

Xenograft tumors were harvested, fixed in 4% paraformaldehyde at room temperature for 24 h, paraffin-embedded, sectioned at a thickness of 4 μ m, and subjected to IHC staining using an anti-CHCHD2 primary antibody. DAB and hematoxylin were used for visualization and counterstaining, respectively, and CHCHD2 staining was quantified by average optical density (AOD) using ImageJ. All animal procedures were approved by the Animal Ethics Committee of Nantong University (approval no. P20250307-034; Nantong, China).

Statistical analysis. Data are presented as the mean ± standard deviation (SD). Comparisons between two groups were performed using paired Student's t-test for matched tumor and

adjacent non-tumorous tissues and unpaired Student's t-test for independent experimental groups, whereas multiple-group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Time-course data were analyzed using two-way ANOVA or repeated-measures two-way ANOVA, as appropriate, followed by Sidak's multiple-comparisons test. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc.; Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference. Unless otherwise stated, *in vitro* experiments were conducted in triplicate and independently repeated at least three times.

Results

Expression levels of CHCHD family genes across cancers. The present analysis revealed distinct expression patterns among the CHCHD family members in the pan-cancer dataset. CHCHD2 was consistently expressed at high levels across a broad range of cancers, whereas CHCHD1, CHCHD3 and CHCHD10 were expressed at moderate levels, and CHCHD4-CHCHD7 were expressed at relatively low levels (Fig. 1A). Further stratification indicated that CHCHD1 was most highly expressed in uterine corpus endometrial carcinoma (UCEC), bladder urothelial carcinoma (BLCA) and cholangiocarcinoma (CHOL). CHCHD2, CHCHD3 and CHCHD5 expression levels peaked in glioblastoma multiforme, whereas the highest expression of CHCHD6 was detected in CHOL (Fig. 1B). Correlation analysis revealed heterogeneity among gene associations: CHCHD5 and CHCHD1 had the strongest positive correlation ($r = 0.44$), whereas CHCHD3 and CHCHD1 had the weakest correlation ($r = -0.01$) (Fig. 1C).

Next, differential expression analysis comparing tumor and normal tissues revealed that CHCHD2 expression was significantly upregulated in multiple cancer types, including BLCA, breast invasive carcinoma (BRCA) and LUAD (Fig. 1D). The expression levels of other members, such as CHCHD1, CHCHD3-CHCHD7 and CHCHD10, were also elevated in most tumor types (Fig. S1A-G).

Genomic alterations of CHCHD family genes across cancers. To characterize the genomic alteration landscape of the CHCHD gene family across cancers, alterations in these genes were analyzed using cBioPortal based on TCGA data. Across 10,967 tumor samples, the average alteration frequency of CHCHD family genes was ~1.5%. Among them, CHCHD2 (2.4%) and CHCHD7 (2.7%) presented relatively high genomic alteration frequencies (Fig. 1I). The 2.4% frequency for CHCHD2 represents its overall genomic alteration frequency, including mutations and copy number alterations, rather than evidence of loss-of-function mutations. Overall, 11% of tumors harbored at least one CHCHD family alteration, with copy-number amplification being the predominant alteration type. Uterine carcinosarcoma had the highest overall alteration frequency (26%), whereas thyroid carcinoma had the lowest (1.5%) (Fig. 1J). Alteration details for individual CHCHD genes are provided in Figs. S2 and S3.

Differential expression of CHCHD2 across cancers. To further investigate CHCHD2, its relationship with pathological stage was examined across cancer types using GEPIA. A positive

correlation between CHCHD2 expression and pathological stage was observed in LUAD (Fig. 1E), liver hepatocellular carcinoma (LIHC) (Fig. 1F), head and neck squamous cell carcinoma (HNSC) (Fig. 1G) and kidney chromophobe (Fig. 1H). At the transcript level, CHCHD2 is consistently overexpressed in numerous cancers. To assess whether this trend extended to the protein level, proteomic data from UALCAN were analyzed. CHCHD2 protein levels were significantly elevated in LIHC, UCEC, BRCA, ovarian serous cystadenocarcinoma, colon adenocarcinoma, lung squamous cell carcinoma and LUAD (Fig. S4A). IHC staining from the HPA further confirmed these findings (Fig. S4B).

Prognostic significance of CHCHD2 expression across cancers. The prognostic significance of CHCHD2 expression across diverse cancer types was evaluated using the KM Plotter database. Increased CHCHD2 expression was significantly correlated with reduced OS in patients with LIHC, HNSC, LUAD, BLCA and ESCA, as evidenced by HRs uniformly exceeding 1 (Fig. 2A-E; $P < 0.05$). By contrast, higher CHCHD2 expression in thymoma was associated with a favorable OS outcome (Fig. 2F, $P < 0.05$). Collectively, these findings suggest that CHCHD2 is overexpressed in diverse malignancies and is associated with an unfavorable prognosis in several tumor types, particularly LUAD. This highlights its potential clinical utility as both a diagnostic biomarker and a therapeutic biomarker. Given the strong prognostic association in LUAD, focus was further addressed on this cancer to investigate CHCHD2-related genes, pathways and cellular functions, aiming to delineate its oncogenic mechanisms in greater detail.

Identification of genes, pathways and cellular functions related to CHCHD2 in LUAD. A co-expression analysis was performed using RNA sequencing data from TCGA and 9,775 genes that were significantly correlated with CHCHD2 expression were identified, including 5,261 negatively correlated genes and 4,514 positively correlated genes. The potential interaction network among the 11 genes most strongly correlated with CHCHD2 is shown in Fig. S5A.

The samples were then stratified into CHCHD2 high- and low-expression groups, revealing 1,150 DEGs, which comprised 156 significantly upregulated genes and 994 significantly downregulated genes. A heatmap was generated to visualize the top 100 DEGs (Fig. S5B).

