Received: 29 October 2023 | Accepted: 19 January 2024

DOI: 10.1111/1759-7714.15234

ORIGINAL ARTICLE

WILEY

FHL2 promotes the aggressiveness of lung adenocarcinoma by inhibiting autophagy via activation of the PI3K/AKT/mTOR pathway

Shuaishuai Wang[1] | **Baomo Liu**[1] | **Yan Su**[1] | **Nian Wang**[2] | **Peixin Dong**[1] | **Xiongye Xu**[1] | **Lixia Huang**[1] | **Shaoli Li**[1] | **Jincui Gu**[1] | **Yanli Qiu**[1] | **Jiating Deng**[1] | **Ziying Lin**[1] | **Yanbin Zhou**[1]

[1]Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

[2]Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China

Correspondence
Ziying Lin and Yanbin Zhou, Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital of Sun Yat-Sen University, No.58, Zhongshan 2nd Road, Guangzhou, 510080, China.
Email: lzying@mail2.sysu.edu.cn and zhouyb@mail.sysu.edu.cn

Funding information
Natural Science Foundation of Guangdong Province, China, Grant/Award Number: 2021A1515010480; Science and Technology Program of Guangzhou, China, Grant/Award Number: 202201010951

Abstract

Background: Increasing evidence indicates that four and a half LIM domains 2 (FHL2) plays a crucial role in the progression of various cancers. However, the biological functions and molecular mechanism of FHL2 in lung adenocarcinoma (LUAD) remain unclear.

Methods: We evaluated the prognostic value of FHL2 in LUAD using public datasets and further confirmed its prognostic value with our clinical data. The biological functions of FHL2 in LUAD were evaluated by in vitro and in vivo experiments. Pathway analysis and rescue experiments were subsequently performed to explore the molecular mechanism by which FHL2 promoted the progression of LUAD.

Results: FHL2 was upregulated in LUAD tissues compared to adjacent normal lung tissues, and FHL2 overexpression was correlated with unfavorable outcomes in patients with LUAD. FHL2 knockdown significantly suppressed the proliferation, migration and invasion of LUAD cells, while FHL2 overexpression had the opposite effect. Mechanistically, FHL2 upregulated the PI3K/AKT/mTOR pathway and subsequently inhibited autophagy in LUAD cells. The effects FHL2 on the proliferation, migration and invasion of LUAD cells are dependent on the inhibition of autophagy, as of induction autophagy attenuated the aggressive phenotype induced by FHL2 overexpression.

Conclusions: FHL2 promotes the progression of LUAD by activating the PI3K/AKT/mTOR pathway and subsequently inhibiting autophagy, which can be exploited as a potential therapeutic target for LUAD.

KEYWORDS
autophagy, FHL2, lung adenocarcinoma, PI3K/AKT/mTOR pathway

INTRODUCTION

Lung cancer is one of the most common malignant tumors and the leading cause of cancer-related death worldwide.[1] Lung adenocarcinoma (LUAD) is the most common pathological type of lung cancer, accounting for 40% of all cases.[2] Unfortunately, most patients are diagnosed at an advanced stage when radical resection is unavailable and the only options left are palliative treatment such as chemotherapy and radiotherapy.[3] In recent decades, the development of targeted therapy and immunotherapy has revolutionized the treatment of LUAD. However, the prognosis of patients with advanced LUAD remains unsatisfactory as therapeutic response varies among different patients and the inevitable development of resistance to currently available treatments.[4,5] According to

Shuaishuai Wang and Baomo Liu contributed equally to this work and share first authorship.

recent reports, the 5-year survival rate of patients with advanced LUAD is only 15%,[6,7] which highlights the need for more effective therapeutic strategies against this deadly disease. Therefore, it is crucial to obtain a more in-depth understanding of the mechanism underlying the aggressiveness of LUAD and identify additional potential therapeutic targets.

Four and a half LIM domains 2 (FHL2) is an important member of the LIM-only protein family.[8] LIM domains are double zinc finger motifs that provide protein–protein binding interfaces,[9] which enable FHL2 to interact with a variety of proteins and play crucial roles in regulating signal transduction, gene expression and cell growth.[10] FHL2 expression is dysregulated in various cancers, but its impact on cancer remains controversial; it can function as either an oncogene or a tumor suppressor depending on the tumor subtype.[11–14] For instance, FHL2 has been found to be upregulated in pancreatic cancer and to promote cell proliferation through the MEK/ERK signaling pathway.[11] It has also been reported that increased expression of FHL2 is associated with a poor prognosis in patients with ovarian cancer and can promote tumorigenesis via the Wnt/β-catenin pathway.[12] In contrast, FHL2 has also been found to be downregulated in hepatocellular carcinoma and to suppress liver cancer cell growth by regulating the expression of cyclin D1 and p21.[15] FHL2 expression has also been found to be significantly decreased in breast cancer, where FHL2 functions as a tumor suppressor through its interaction with Smad4, subsequently decreasing the transcriptional activity of Era and suppressing the development of breast cancer.[16,17] In summary, FHL2 is a multifunctional protein involved in the regulation of various molecular pathways that either promotes tumor progression or suppresses tumorigenesis, and it exerts its biological functions in a context-depending manner.[10] However, studies investigating the biological role of FHL2 in lung cancer are rare. A recent study reported that high FHL2 expression in lung cancer was an independent predictor of a poor prognosis, yet the underlying mechanism remains unclear.[18]

In this study, we first evaluated the prognostic relevance of FHL2 in LUAD using clinical data and subsequently confirmed its impact on tumor progression through in vitro and in vivo studies. We further explored the underlying mechanism and revealed that upregulation of the PI3K/AKT/mTOR signaling pathway and subsequent suppression of autophagy are the key mechanisms by which FHL2 promoted LUAD tumorigenesis. Our study is the first to fully clarify the molecular function of FHL2 in LUAD and the first to discover the regulatory effect of FHL2 on autophagy via the PI3K/AKT/mTOR signaling pathway. Our findings provide a more in-depth understanding of the role of FHL2 in cancer biology and indicate that FHL2 can be exploited for the development of therapeutic strategies against LUAD.

METHODS

Patient specimens

Paraffin-embedded specimens of surgically resected tumor tissues from 60 patients diagnosed with LUAD were obtained from the Pathology Department of the First Affiliated Hospital of Sun Yat-Sen University and subjected to immunohistochemistry (IHC) assays. Additionally, 12 pairs of fresh-frozen LUAD tissues and cancer-adjacent normal lung tissues were collected for analysis via western blotting. None of the patients received any treatment before surgery. This study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University ([2023]303).

Data mining and bioinformatic analysis

The gene expression profiles and clinical data of the patients in the GSE31210 dataset were obtained from the Gene Expression Omnibus (GEO) database. This dataset included data of 226 tumor tissues from LUAD patients and 20 normal lung tissues. The differential expression of FHL2 between the two groups was evaluated using the "limma" R package. Patients with LUAD were also categorized into two groups (FHL2-high and FHL2-low), with the median expression value of FHL2 serving as the cutoff point. The Kaplan–Meier (K-M) survival curves were plotted using the "survival" R package to analyze the prognostic value of FHL2 in LUAD. In addition, the expression of FHL2 and its prognostic value in LUAD patients were further verified via the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) database.

Reagents

Lentiviral vector containing the following plasmids, FHL2 complementary cDNA, a negative control, three shRNAs designed to interfere FHL2 expression (shFHL2#1, #2, #3) and the negative control shRNA (shControl), were all synthesized by GeneCopoeia. The target sequences of FHL2 shRNAs are listed in Table S1. SC79 (#S7863), MK-2206 (#S1078), MHY1485 (#S7811) and rapamycin (#S1039) were purchased from Selleck Chemicals.

Cell culture and transfection

The human normal bronchial epithelial cell line BEAS-2B and LUAD cell lines A549, H1299 and PC9 were purchased from iCell Bioscience Inc. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an incubator at 37°C with 5% CO_2. All cells were authenticated by short tandem repeat analysis and tested for mycoplasma contamination.

For cell transfection, cells were seeded into 24-well plates and cultured to 80% confluence. Then, lentiviral particles were added to the cells according to the manufacturer's instructions. After transfection for 12 h, the cells were washed three times with phosphate buffered solution (PBS)

and cultured in fresh medium at 37°C and 5% CO_2. Finally, the cells were treated with puromycin (2 μg/mL) to select for successfully transfected cells.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from the cells using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme) and reverse-transcribed into cDNA using the HiScript III RT SuperMix kit (Vazyme). RT-qPCR analysis was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a CFX96 Real-Time PCR system (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. The relative expression of the target genes was analyzed using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers were listed in Table S1.

Western blotting

Radioimmunoprecipitation assay (RIPA) lysis buffer (Epizyme) containing protease and phosphate inhibitors (Epizyme) was used to lyse cells and fresh-frozen tissues for protein extraction. The protein concentration was measured using the BCA protein assay kit (Epizyme). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% defatted milk at room temperature for 1 h, the membranes were incubated with primary antibodies overnight at 4°C and incubated with the corresponding secondary antibodies at room temperature for 1 h. Finally, the enhanced chemiluminescence reagent (Epizyme) was used to detect the protein bands. The antibodies used in this study are listed Table S2.

Cell proliferation and colony formation assays

Cell proliferation was assessed using the cell counting kit-8 (CCK-8; FUDE) according to the manufacturer's instructions. The absorbance was determined at a wavelength of 450 nm. For the colony formation assay, cells (1×10^3 per well) were seeded in six-well plates for 10–14 days. The colonies were fixed with 4% paraformaldehyde and stained with crystal violet. The number of colonies was counted.

Wound healing assay

For wound healing assay, the cells were seeded into six-well plates and cultured to 90% confluence. The plates were scratched with sterile 200 μL pipette tips and then washed three times with PBS. After that, the cells were cultured in FBS-free medium at 37°C and 5% CO_2. Images were taken at 0 h and 48 h, and the wound area was measured using ImageJ software.

Transwell assay

The migration and invasion ability of the cells were evaluated using transwell chambers (Corning) coated with or without Matrigel (ABW). The cells were resuspended in 200 μL FBS-free medium and seeded in the upper chamber, and 800 μL medium supplemented with 10% FBS was added to the lower chamber. After incubation for 36 h at 37°C, the cells that passed through the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The stained cells were counted under a microscope.

RNA-sequencing analysis

Total RNA was isolated from the cells and subsequently sent to Annoroad Corporation for transcriptome sequencing (3 samples per cell line). Analysis of differential gene expression was performed using the "limma" R package. Genes with $|\log_2 FC| > 0.5$ and $p < 0.05$ were considered differentially expressed genes (DEGs) and were subjected to further pathway analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using the "clusterProfiler" R package.

IHC assay

For IHC staining, the tissue sections were incubated with specific primary antibody overnight at 4°C after deparaffinization, hydration, antigen retrieval and goat serum blocking. Then, the sections were subsequently incubated with the corresponding secondary antibody at room temperature for 1 h. After that, positive staining was visualized using 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin.

The IHC score was calculated as the product of the score for staining intensity and the score for the percentage of positively stained cells. The staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positively stained cells was scored as follows: 1, 0%–25%; 2, 26%–50%; 3, 51%–75%; and 4, >75%. A total score of ≤6 or >6 was regarded as low or high FHL2 expression, respectively. The IHC results were independently scored by two pathologists who were blinded to the clinical information.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde, permeated with 0.5% Triton-100 and blocked with 0.5% bovine serum albumin (BSA). Next, the cells were incubated with primary

antibody overnight at 4°C and then with secondary antibody (Affinity) at room temperature for 1 h. The nuclei were stained with DAPI (Beyotime). Images were obtained with an inverted fluorescence microscope (Leica DMI8).

Transmission electron microscopy (TEM)

The cells were harvested, fixed with fixative (Servicebio) at 4°C, washed three times with 0.1 M phosphate buffer (PB) and wrapped in agarose. Then, the cells were post fixed with 1% OsO4 buffer at room temperature for 2 h, washed three times with 0.1 M PB, dehydrated with ethanol, embedded in Epon812 epoxy resin, cut into 80 nm thin on the ultra-microtome (Leica UC7) and collected on the cuprum grids. Next, the cuprum grids were stained with 2% uranium acetate saturated alcohol solution and 2.6% lead citrate and observed using a transmission electron microscope (Hitachi).

Xenograft model

Five-week-old female BALB/c nude mice were used for the generation of tumor xenograft models. A total of 2×10^5 FHL2-overexpressing A549 cells and control cells were resuspended in 200 μL basal medium and injected into the right flank of nude mice ($n = 5$/group). The tumor size was measured every 4 days with a caliper, and the tumor volume was calculated according to the following formula: tumor volume = length × width^2/2. The mice were euthanized on the 28th day. The xenograft tumors were removed, weighed and photographed. All animals used in the present study were approved by the ethical committee of the First Affiliated Hospital of Sun Yat-Sen University ([2022]379).

Statistical analysis

R 4.1.2 and GraphPad Prism 8.0 software were utilized for data analysis and visualization. Intergroup comparisons were performed using Student's *t* test. K-M curves were used for survival analysis. All data are presented as mean ± SD. $p < 0.05$ was considered statistically significant.

RESULTS

FHL2 was upregulated in LUAD and associated with a poor prognosis

We first evaluated the expression and prognostic relevance of FHL2 in LUAD using public datasets. FHL2 was significantly upregulated in LUAD tumor tissues compared to normal lung tissues (Figure 1a,b). FHL2 overexpression was significantly associated with reduced overall survival (OS) in patients with LUAD in the GSE31210 and GEPIA cohorts (Figure 1c,d). Analysis of clinical samples obtained from our clinical center also confirmed that FHL2 was overexpressed in LUAD tissues compared to tumor-adjacent tissues (Figure 1e). We also detected FHL2 expression in cell lines by RT-qPCR and western blotting and found that FHL2 was more highly expressed in LUAD cell lines (H1299, PC9 and A549) than in a nonmalignant bronchial epithelial cell line (BEAS-2B) (Figure 1f,g). To further confirm the prognostic significance of FHL2 in clinical samples from our center, we detected FHL2 expression in 60 LUAD tissues by IHC (Figure 1h) and collected corresponding clinical data. K-M survival curves revealed that patients with lower FHL2 expression ($n = 31$) had longer OS than those with high FHL2 expression ($n = 29$) (Figure 1i). These results indicated that FHL2 was upregulated in LUAD and associated with a poor prognosis.