Functional annotation via GO enrichment analysis revealed that in the BP category there was significant enrichment in terms such as potassium ion transport and potassium ion transmembrane transport (Fig. S5C). In the cellular component category, the enriched terms included collagen-containing extracellular matrix, neuronal cell body, and glutamatergic synapse. In the molecular function category, the most enriched terms were metal ion transmembrane transporter activity, voltage-gated monoatomic cation channel activity, and potassium ion transmembrane transporter activity. KEGG pathway analysis revealed significant enrichment in pathways such as PI3K-Akt signaling, neuroactive ligand-receptor interactions, cAMP signaling and MAPK signaling (Fig. S5D), suggesting that these pathways may be associated with CHCHD2-related transcriptional changes in LUAD. However, these enrichment

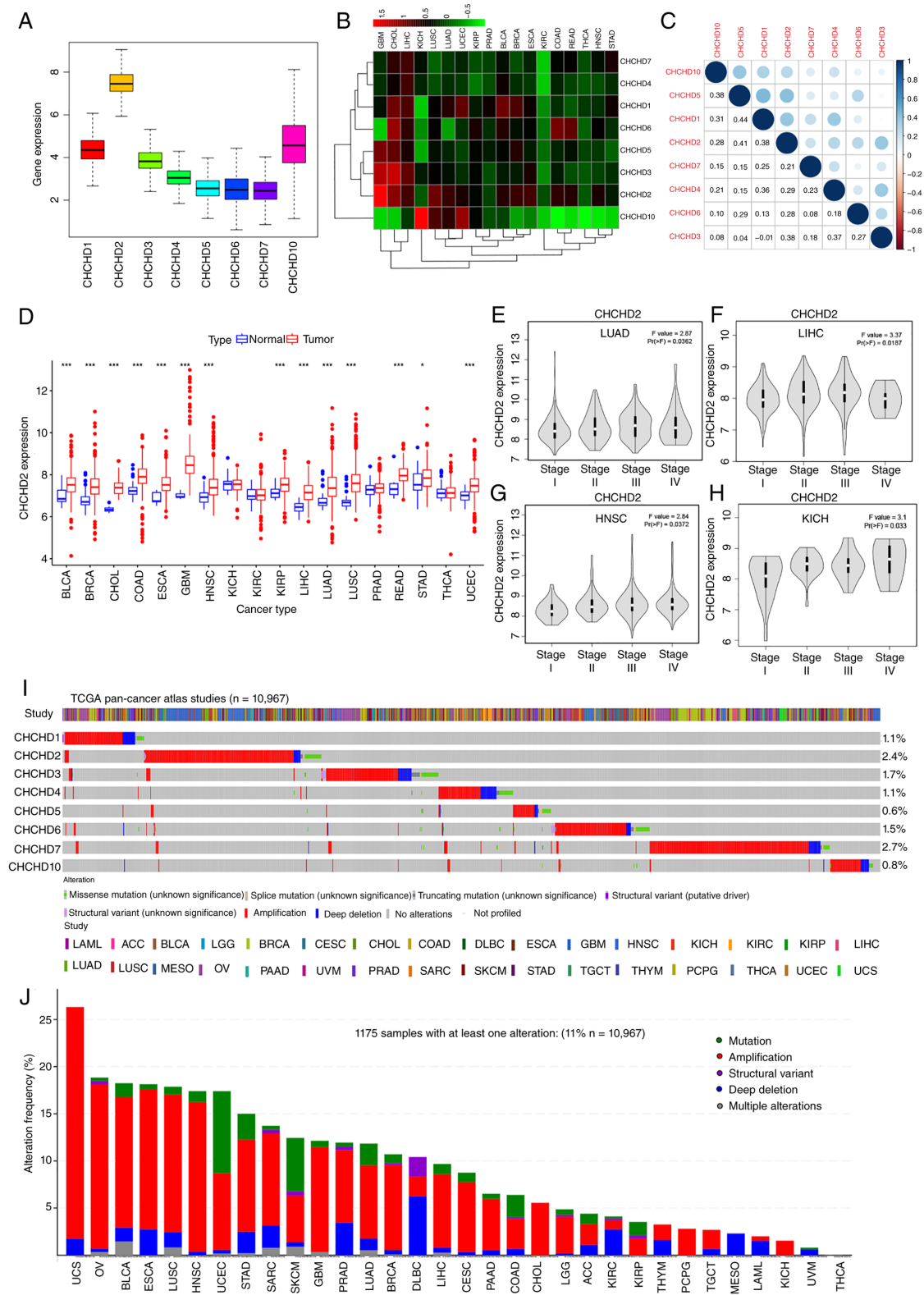


Figure 1. Pan-cancer expression patterns and genetic alteration landscape of the CHCHD family. (A) Boxplots showing the overall mRNA expression distribution of CHCHD family members (CHCHD1-7 and CHCHD10) across TCGA pan-cancer types. (B) Heatmap illustrating the relative expression levels of CHCHD family genes across different cancer types (normalized expression values), with unsupervised hierarchical clustering applied to both genes and tumor types. (C) Pairwise expression correlation matrix of CHCHD family members based on Spearman's correlation analysis; dot size and color indicate the strength and direction of correlations, with numerical values representing correlation coefficients. (D) Differential expression analysis of CHCHD2 between tumor tissues (red) and corresponding normal tissues (blue) across TCGA cancer types. (E-H) Pathological stage-associated expression patterns of CHCHD2 across tumor stages (I-IV), generated using the GEPIA2 'Stage Plot' module and evaluated by one-way ANOVA: (E) LUAD, (F) LIHC, (G) HNSC and (H) KICH. (I) OncoPrint summarizing the spectrum and frequency of genetic alterations in CHCHD family genes across 10,967 TCGA tumors using cBioPortal. (J) Cancer-type-specific summary of overall CHCHD family alteration frequencies, indicating the proportion of tumors harboring at least one CHCHD-family alteration; copy-number amplification represents the predominant alteration event. *P<0.05 and ***P<0.001. CHCHD, coiled-coil-helix-coiled-coil-helix domain; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; LIHC, liver hepatocellular carcinoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe.

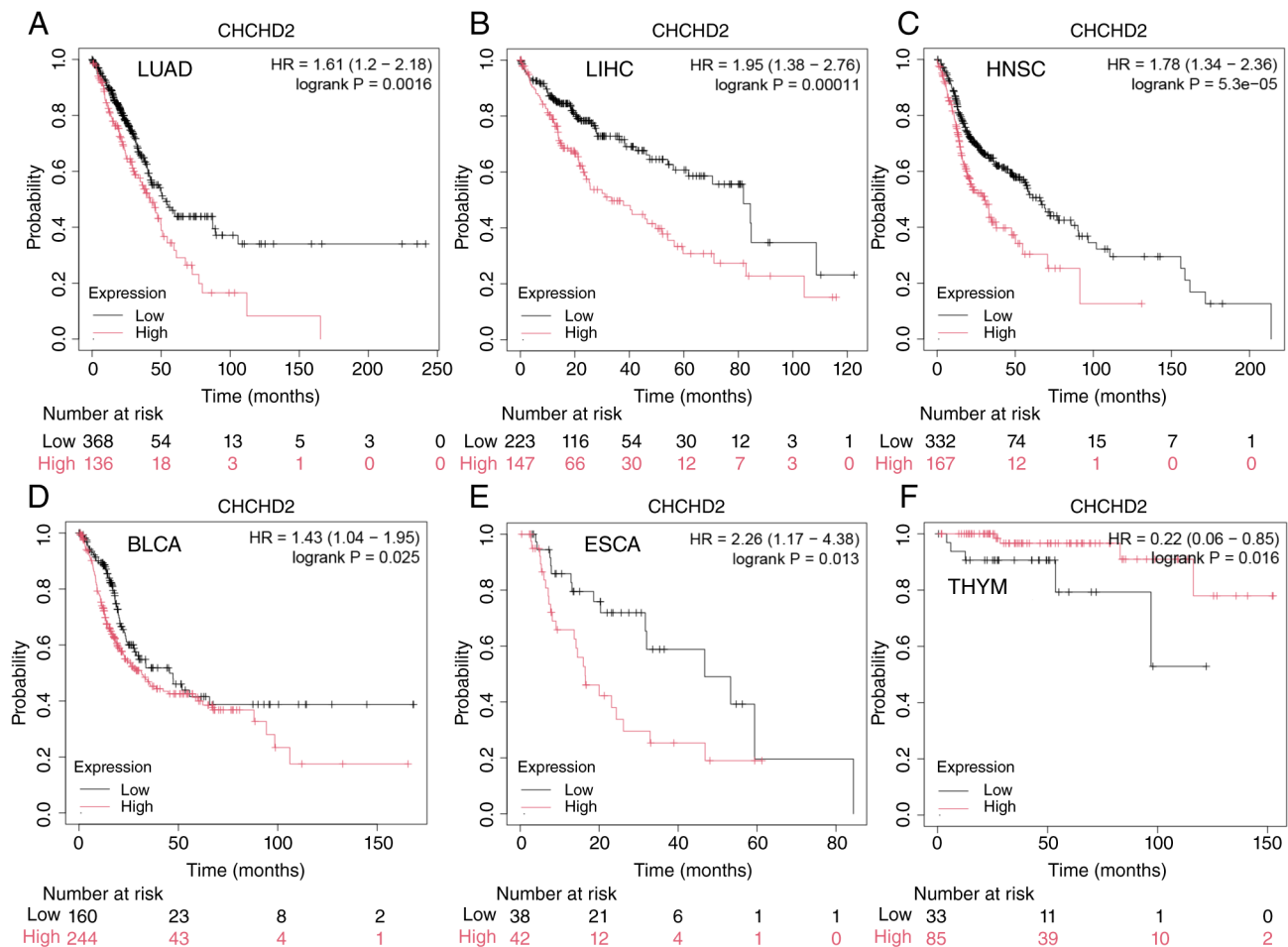


Figure 2. Association between CHCHD2 expression and OS across multiple cancers (Kaplan-Meier Plotter). (A) In LUAD, patients with high CHCHD2 expression (red) exhibited poorer OS than those with low expression (black). (B) In LIHC, patients with high CHCHD2 expression (red) exhibited poorer OS than those with low expression (black). (C) In HNSC, patients with high CHCHD2 expression (red) exhibited poorer OS than those with low expression (black). (D) In BLCA, patients with high CHCHD2 expression (red) exhibited poorer OS than those with low expression (black). (E) In ESCA, patients with high CHCHD2 expression (red) exhibited poorer OS than those with low expression (black). (F) In THYM, high CHCHD2 expression was associated with improved OS. CHCHD, coiled-coil-helix-coiled-coil-helix domain; OS, overall survival; LUAD, lung adenocarcinoma; LIHC, liver hepatocellular carcinoma; HNSC, head and neck squamous cell carcinoma; BLCA, bladder urothelial carcinoma; ESCA, esophageal carcinoma; THYM, thymoma; HR, hazard ratio.

results should be interpreted as hypothesis-generating rather than direct mechanistic evidence. These pathway associations were subsequently evaluated experimentally by assessing AKT and ERK phosphorylation following CHCHD2 knockdown.

Correlations between CHCHD2 expression and TME/stemness scores in LUAD. Although the role of CHCHD2 in tumor immunity has not been extensively studied, mitochondria are increasingly recognized as key regulators of the TME and play important roles in tumor progression, immune evasion and therapeutic responses (30). To further elucidate the potential association between CHCHD2 expression levels and TME characteristics, as well as stemness scores, Spearman's rank correlation coefficient was used for nonparametric correlation analysis. The results revealed a significant positive correlation between CHCHD2 expression and both the RNAss ($R=0.37$, $P<0.001$) and the DNAss ($R=0.13$, $P<0.001$) (Fig. S6A), suggesting that CHCHD2 may play a role in maintaining or enhancing the stemness phenotype. On the other hand, CHCHD2 expression was negatively correlated with several TME-related scoring metrics, including the stromal score

($R=-0.19$, $P<0.001$), immune score ($R=-0.17$, $P<0.001$) and the composite ESTIMATE score ($R=-0.19$, $P<0.001$) (Fig. S6A), indicating that increased CHCHD2 expression may be associated with reduced immune infiltration and stromal content, further suggesting a potential immunosuppressive role within the TME.

Association between CHCHD2 expression and immune subtype in LUAD. To evaluate the differential expression of CHCHD2 across various immune subtypes, the Kruskal-Wallis H test was applied to LUAD samples, and a non-parametric comparison of CHCHD2 expression among six predefined immune subtypes was conducted (that is, C1, wound healing; C2, IFN- γ dominant; C3, inflammatory; C4, lymphocyte-depleted; C5, immunologically quiet; and C6, TGF- β dominant) (13,25). The statistical results indicated that CHCHD2 expression was relatively higher in the C1 subtype, whereas the lowest expression was observed in the C3 subtype (Fig. S6B), suggesting that CHCHD2 may perform distinct regulatory functions in different immune-inflammatory microenvironmental contexts.

Correlations between CHCHD2 expression and immune cell infiltration in LUAD. The relationship between CHCHD2 expression and 50 immune checkpoint-related genes was first analyzed to assess its potential association with immune checkpoint inhibitors. The results revealed that the expression of CHCHD2 was significantly correlated with that of 10 immune checkpoint-related genes ($P < 0.001$). Specifically, CHCHD2 expression was negatively correlated with CD40LG, TNFSF15, TNFSF14, CD28, BTLA, ADORA2A, CD160, TIGIT and NRP1 expression but positively correlated with TNFSF18 expression (Fig. S6C). Given the observed correlations with immune checkpoint-related genes, it was next evaluated whether CHCHD2 expression was also associated with differences in tumor-infiltrating immune cell composition.