FHL2 promoted the proliferation, migration and invasion of LUAD tumor cells

According to the expression pattern of FHL2 in LUAD cell lines, H1299 and PC9 cells with high FHL2 expression were selected for FHL2 knockdown, and A549 cells with low FHL2 expression were selected for FHL2 overexpression. The knockdown and overexpression efficiency were verified by RT-qPCR and western blotting (Figure 2a,b). CCK-8 assays indicated that FHL2 knockdown or overexpression significantly suppressed or promoted cell growth, respectively, compared to that in the control groups (Figure 2c,d). In addition, FHL2 knockdown significantly decreased the colony-forming ability of H1299 and PC9 cells (Figure 2e), while FHL2 overexpression promoted the colony formation of A549 cells (Figure 2f). Transwell assays showed that FHL2 knockdown inhibited the migration and invasion of H1299 and PC9 cells (Figure 2g), whereas FHL2 overexpression had the opposite effect on A549 cells (Figure 2h). The impact of FHL2 on tumorigenesis was further evaluated using a xenograft tumor model. FHL2-overexpressing A549 cells and control cells were injected into the right flank of nude mice. Tumors derived from the FHL2-overexpressing cell line grew faster than that of the control ones (Figure 2i,j). In addition, the tumor weight in the FHL2-overexpressing group was significantly greater than that in the control group (Figure 2k). These findings suggested that FHL2 could act as an oncogene to promote tumor proliferation and metastasis in LUAD.

PI3K/AKT/mTOR pathway was activated by FHL2

RNA-sequencing was performed to investigate the molecular mechanism by which FHL2 promoted the progression of LUAD. A total of 379 DEGs were identified between FHL2-knockdown H1299 cells and corresponding control cells, among which 217 genes were upregulated and

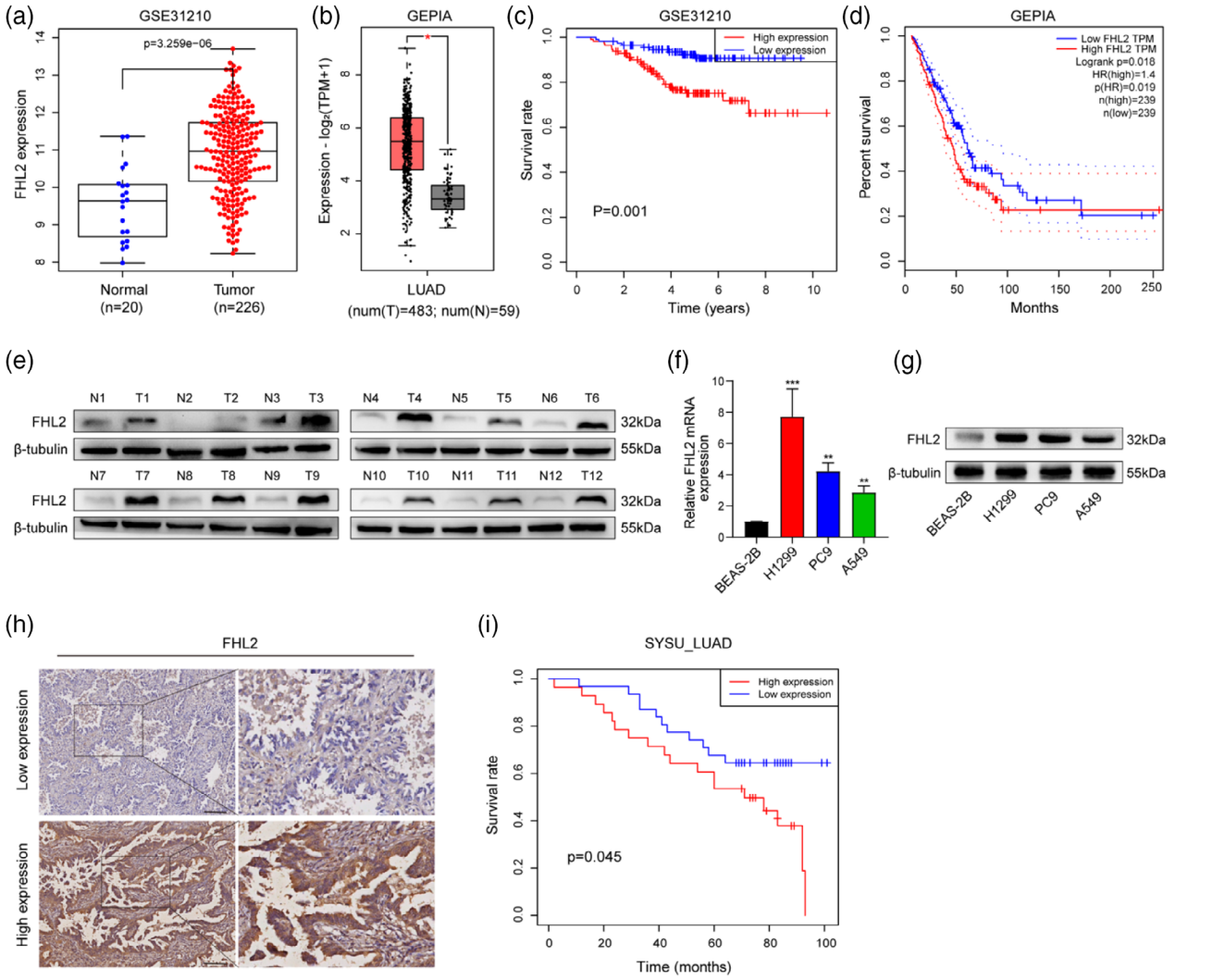

FIGURE 1 Increased FHL2 expression is associated with poor prognosis of patients with lung adenocarcinoma (LUAD). (a, b) The expression of FHL2 was compared between lung adenocarcinoma (LUAD) tissues and normal lung tissues (GSE31210 dataset and GEPIA database). (c, d) Kaplan–Meier (K-M) survival curves of overall survival (OS) in GSE31210 dataset and GEPIA database. (e) The expression of FHL2 in LUAD tissues ($n = 12$) and cancer-adjacent tissues ($n = 12$) was detected by western blotting. (f, g) The mRNA and protein expression of FHL2 in human normal bronchial epithelial cell line (BEAS-2B) and LUAD cell lines (H1299, PC9 and A549) was detected by reverse transcription quantitative PCR (RT-qPCR) and western blotting. (h) Representative immunohistochemistry (IHC) staining images of low and high FHL2 expression in LUAD tissues. scale bar = 50 μm. (i) K-M survival curves of OS in 60 patients with LUAD stratified by IHC score (low FHL2 expression, $n = 31$ vs. high FHL2 expression, $n = 29$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

162 genes were downregulated after knockdown of FHL2 (Figure S1, Table S3). We also identified a total of 654 DEGs (471 upregulated genes and 183 downregulated genes) in FHL2-overexpressing A549 cells compared to control cells (Figure S1, Table S4). The DEGs were further subjected to KEGG pathway analysis. The top 15 pathways that were found to be upregulated by FHL2 are presented in Figure 3a,b, among which the PI3K/AKT pathway was one of the predominant pathways in both cell lines.

To confirm the findings derived from pathway analysis, we next detected the protein levels of the key markers in this pathway by western blotting. After FHL2 knockdown, the protein levels of PI3K, phospho-AKT (p-AKT) and phospho-mTOR (p-mTOR) were significantly decreased in H1299 and PC9 cells, while the protein levels of total AKT and mTOR were not significantly changed (Figure 3c). Furthermore, FHL2 overexpression significantly increased the protein levels of PI3K, p-AKT and p-mTOR in A549 cells (Figure 3d). These results suggested that FHL2 promoted the proliferation, migration and invasion of LUAD cells via activation of the PI3K/AKT/mTOR pathway.

FHL2 regulated the autophagy of LUAD tumor cells through the PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is a vital regulator of autophagy.[19] Therefore, we explored whether FHL2 could regulate

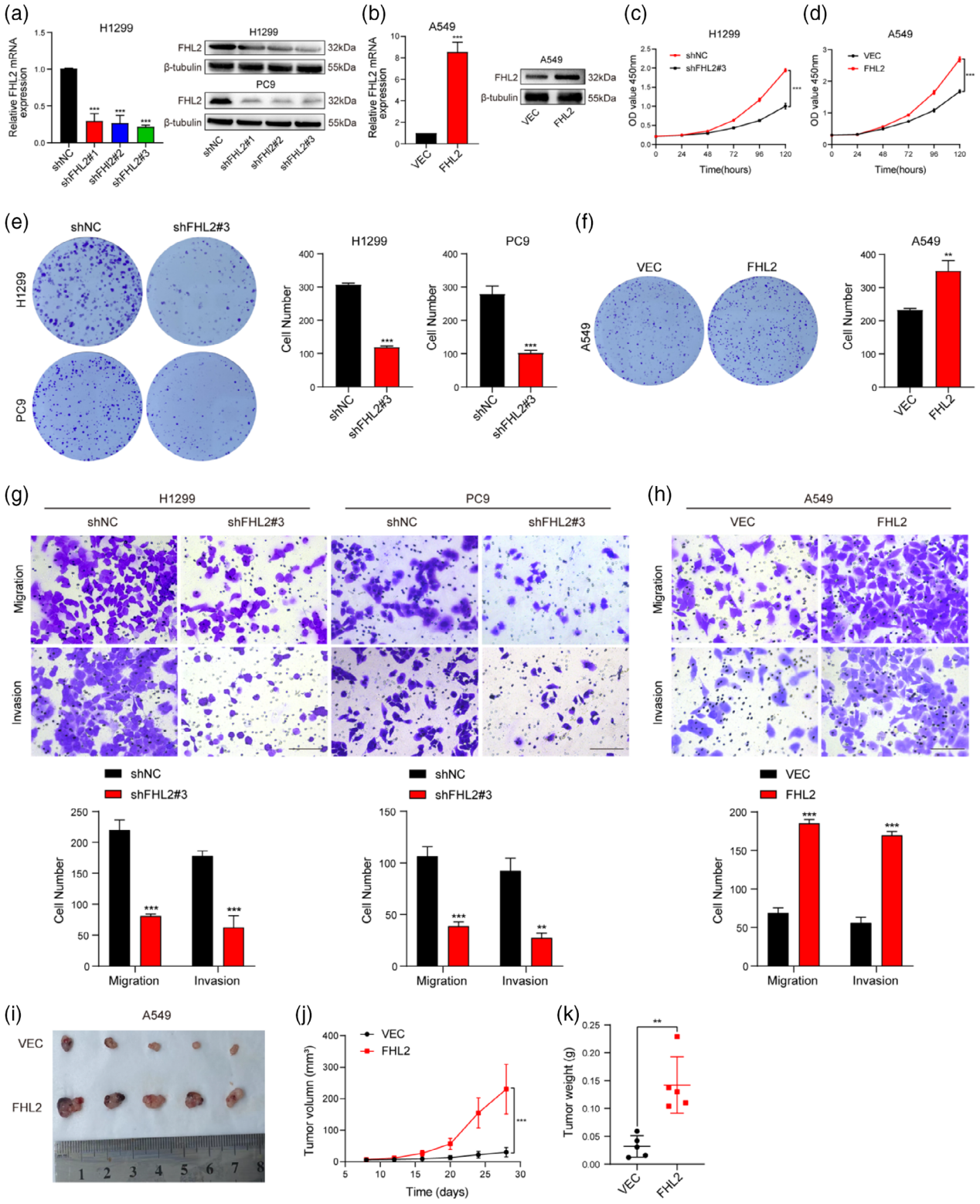

FIGURE 2 FHL2 promotes the tumorigenesis of lung adenocarcinoma (LUAD) in vitro and in vivo. (a, b) The mRNA and protein expression of FHL2 in FHL2-knockdown or -overexpressing LUAD tumor cells and corresponding control cells. (c, d) Cell growth curves of FHL2-knockdown H1299 cells, FHL2-overexpressing A549 cells and their control cells. (e) Colony formation assay after FHL2 knockdown in H1299 and PC9 cells. (f) Colony formation assay after FHL2 overexpression in A549 cells. (g) The migration and invasion ability of FHL2-knockdown H1299 and PC9 cells was detected by transwell assay. scale bar = 100 μm. (h) The migration and invasion ability of FHL2-overexpressing A549 cells was detected by transwell assay. scale bar = 100 μm. (i) Xenograft tumors in FHL2-overexpressing and control groups. The mice were executed on 28 days. (j) Tumor growth curves after the injection of FHL2-overexpressing A549 cells and control cells. (k) Tumor weight in FHL2-overexpressing and control groups was measured. **, $p < 0.01$; ***, $p < 0.001$.