To further investigate the association between CHCHD2 and the tumor immune microenvironment, the CIBERSORT algorithm was used to evaluate immune cell subpopulations in LUAD samples with different CHCHD2 expression levels. A comparison of the high- and low-expression groups revealed that CHCHD2 expression was positively correlated with the numbers of follicular helper T cells, activated CD4 memory T cells and gamma delta T cells but negatively correlated with the numbers of resting CD4 memory T cells and resting mast cells (Fig. S6D and E).

Single-cell analysis of CHCHD2 expression in an LUAD-derived dataset. Single-cell RNA sequencing analysis was performed using multiple TISCH2 datasets, with NSCLC_GSE131907 as the primary LUAD-derived validation cohort. Although categorized under NSCLC in TISCH2, NSCLC_GSE131907 was derived from patients with pathologically diagnosed LUAD. In this dataset, CHCHD2 expression was detectable across multiple cellular compartments, including epithelial cells, T cells, B cells, fibroblasts and plasma cells (Fig. S7A-D). Other NSCLC datasets, including NSCLC_GSE117570, NSCLC_GSE127465, and NSCLC_GSE99254, were used only as supportive references and were not interpreted as LUAD-specific evidence.

Drug sensitivity analysis of CHCHD2. To investigate the potential association between CHCHD2 expression and drug sensitivity in human cancer cell lines, correlation analysis was performed using the CellMiner™ database. The results (Fig. S8A-P) revealed a significant negative correlation between CHCHD2 expression and sensitivity to Irofulven. By contrast, CHCHD2 expression was positively correlated with sensitivity to multiple agents, including uracil mustard, dexamethasone (Decadron), asparaginase, acrichine, fluphenazine, vorinostat, PX-316, chelerythrine, nelarabine, imexon, fenretinide, hydroxyurea, carmustine, lomustine and ifosfamide ($P < 0.05$).

Experimental validation of CHCHD2 upregulation in LUAD tissues and cell lines. Based on these multi-omics and database-derived findings, LUAD-specific experimental validation was next performed at the tissue, cellular and animal levels. Tumor tissues and matched adjacent normal tissues from nine patients with stage II LUAD were used to assess CHCHD2 expression levels. Western blotting and RT-qPCR results revealed that the protein and mRNA levels

of CHCHD2 were significantly higher in LUAD tissues than in adjacent normal tissues (Fig. 3A and B), which is consistent with the findings of the aforementioned bioinformatics analysis. A total of five LUAD cell lines were selected to examine CHCHD2 expression levels, and the results revealed that the protein and mRNA levels of CHCHD2 were markedly higher in PC9 and H1975 cells than in H1299 and H1650 cells (Fig. 3C and D).

CHCHD2 knockdown suppresses malignant phenotypes in PC9 and H1975 cells. Two LUAD cell lines with high CHCHD2 expression, PC9 and H1975, were chosen to establish transient knockdown models using shRNA. Western blotting and RT-qPCR analyses showed that all three CHCHD2-targeting shRNAs reduced CHCHD2 expression, with shCHCHD2-1 producing the strongest and most consistent knockdown across both cell lines (Fig. 4A-D). Direct comparisons showed significantly greater protein suppression by shCHCHD2-1 than by shCHCHD2-2 or shCHCHD2-3 in both PC9 and H1975 cells. At the mRNA level, shCHCHD2-1 also demonstrated greater knockdown in PC9 cells and was significantly more effective than shCHCHD2-2 in H1975 cells, whereas the difference from shCHCHD2-3 in H1975 cells was not significant. Therefore, shCHCHD2-1 was selected for subsequent functional assays.

CCK-8 assays revealed that CHCHD2 knockdown significantly reduced cell proliferation in PC9 and H1975 cells (Fig. 4E and F). Flow cytometry revealed significantly increased apoptosis following CHCHD2 knockdown (Fig. 4G). Transwell and wound-healing assays further demonstrated impaired migration in both cell lines (Fig. 4H-J).

CHCHD2 overexpression enhances malignant phenotypes in H1299 and H1650 cells. CHCHD2-overexpressing cell models were established in H1299 and H1650 cells, which exhibit low endogenous CHCHD2 expression. Western blotting and RT-qPCR analyses confirmed a significant increase in CHCHD2 expression levels in the overexpression group (Fig. 5A and B). CCK-8 assays revealed that CHCHD2 overexpression significantly increased the proliferation rates of H1299 and H1650 cells (Fig. 5C and D). Flow cytometric analysis revealed that CHCHD2 overexpression significantly reduced both early and late apoptosis in H1299 and H1650 cells (Fig. 5E). Wound healing and Transwell assays revealed that CHCHD2 overexpression significantly promoted cell migration (Fig. 5F-H). These results indicate that both knockdown and overexpression of CHCHD2 can alter LUAD cell proliferation, migration and apoptosis, suggesting a potential oncogenic role for CHCHD2 in LUAD.

CHCHD2 knockdown reduces AKT/ERK phosphorylation and suppresses LUAD xenograft tumor growth in vivo. To further evaluate the signaling pathways suggested by the KEGG enrichment analysis, AKT and ERK phosphorylation were examined following CHCHD2 knockdown. Western blot analysis showed that CHCHD2 knockdown reduced the levels of phosphorylated AKT and ERK in both PC9 and H1975 cells, as reflected by decreased p-AKT/t-AKT and p-ERK/t-ERK levels (Fig. 6A and B). These findings provide experimental support for the involvement of PI3K-AKT and MAPK signaling in CHCHD2-associated LUAD phenotypes.