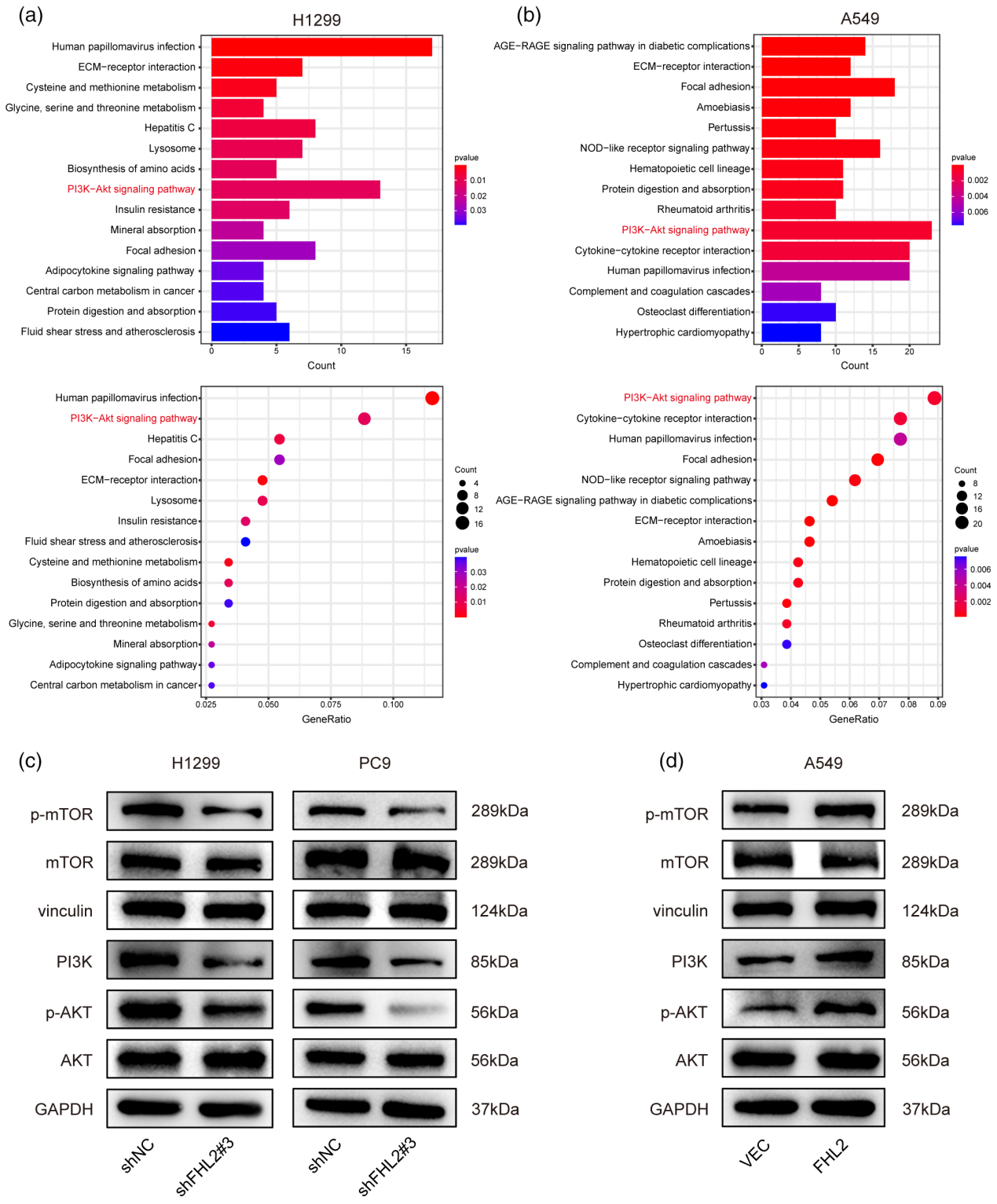

FIGURE 3 FHL2 activates the PI3K/AKT/mTOR pathway in lung adenocarcinoma (LUAD). (a) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes (DEGs) between FHL2-knockdown H1299 cells and control cells. (b) KEGG analysis of DEGs between FHL2-overexpressing A549 cells and control cells. (c, d) The protein level of PI3K, AKT, p-AKT, mTOR and p-mTOR in FHL2-knockdown H1299 cells, FHL2-knockdown PC9 cells, FHL2-overexpressing A549 cells and their control cells.

the autophagy of LUAD cells by detecting the protein levels of autophagy-related markers. As shown by western blotting, FHL2 knockdown increased the LC3 II/LC3 I ratio and the Beclin1 expression level but decreased the P62 expression level in H1299 and PC9 cells (Figure 4a). In contrast, FHL2 overexpression decreased the LC3 II/ LC3 I ratio and

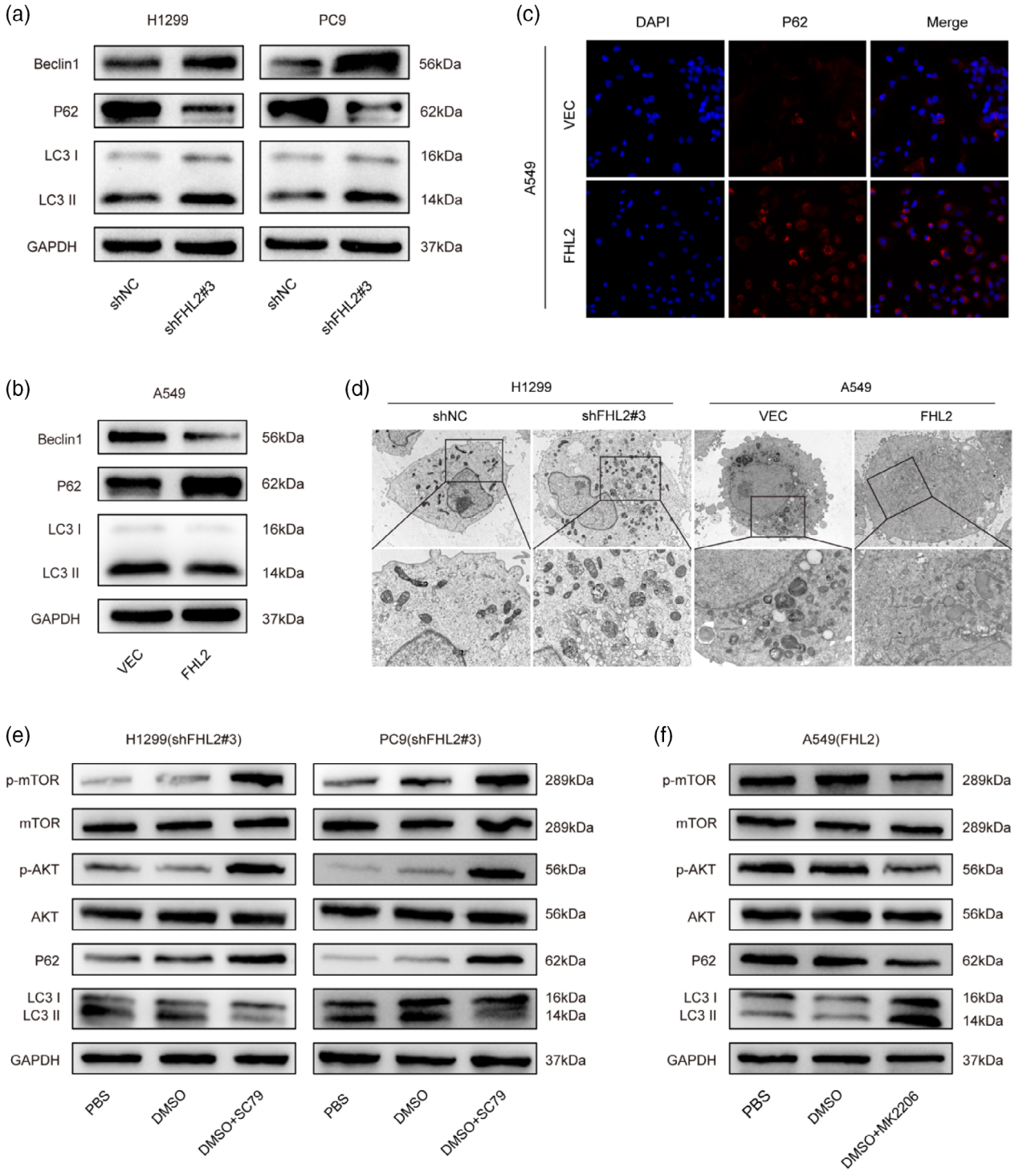

FIGURE 4 FHL2 regulates the autophagy of lung adenocarcinoma (LUAD) tumor cells through PI3K/AKT/mTOR pathway. (a, b) The protein level of Beclin1, LC3 II/LC3 I and P62 in FHL2-knockdown H1299 cells, FHL2-knockdown PC9 cells, FHL2-overexpressing A549 cells and their control cells was detected by western blotting. (c) Immunofluorescence images of P62 (red) staining in FHL2-overexpressing A549 cells and control cells. Nuclei were stained with DAPI (blue). (d) Autophagosome in FHL2-knockdown H1299 cells, FHL2-overexpressing A549 cells and their control cells was detected by transmission electron microscopy. (e) FHL2-knockdown H1299 and PC9 cells treatment with or without SC79 (5 μg) for 24 h, respectively. The protein level of AKT, p-AKT, mTOR, p-mTOR, LC3 II/LC3 I and P62 was detected by western blotting. (f) FHL2-overexpressing A549 cells treatment with or without MK-2206 (4 μM) for 24 h, respectively. The protein level of AKT, p-AKT, mTOR, p-mTOR, LC3 II/LC3 I and P62 was detected by western blotting.

the expression of Beclin1 in A549 cells, while the expression of P62 was significantly increased (Figure 4b). Immunofluorescence analysis also showed that FHL2 overexpression increased the expression of P62 in A549 cells compared to that in the control cells (Figure 4c). Furthermore, we found that FHL2 knockdown significantly induced autophagosome

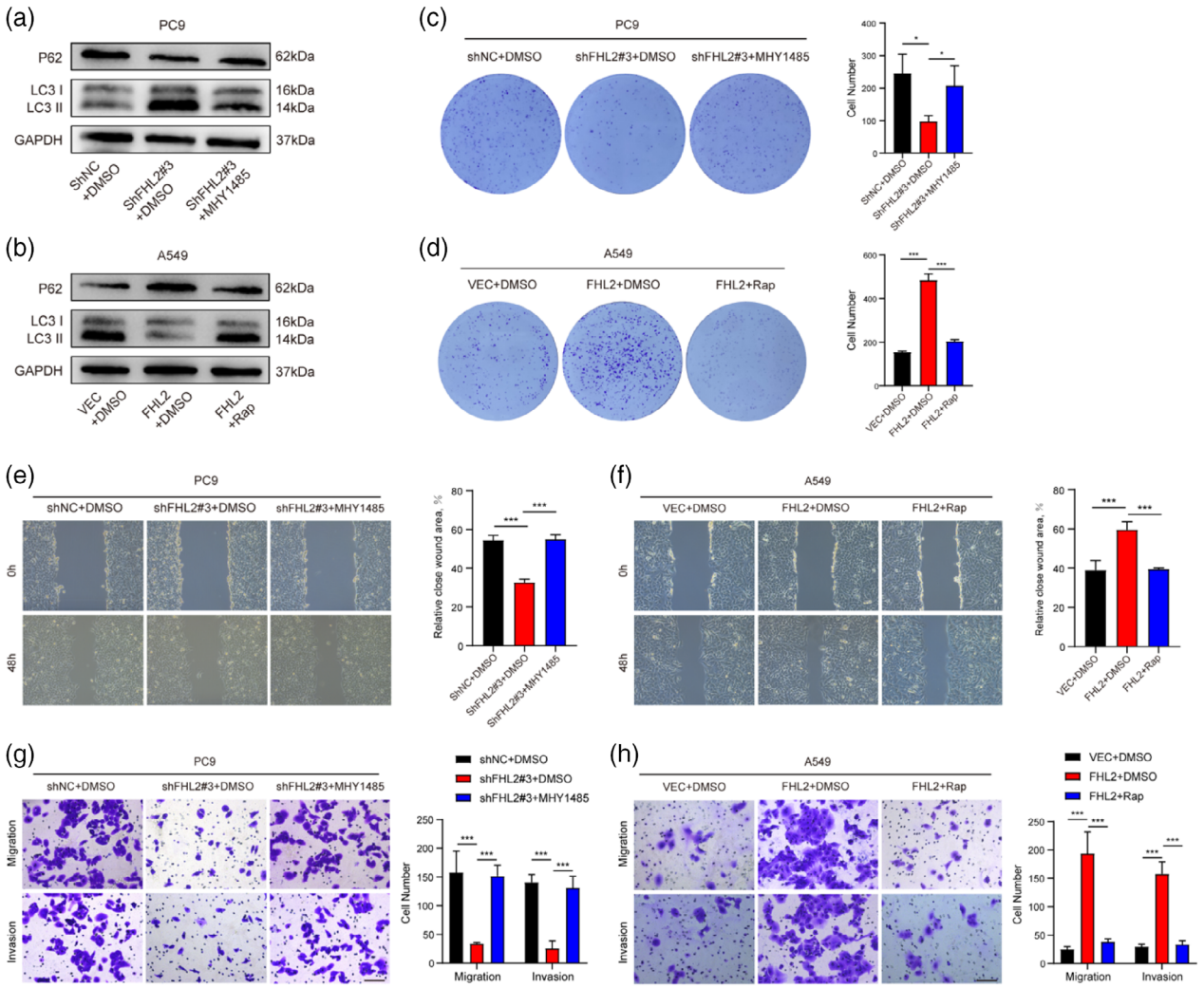

FIGURE 5 FHL2 promotes aggressiveness of lung adenocarcinoma (LUAD) cells through suppression of autophagy. (a) FHL2-knockdown PC9 cells treatment with or without MHY1485 (5 μM) for 24 h, respectively. The protein level of LC3 II/LC3 I and P62 was detected by western blotting. (b) FHL2-overexpressing A549 cells treatment with or without rapamycin (100 nM) for 24 h, respectively. The protein level of LC3 II/LC3 I and P62 was detected by western blotting. (c) The colony formation ability of shNC + DMSO, shFHL2#3 + DMSO and shFHL2#3 + MHY1485 cell groups in PC9 cells. (d) The colony formation ability of VEC + DMSO, FHL2 + DMSO and FHL2 + Rapamycin cell groups in A549 cells. (e) Wound-healing assay was used to evaluate the migration ability of shNC + DMSO, shFHL2#3 + DMSO and shFHL2#3 + MHY1485 cell groups in PC9 cells. (f) Wound-healing assay was used to evaluate the migration ability of VEC + DMSO, FHL2 + DMSO and FHL2 + Rapamycin cell groups in A549 cells. (g) Transwell assay was used to evaluate the migration and invasion ability of shNC + DMSO, shFHL2#3 + DMSO and shFHL2#3 + MHY1485 cell groups in PC9 cells. scale bar = 100 μm. (h) Transwell assay was used to evaluate the migration and invasion ability of VEC + DMSO, FHL2 + DMSO and FHL2 + Rapamycin cell groups in A549 cells. scale bar = 100 μm. *, $p < 0.05$; ***, $p < 0.001$.

formation in H1299 cells, whereas FHL2 overexpression reduced this phenomenon in A549 cells (Figure 4d). These findings revealed that FHL2 suppressed autophagy in LUAD tumor cells.