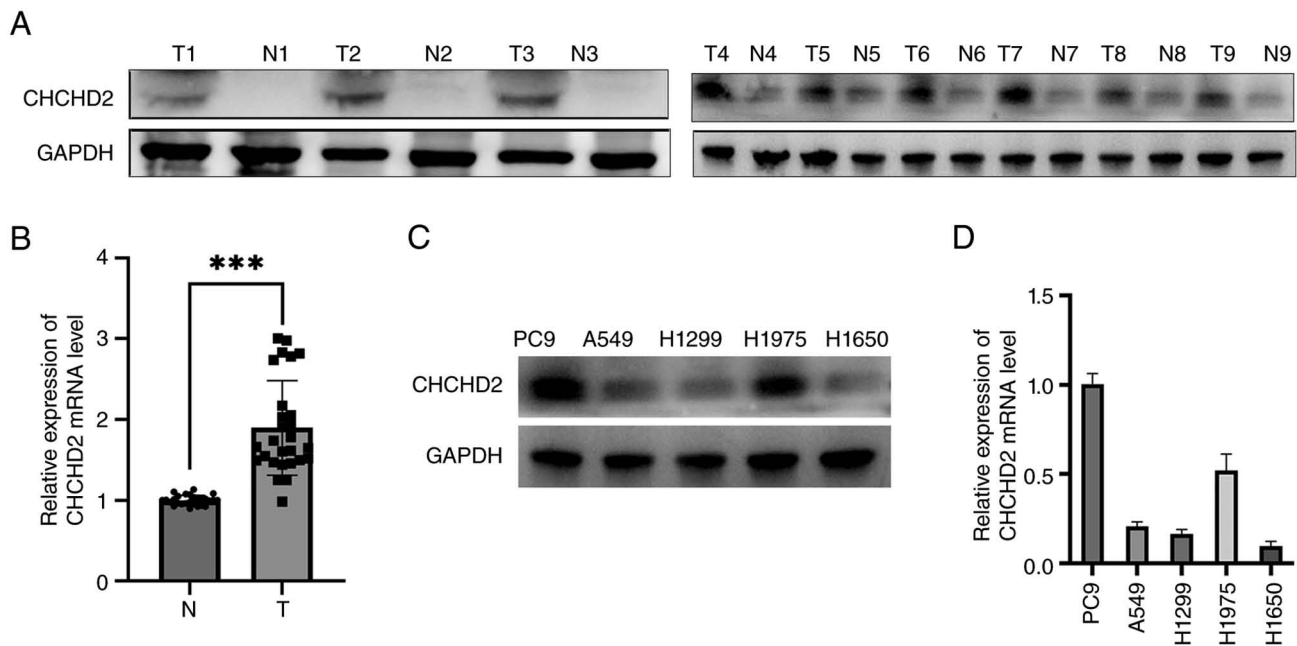


Figure 3. CHCHD2 is upregulated in LUAD tissues and differentially expressed across LUAD cell lines. (A) Western blot analysis of CHCHD2 protein expression in nine paired LUAD tumor tissues (T1-T9) and adjacent non-tumorous tissues (N1-N9). GAPDH was used as the loading control. (B) RT-qPCR analysis of CHCHD2 mRNA expression in paired LUAD tumor tissues (T) and adjacent non-tumorous tissues (N). (C) Western blot analysis of CHCHD2 protein expression in LUAD cell lines, including PC9, A549, H1299, H1975 and H1650. (D) RT-qPCR analysis of relative CHCHD2 mRNA expression in the indicated LUAD cell lines. Data are presented as the mean $\pm$ SD. *** $P < 0.001$. CHCHD, coiled-coil-helix-coiled-coil-helix domain; LUAD, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR.

To further evaluate the effect of CHCHD2 knockdown *in vivo*, PC9-shCHCHD2-1 and PC9-shNC cells were subcutaneously injected into nude mice. Tumor growth was monitored every 3 days. At Day 30, xenograft tumors were excised, and representative tumor images are shown in Fig. 6C. Tumor growth curves demonstrated that CHCHD2 knockdown significantly reduced xenograft tumor volume compared with the shNC group (Fig. 6D). IHC staining further showed reduced CHCHD2 expression in xenograft tumors from the shCHCHD2-1 group (Fig. 6E), which was confirmed by AOD quantification (Fig. 6F). These findings indicate that CHCHD2 knockdown suppresses LUAD tumor growth *in vivo* and are consistent with the inhibitory effects observed *in vitro*.

Discussion

The present study aimed to delineate the expression landscape and prognostic relevance of the CHCHD family across cancers, with an emphasis on the biological role and clinical significance of CHCHD2 in LUAD. It was revealed that CHCHD2 is broadly upregulated across tumor types and is associated with adverse outcomes; in LUAD, high expression of CHCHD2 is correlated with advanced disease stage and poor survival, and it functionally enhances proliferation and migration, suppresses apoptosis, and accelerates xenograft growth. Multilayer analyses of the TME, stemness features, immune subtypes and drug sensitivity support an oncogenic role for CHCHD2, highlighting its potential as a biomarker candidate and motivating further mechanistic investigation. Importantly, because CHCHD2 has previously been reported to be coamplified with EGFR and to regulate mitochondrial function and migration in NSCLC,

the present findings should be interpreted as integrative and supportive evidence that extends prior observations to LUAD-specific multi-omics and experimental contexts rather than as definitive evidence of a completely new oncogenic mechanism.

Integrating bioinformatics and experimental evidence, the present findings indicate that the expression of CHCHD2, a biomarker linked to prognosis and disease stage, may facilitate risk stratification and patient management in LUAD. Pathway enrichment analyses in LUAD revealed associations with PI3K-AKT and MAPK signaling-related gene sets. Consistent with these bioinformatic findings, additional western blot analyses showed that CHCHD2 knockdown reduced AKT and ERK phosphorylation in both PC9 and H1975 cells, providing experimental support for the involvement of PI3K-AKT/MAPK signaling in CHCHD2-associated LUAD phenotypes. Nevertheless, these findings do not establish that CHCHD2 directly regulates these signaling cascades, and the precise molecular mechanisms linking CHCHD2 to AKT and ERK activation remain to be elucidated. Prior studies have reported that CHCHD2 interacts with Bcl-XL and inhibits BAX oligomerization, thereby attenuating mitochondrial apoptosis (31), which is consistent with the observation of attenuated apoptotic programs. Moreover, CHCHD2 is located on chromosome 7p11.2 and is frequently coamplified with EGFR in NSCLC, where it promotes mitochondrial respiration and migration (11).

Positive correlations were observed between CHCHD2 and tumor stemness indices (RNass and DNass), coupled with lower ESTIMATE, immune and stromal scores. CHCHD2 expression was higher in the 'wound-healing' (C1) immune subtype and lower in the 'inflammatory' (C3)

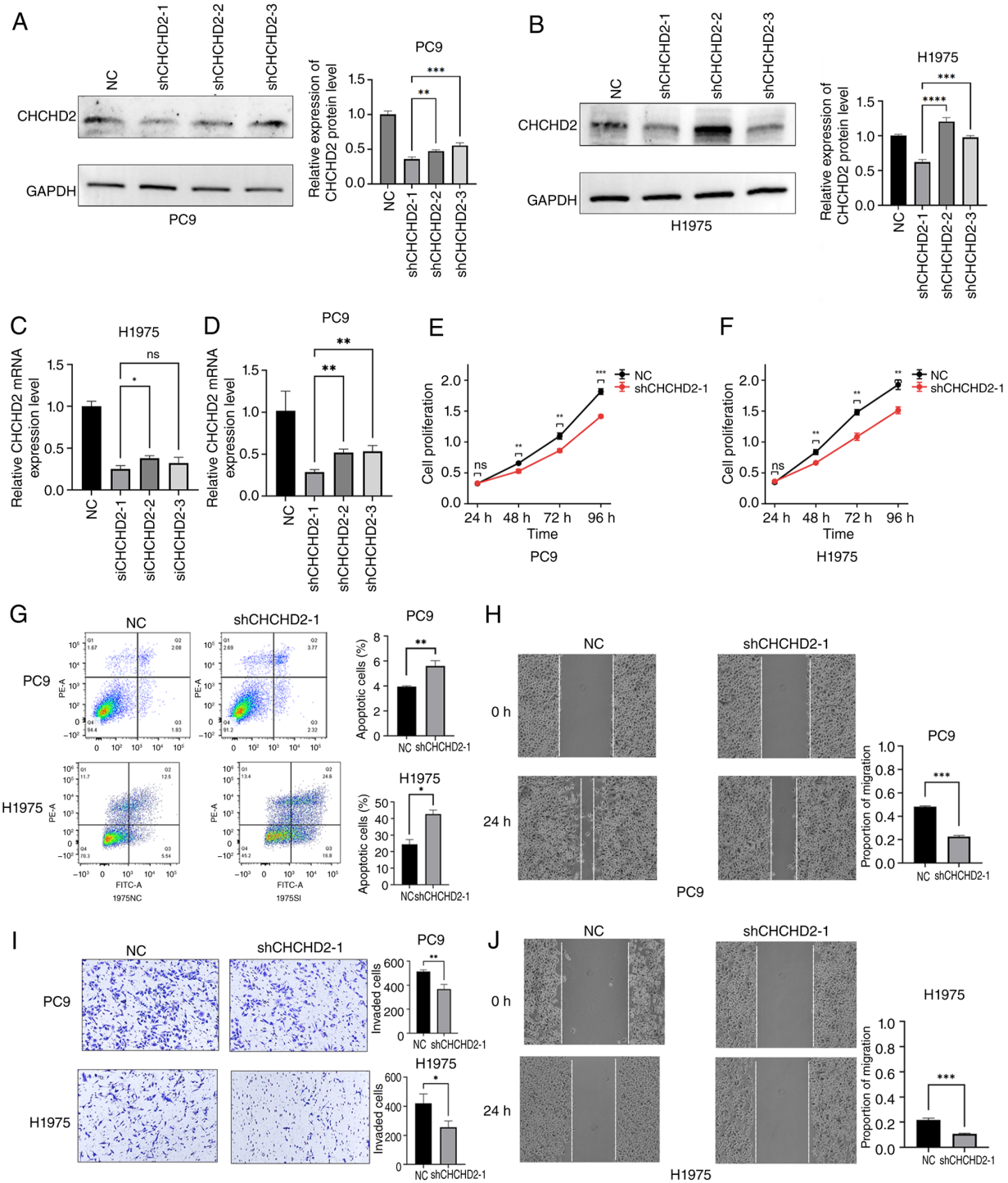


Figure 4. CHCHD2 knockdown suppresses proliferation and migration and promotes apoptosis in PC9 and H1975 cells. (A and B) Western blot analyses of CHCHD2 knockdown efficiency in PC9 (A) and H1975 (B) cells transfected with shCHCHD2-1, shCHCHD2-2, shCHCHD2-3, or negative-control shRNA (shNC), with corresponding densitometric quantification of CHCHD2 protein expression. (C and D) Reverse transcription-quantitative PCR analyses of CHCHD2 mRNA expression following transfection with shNC or the three CHCHD2-targeting shRNAs in H1975 (C) and PC9 (D) cells. Direct statistical comparisons among the three shRNAs were performed to evaluate knockdown efficiency. (E and F) CCK-8 assays showing that CHCHD2 knockdown reduced cell proliferation in PC9 (E) and H1975 (F) cells. (G) Flow cytometric analysis showing increased apoptosis following CHCHD2 knockdown in PC9 and H1975 cells. (H) Wound-healing assay showing reduced migration after CHCHD2 knockdown in PC9 cells. (I) Transwell migration assays showing reduced migratory ability after CHCHD2 knockdown in PC9 and H1975 cells. (J) Wound-healing assay showing reduced migration after CHCHD2 knockdown in H1975 cells. White dashed lines indicate the wound edges used for wound-width measurement. For the CCK-8 time-course data, statistical significance was assessed using two-way ANOVA followed by Sidak's multiple-comparisons test. Data are presented as the mean $\pm$ SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CHCHD, coiled-coil-helix-coiled-coil-helix domain; sh-, short hairpin; NC, negative control; CCK-8, Cell Counting Kit-8; ns, not significant.

subtype; CIBERSORT deconvolution linked CHCHD2 to the enrichment of follicular helper and activated memory CD4⁺ T cells, with depletion of resting immune compartments.

This pattern points to a TME skewed towards proliferation and matrix remodeling with dampened inflammatory tonicity, an arrangement aligned with the prognostic and