To further prove that FHL2 regulated autophagy in LUAD cells via activation of the PI3K/AKT/mTOR pathway, an AKT agonist (SC79) and an AKT inhibitor (MK-2206) were used to manipulate the AKT pathway in subsequent analyses. As shown in Figure 4e, treating FHL2-knockdown H1299 and PC9 cells with SC79 significantly promoted the phosphorylation of AKT and mTOR, which simultaneously reduced the LC3 II/LC3 I ratio and increased P62 expression. On the other hand, an increase in the LC3 II/LC3 I ratio and a reduction in P62 expression were observed in FHL2-overexpressing A549 cells after treatment with MK-2206, which suppressed the AKT/mTOR signaling pathway as indicated by decreased phosphorylation of AKT and mTOR (Figure 4f). The above findings suggested that FHL2 suppressed autophagy in LUAD tumor cells via activation of the PI3K/AKT/mTOR pathway.

Autophagy induction regulated the progression of LUAD

To explore whether FHL2 promoted the progression of LUAD via the suppression of autophagy, an autophagy inhibitor

(MHY1485) and an autophagy activator (rapamycin) were applied to modulate autophagy in FHL2-knockdown PC9 cells and FHL2-overexpressing A549 cells. As shown in Figure 5a, the LC3 II/LC3 I ratio was significantly reduced in FHL2-knockdown PC9 cells after treatment with MHY1485, while the protein level of P62 was significantly increased. Conversely, rapamycin treatment significantly increased the LC3 II/LC3 I ratio but inhibited the expression of P62 in FHL2-overexpressing A549 cells (Figure 5b).

Interestingly, the colony formation ability of FHL2-knockdown PC9 cells was reversed when autophagy was blocked by MHY1485 treatment (Figure 5c), while the induction of autophagy with rapamycin abrogated the effect of FHL2 overexpression on the colony formation of A549 cells (Figure 5d). Wound-healing assay showed that the migration ability of FHL2-knockdown PC9 cells was reversed after MHY1485 treatment (Figure 5e), whereas the migration ability of A549 cells was attenuated after rapamycin treatment (Figure 5f). Furthermore, transwell assay showed that the migration and invasion ability of FHL2-knockdown PC9 cells treated with MHY1485 was significantly increased (Figure 5g), whereas those of FHL2-overexpressing A549 cells were significantly inhibited by rapamycin treatment (Figure 5h). These findings indicated that FHL2 promoted the progression of LUAD by inhibiting autophagy.

DISCUSSION

FHL2 is aberrantly expressed in various types of cancer, and it has been shown to exert either oncogenic or cancer-suppressive effects in a cancer-type dependent manner.[12–15] However, the biological functions of FHL2 in lung cancer are still not fully understood. We not only thoroughly evaluated the prognostic impact of FHL2 in LUAD but also carried out an in-depth investigation of its biological functions within the context of LUAD. Mechanistically, FHL2 promoted LUAD progression through activation of the PI3K/AKT/mTOR pathway and the subsequent suppression of tumor autophagy. Our findings broaden the understanding of the role of FHL2 in cancer biology and might facilitate the development of more effective anticancer treatments.

Jiao et al. investigated the role of FHL2 in lung cancer and reported that increased FHL2 expression was an independent predictor of a poor prognosis based on analysis of the TCGA dataset.[18] Consistently, our study further proved that FHL2 expression was significantly associated with an unfavorable prognosis in patients with LUAD according to analysis of both public datasets and data derived from our own clinical center. Function loss and function gain experiments further demonstrated the important role of FHL2 in promoting the proliferation, migration and invasion of LUAD tumor cells. These findings suggested that FHL2, as an oncogene, could be exploited as a prognostic biomarker in LUAD. Additionally, the critical role of FHL2 in tumorigenesis suggested that FHL2 might be a potential therapeutic target for LUAD.

Numerous studies have indicated that FHL2 can act as an oncoprotein or tumor suppressor to influence the progression of various cancers through multiple different mechanisms.[11,15,20] However, the molecular mechanism by which FHL2 promotes the aggressiveness of LUAD has never been explored. RNA-sequencing and pathway analysis facilitated the investigation of the underlying mechanism. Based on the results of pathway analysis, we speculated that FHL2 may enhance the malignant phenotype of LUAD tumor cells by activating the PI3K/AKT pathway, which controls many cellular functions, such as cell survival, proliferation, apoptosis and autophagy.[21,22] Numerous studies have shown that the PI3K/AKT pathway is aberrantly activated in various cancers and plays an important role in promoting tumor progression.[22,23] Hence, we detected the expression of key proteins involved in this pathway to validate our hypothesis. Consistent with these findings, FHL2 knockdown inhibited the expression of PI3K, p-AKT and p-mTOR, while FHL2 overexpression had the opposite effects. Collectively, these results demonstrated that FHL2 may promote the progression of LUAD by activating the PI3K/AKT/mTOR pathway.

Previous studies have demonstrated that the PI3K/AKT/mTOR pathway is a key regulator of autophagy.[19] Autophagy is a highly conserved intracellular process in which damaged proteins and organelles are digested by lysosomal hydrolases, and degradative products such as amino acids and sugars are ultimately recycled into the cytoplasm to maintain cellular homeostasis.[24–26] Emerging evidence indicates that autophagy plays a dual role in cancer.[27] On one hand, autophagy plays a critical role in suppressing tumorigenesis by preventing cell injury and maintaining genome stability.[28,29] On the other hand, autophagy can help cancer cells meet high metabolic demands and promote cell growth.[30] However, unrestrained autophagy might induce cell death.[31] The association between FHL2 and the PI3K/AKT/mTOR pathway identified in our study prompted us to explore the novel role of FHL2 in autophagy. Interestingly, we found that FHL2 knockdown inhibited the PI3K/AKT/mTOR pathway, thereby inducing autophagy in LUAD tumor cells, while FHL2 overexpression had the opposite effect. Furthermore, rescue experiments showed that inhibiting or inducing autophagy reversed the effect of FHL2 knockdown or overexpression on the malignant behavior of LUAD tumor cells. Taken together, our findings suggested that FHL2 inhibited the autophagy of LUAD tumor cells by activating the PI3K/AKT/mTOR pathway, thereby promoting LUAD progression.

This study had several limitations. First, the data used for prognostic validation were derived from a retrospective study conducted at a single clinical center, and the sample size was relatively small. Second, we studied the mechanism by which FHL2 promoted LUAD progression through cell experiments but did not further verify this phenomenon in

animal models. Finally, the upstream molecular mechanism underlying the abnormal expression of FHL2 in LUAD needs further investigation.