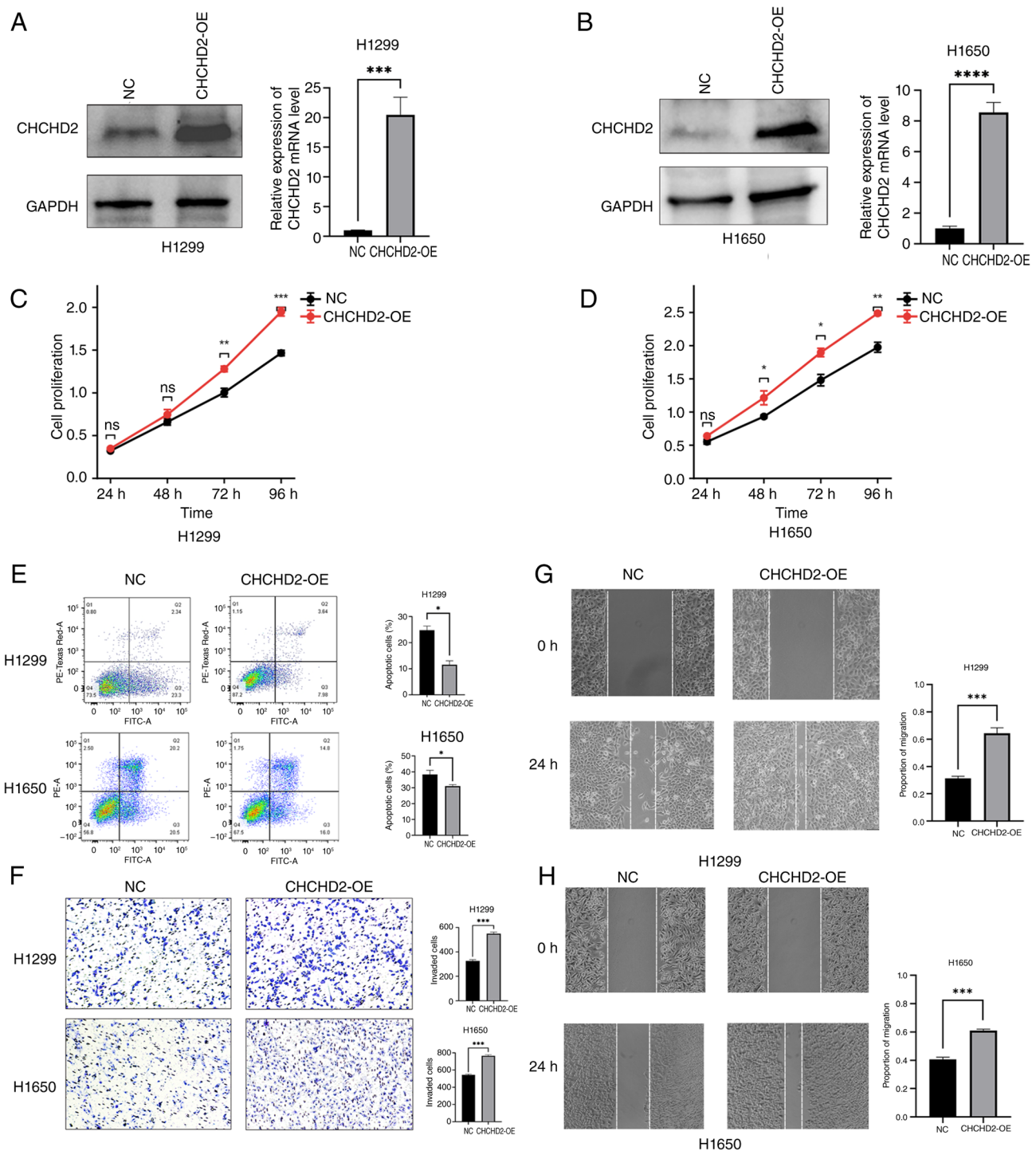


Figure 5. CHCHD2 overexpression promotes proliferation and migration and inhibits apoptosis in H1299 and H1650 cells. (A) Western blot and RT-qPCR analyses confirming CHCHD2 overexpression efficiency in H1299 cells transfected with CHCHD2-overexpression plasmid (CHCHD2-OE) or NC plasmid. (B) Western blot and RT-qPCR analyses confirming CHCHD2 overexpression efficiency in H1650 cells transfected with CHCHD2-OE or NC. (C and D) CCK-8 assays showing that CHCHD2 overexpression enhanced cell proliferation in H1299 (C) and H1650 (D) cells. (E) Flow cytometric analysis showing decreased apoptosis after CHCHD2 overexpression in H1299 and H1650 cells. (F) Transwell migration assays showing increased migratory ability after CHCHD2 overexpression in H1299 and H1650 cells. (G and H) Wound-healing assays showing enhanced migration after CHCHD2 overexpression in H1299 (G) and H1650 (H) cells. White dashed lines indicate the wound edges used for wound-width measurement. For the CCK-8 time-course data, statistical significance was assessed using two-way ANOVA followed by Sidak's multiple-comparisons test. Data are presented as the mean $\pm$ SD. ns, not significant; * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. CHCHD, coiled-coil-helix-coiled-coil-helix domain; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; CCK-8, Cell Counting Kit-8.

inflammatory contrasts between C1 and C3 reported by Thorsson *et al* (25).

Drug sensitivity analysis revealed that high CHCHD2 expression was negatively correlated with sensitivity to

the DNA-damaging agent Irofulven, suggesting a potential role for CHCHD2 in modulating cellular responses to this compound. By contrast, high CHCHD2 expression was positively correlated with sensitivity to a range of other

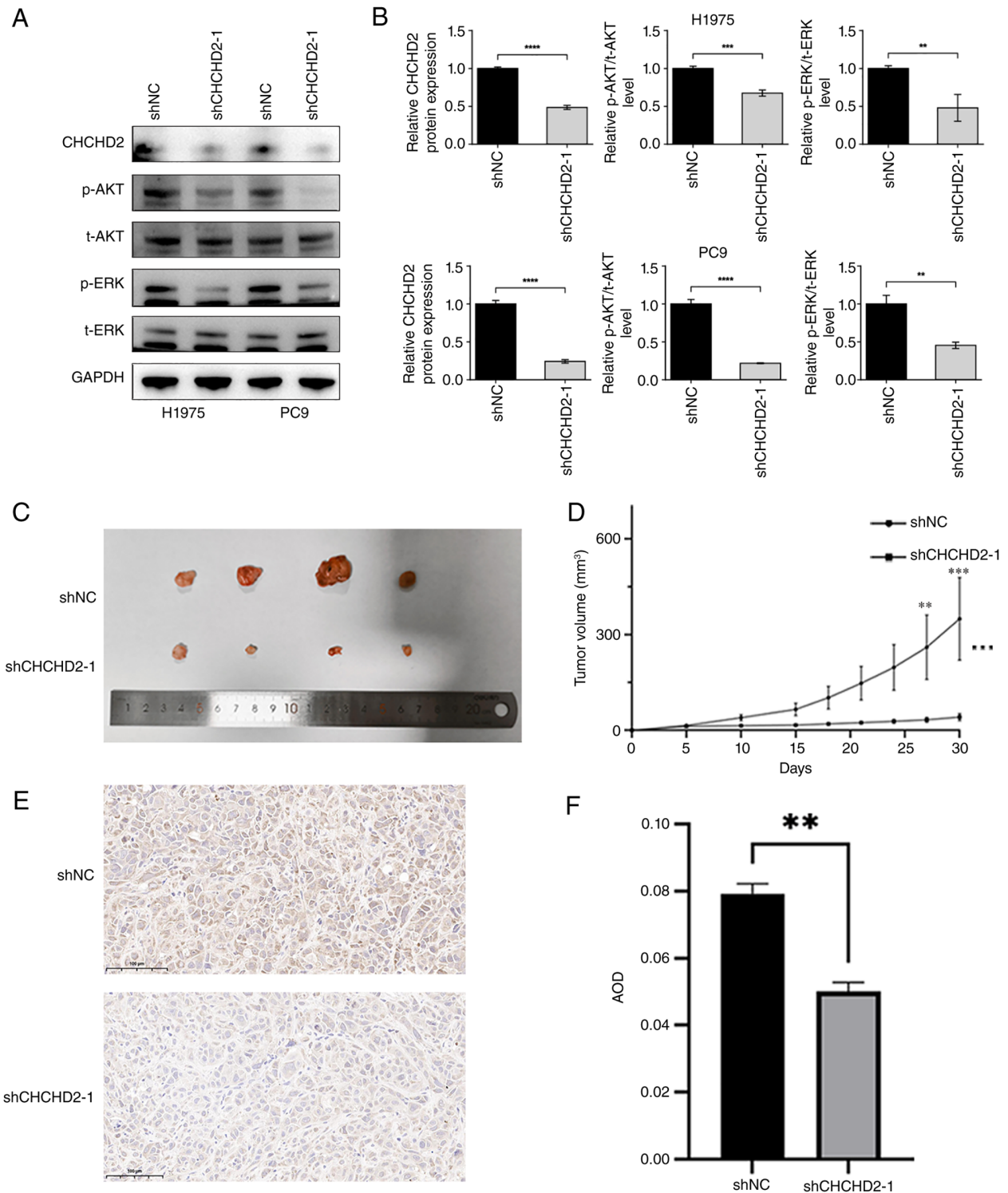


Figure 6. CHCHD2 knockdown inhibits AKT/ERK phosphorylation and suppresses lung adenocarcinoma xenograft tumor growth. (A) Representative western blot analysis of CHCHD2, p-AKT, t-AKT, p-ERK and t-ERK in H1975 and PC9 cells transfected with shNC or shCHCHD2-1. GAPDH was used as the loading control. (B) Densitometric quantification of CHCHD2 expression and the p-AKT/t-AKT and p-ERK/t-ERK ratios in H1975 and PC9 cells. (C) Representative images of excised xenograft tumors from the shNC and shCHCHD2-1 groups. (D) Tumor growth curves showing xenograft tumor volume changes over time in the shNC and shCHCHD2-1 groups. Tumor growth curves were analyzed using repeated-measures two-way ANOVA followed by Sidak's multiple-comparisons test. (E) Representative IHC images of CHCHD2 staining in xenograft tumor tissues from the shNC and shCHCHD2-1 groups. Scale bar, 100 μ m. (F) Quantification of CHCHD2 IHC staining by average optical density, showing decreased CHCHD2 expression in the shCHCHD2-1 group. shNC, negative-control shRNA; shCHCHD2-1, CHCHD2-targeting shRNA-1. Data are presented as the mean $\pm$ SD. ** P <0.01, *** P <0.001 and **** P <0.0001. CHCHD, coiled-coil-helix-coiled-coil-helix domain; p-, phosphorylated; t-, total; sh-, short hairpin; NC, negative control; IHC, immunohistochemical.

therapeutic agents, including alkylating agents (such as uracil mustard, lomustine and carmustine), metabolic inhibitors (such as asparaginase, hydroxyurea and fenretinide),

and epigenetic regulators (such as the HDAC inhibitor vorinostat). These preliminary findings suggest that rather than reflecting a uniform response to DNA-damaging therapies,

CHCHD2 expression may have drug-specific predictive value. Notably, elevated CHCHD2 levels could predict reduced sensitivity to Irofulven while being associated with increased sensitivity to agents with distinct mechanisms of action. Notably, these observations are derived from the CellMiner database (28) and warrant further validation in LUAD-specific pharmacogenomic models and clinical cohorts.

Multiple cancer-specific studies have linked CHCHD2 to oncogenic phenotypes, such as co-amplification with EGFR and promotion of migration/respiration in NSCLC (11), upregulation in hepatocellular carcinoma with angiogenic signaling and poor prognosis (32), and reduced docetaxel sensitivity via MMP2 activation in breast cancer (33). Against this backdrop, the present study provides several layers of additional evidence. First, it places CHCHD2 within a broader pan-cancer framework by integrating expression, survival, genomic alteration, pathway enrichment, TME, stemness, single-cell and drug-response analyses. Second, it focuses on LUAD and combines public multi-omics evidence with clinical tissue validation, *in vitro* gain- and loss-of-function assays, and an *in vivo* xenograft model. Third, it reveals potential associations between CHCHD2 expression and immune/stemness-related features, thereby generating testable hypotheses for future mechanistic studies. Nevertheless, the present work should be regarded as an integrative and functional validation study rather than a complete mechanistic dissection of CHCHD2 downstream signaling. Further studies are needed to define the direct molecular interactors and signaling cascades regulated by CHCHD2 in LUAD. Because functional assays for individual CHCHD2 variants were not performed, the biological consequences of CHCHD2 genomic alterations require further validation.

The limitations of the present study include the reliance on TCGA data, which may lead to an underestimation of the representativeness of diverse ethnicities and patients with advanced disease stage, thereby affecting the generalizability of pan-cancer inferences; however, the integration of GTEx and CPTAC data partially mitigates the deficiencies in normal tissue comparisons. The experimental models primarily employed cell lines and xenograft mice, which do not fully capture patient heterogeneity and *in situ* TME dynamics, although single-cell and *in vivo* data compensate for this limitation to some extent. Although the present western blot findings provide experimental support for the involvement of PI3K-AKT/MAPK signaling, pathway-rescue experiments and studies of direct molecular interactions are still required to establish causality and clarify the precise molecular mechanisms by which CHCHD2 modulates these signaling pathways. In addition, despite expansion of the validation cohort to nine paired LUAD specimens, the sample size remains relatively small and is derived from a single center, which may limit statistical power and generalizability. Validation in larger, independent clinical cohorts is therefore warranted. These shortcomings do not undermine the core conclusions but indicate the need for further validation of its translational potential in syngeneic models and prospective studies.

In conclusion, the results of the present study support the role of CHCHD2 as a potential oncogenic regulator in LUAD. Pan-cancer and LUAD-focused analyses linked CHCHD2 to adverse prognosis, genomic alterations, TME features, stemness-related indices, single-cell expression patterns, and drug-response correlations. Functional validation further revealed that CHCHD2 promotes LUAD cell proliferation and migration, inhibits apoptosis, and supports xenograft tumor growth, while CHCHD2 knockdown reduced AKT and ERK phosphorylation. Future studies should clarify the direct molecular mechanisms linking CHCHD2 to PI3K-AKT/MAPK signaling and evaluate its therapeutic relevance in LUAD models.

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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

ZKX and ZY conceived the study, performed the data curation, formal analysis, investigation, methodology, software analysis, validation, and visualization, and wrote the original draft of the manuscript. JS, YZL, HTS and CZ contributed to methodology and validation. ZWZ and XYZ supervised the study and contributed to conceptualization, methodology, project administration, resources, validation, and manuscript review and editing. XYZ also acquired funding. ZKX and ZY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The human study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (approval no. 2023-K010-01; Nantong, China). Written informed consent was obtained from all participants. All animal procedures were approved by the Animal Ethics Committee of Nantong University (approval no. P20250307-034; Nantong, China). All experiments were conducted in accordance with relevant institutional guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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