In conclusion, we demonstrated that high FHL2 expression was correlated with a poor prognosis in patients with LUAD. Functionally, we discovered that FHL2 could promote the proliferation, migration and invasion of LUAD tumor cells. Mechanistically, FHL2 may promote the progression of LUAD through activation of the PI3K/AKT/mTOR pathway and subsequent inhibition of autophagy. Targeting FHL2 is expected to provide a new therapeutic strategy for patients with LUAD.

AUTHOR CONTRIBUTIONS

Yanbin Zhou, Ziying Lin and Shuaishuai Wang designed the study. Shuaishuai Wang and Baomo Liu performed the majority of experiments and drafted the manuscript. Xiongye Xu and Peixin Dong collected the tissue samples. Yan Su, Nian Wang, Yanli Qiu and Jiating Deng performed the statistical analysis. Lixia Huang, Jincui Gu and Shaoli Li interpreted the results. Yanbin Zhou and Ziying Lin revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We are very grateful for the data provided by GEO and GEPIA databases. This work was supported by the Natural Science Foundation of Guangdong Province, China (grant no.: 2021A1515010480) and Science and Technology Program of Guangzhou, China (grant no.: 202201010951).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Yanbin Zhou https://orcid.org/0000-0002-0099-4258

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–49.
2. Denisenko TV, Budkevich IN, Zhivotovsky B. Cell death-based treatment of lung adenocarcinoma. Cell Death Dis. 2018;9(2):117.
3. Arbour KC, Riely GJ. Systemic therapy for locally advanced and metastatic non-small cell lung cancer: a review. JAMA. 2019;322(8):764–74.
4. Rosell R, Cardona AF, Arrieta O, Aguilar A, Ito M, Pedraz C, et al. Coregulation of pathways in lung cancer patients with EGFR mutation: therapeutic opportunities. Br J Cancer. 2021;125(12):1602–11.
5. Lahiri A, Maji A, Potdar PD, Singh N, Parikh P, Bisht B, et al. Lung cancer immunotherapy: progress, pitfalls, and promises. Mol Cancer. 2023;22(1):40.
6. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, et al. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin. 2019;69(5):363–85.
7. Duma N, Santana-Davila R, Molina JR. Non-small cell lung cancer: epidemiology, screening, diagnosis, and treatment. Mayo Clin Proc. 2019;94(8):1623–40.
8. Cao CY, Mok SW, Cheng VW, et al. The FHL2 regulation in the transcriptional circuitry of human cancers. Gene. 2015;572(1):1–7.
9. Johannessen M, Møller S, Hansen T, Moens U, Ghelue MV. The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol Life Sci. 2006;63(3):268–84.
10. Zhang J, Zeng Q, She M. The roles of FHL2 in cancer. Clin Exp Med. 2023;23:3113–24.
11. Zienert E, Eke I, Aust D, Cordes N. LIM-only protein FHL2 critically determines survival and radioresistance of pancreatic cancer cells. Cancer Lett. 2015;364(1):17–24.
12. Huang Z, Li Q, Luo K, Zhang Q, Geng J, Zhou X, et al. miR-340-FHL2 axis inhibits cell growth and metastasis in ovarian cancer. Cell Death Dis. 2019;10(5):372.
13. Wu M, Wang J, Tang W, Zhan X, Li Y, Peng Y, et al. FOXK1 interaction with FHL2 promotes proliferation, invasion and metastasis in colorectal cancer. Oncogenesis. 2016;5(11):e271.
14. Kinoshita M, Nakagawa T, Shimizu A, et al. Differently regulated androgen receptor transcriptional complex in prostate cancer compared with normal prostate. Int J Urol. 2005;12(4):390–7.
15. Ng CF, Ng PK, Lui VW, et al. FHL2 exhibits anti-proliferative and anti-apoptotic activities in liver cancer cells. Cancer Lett. 2011;304(2):97–106.
16. Xiong Z, Ding L, Sun J, Cao J, Lin J, Lu Z, et al. Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. IUBMB Life. 2010;62(9):669–76.
17. Ding L, Wang Z, Yan J, Yang X, Liu A, Qiu W, et al. Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-beta-like signaling pathway. J Clin Invest. 2009;119(2):349–61.
18. Jiao Y, Wei J, Li Z, Zhou J, Liu Y. High FHL2 mRNA expression and its prognostic value in lung cancer. Aging. 2022;14(19):7986–8000.
19. Xu Z, Han X, Ou D, Liu T, Li Z, Jiang G, et al. Targeting PI3K/AKT/mTOR-mediated autophagy for tumor therapy. Appl Microbiol Biotechnol. 2020;104(2):575–87.
20. Gao X, Yang L. HBXIP knockdown inhibits FHL2 to promote cycle arrest and suppress cervical cancer cell proliferation, invasion and migration. Oncol Lett. 2023;25(5):186.
21. Tewari D, Patni P, Bishayee A, Sah AN, Bishayee A. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: a novel therapeutic strategy. Semin Cancer Biol. 2022;80:1–17.
22. Xue C, Li G, Lu J, Li L. Crosstalk between circRNAs and the PI3K/AKT signaling pathway in cancer progression. Signal Transduct Target Ther. 2021;6(1):400.
23. Aziz AUR, Farid S, Qin K, Wang H, Liu B. PIM kinases and their relevance to the PI3K/AKT/mTOR pathway in the regulation of ovarian cancer. Biomolecules. 2018;8(1):7.
24. Li YJ, Lei YH, Yao N, Wang CR, Hu N, Ye WC, et al. Autophagy and multidrug resistance in cancer. Chin J Cancer. 2017;36(1):52.
25. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. Mol Cancer. 2020;19(1):12.
26. Poillet-Perez L, White E. Role of tumor and host autophagy in cancer metabolism. Genes Dev. 2019;33(11–12):610–9.
27. Marinković M, Šprung M, Buljubašić M, Novak I. Autophagy modulation in cancer: current knowledge on action and therapy. Oxid Med Cell Longev. 2018;2018:8023821.
28. Singh SS, Vats S, Chia AY, et al. Dual role of autophagy in hallmarks of cancer. Oncogene. 2018;37(9):1142–58.
29. Verma AK, Bharti PS, Rafat S, Bhatt D, Goyal Y, Pandey KK, et al. Autophagy paradox of cancer: role, regulation, and duality. Oxid Med Cell Longev. 2021;2021:8832541.

30. Xu HM, Hu F. The role of autophagy and mitophagy in cancers. Arch Physiol Biochem. 2022;128(2):281–9.
31. Xiao L, Li Y, Zeng X, Zhou Z, Hu S, Zhang S, et al. Silencing of LOC389641 impairs cell proliferation and induces autophagy via EGFR/MET signaling in lung adenocarcinoma. Aging. 2020;13(2):2539–52.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wang S, Liu B, Su Y, Wang N, Dong P, Xu X, et al. FHL2 promotes the aggressiveness of lung adenocarcinoma by inhibiting autophagy via activation of the PI3K/AKT/mTOR pathway. Thorac Cancer. 2024;15(8):630–41. https://doi.org/10.1111/1759-7714.15234