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ORIGINAL ARTICLE

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High level of FHL2 exacerbates the outcome of non-small cell lung cancer (NSCLC) patients and the malignant phenotype in NSCLC cells

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Abstract

Non-small cell lung cancer (NSCLC) is a malignant tumour with high mortality. FHL2 has been identified as a biomarker of lung cancer. This research explored the effects of FHL2 expression on NSCLC. NSCLC-associated data sets were collected from the assistant for clinical bioinformatics and TCGA databases respectively. The association between FHL2 and clinical characteristics, the prognostic significance of FHL2 and the influences of various variables on NSCLC were determined by Pearson's chi-squared test, the Kaplan–Meier curve and the Cox regression model respectively. FHL2 level was altered by cell transfection and was measured by qRT-PCR. Tumour xenograft formation was completed by inoculating sh-FHL2/pcDNA-FHL2 transfected cells into BALB/c nude mice. Protein expression was assessed by western blot. Cell apoptosis, proliferation and epithelial - mesenchymal transition (EMT) characteristics were evaluated employing TUNEL, $BrdU^{+}$ and microscopic observation respectively. The expression of Ki67 and N-cadherin was assessed by immunohistochemistry. The results showed that FHL2 was highly expressed in NSCLC tissues. Patients with high FHL2 expression experienced lower overall survival probability. FHL2 knockdown promoted apoptosis, but inhibited EMT of A549 and NCI-H460 cells, which was verified by the increased ratios of cleaved caspase 9/caspase 9 and cleaved caspase 3/caspase 3, as well as augmented E-cadherin and reduced N-cadherin. In an in vivo assay FHL2 knockdown decreased tumour volume and weight, repressed EMT, but enhanced apoptosis. FHL2 upregulation showed the opposite effects of FHL2 knockdown. Furthermore, FHL2 upregulation facilitated cell proliferation both in in vitro and in vivo assays. These outcomes indicated that high level of FHL2 facilitated tumorigenesis, as well as the proliferation and EMT of NSCLC cells.

KEYWORDS
cell proliferation, EMT, FHL2, NSCLC, tumorigenesis

Na Li and Ling Xu contributed equally to this work.

1 | INTRODUCTION

In recent years, lung cancer (LC) has become one of the commonest malignancies in the world and has been recognized as the dominant reason for cancer-related deaths in 2020.[1] Among all types of LC, non-small cell lung cancer (NSCLC) accounts for about 85%, and currently, the most effective treatment strategy is surgical resection.[2] However, when diagnosed with NSCLC, nearly 70% of the patients have developed metastatic and/or locally advanced disease.[3] Although great progress has been achieved in the diagnosis and treatment of NSCLC in the last few years, outcomes remain unsatisfactory due to lack of adequate tumor biomarkers for early diagnosis and metastasis identification.[4,5] The 5-year survival rate of NSCLC patients is still <16%.[6] Hence, it is still pressing to identify novel effective diagnostic and metastatic biomarkers for NSCLC treatment.

Four and a half LIM domains protein 2 (FHL2), also known as downregulated in rhabdomyosarcoma LIM (DRAL) domain protein, is a member of the FHL family and is composed of one N-terminal half- and four-LIM domains.[7,8] Previous studies have found that FHL2 can play different roles as tumor suppressor genes or oncogene proteins in different cancers.[9-11] Previous investigations have also revealed that FHL2 participates in various cellular processes, including cell differentiation, proliferation, motility and adhesion, via regulating gene expression.[12-14] For example, Zienert et al reported that FHL2 depletion notably repressed proliferation, but promoted apoptosis of pancreatic cancer cells.[15] Besides, Al-Nomani et al found that there was a close association between lymphatic metastasis and enhanced FHL2 expression in sporadic colorectal cancer.[16] Importantly, FHL2 was described previously as a biomarker of lung adenocarcinoma and was recorded to be working as an invasion-promoting gene facilitating the progression and metastasis of human LC.[17,18] Nevertheless, the effects of FHL2 on NSCLC still remains under investigated to date.

In this research, we explored the roles of FHL2 in NSCLC by performing bioinformatic and statistical analysis, as well as various experimental procedures. We found that FHL2 was highly expressed in NSCLC tissues. Besides, NSCLC patients with high FHL2 expression experienced a dramatically shorter overall survival of 20 years than those with low FHL2 expression. Moreover, FHL2 knockdown facilitated cell apoptosis and repressed epithelial-mesenchymal transition (EMT) , while FHL2 upregulation promoted cell proliferation and EMT both in in vitro and in vivo assays. These discoveries might provide a theoretical basis for finding a promising biomarker and target for NSCLC treatment in the future.

2 | METHODS

2.1 | Data analysis

For this research the NSCLC-related lncRNA microarray data sets were obtained from assistant for clinical bioinformatics (https://www.aclbi.com/static/index.html#/) (aclbi) and TCGA (https://portal.gdc.com) databases respectively. The relationship between FHL2 expression and clinical characteristics were explored by analysis of the aclbi database. The relative expression of FHL2 in NSCLC tissues ($n = 1014$) and normal tissues ($n = 49$) was determined by analysis of the TCGA database. Cancer-specific survival (CSS) analysis of FHL2 in NSCLC was performed utilizing the Kaplan–Meier method, and the differences were analyzed using the log-rank test. Furthermore, the Cox regression models of FHL2, four pathway-related genes (AKT1, TCF4, MAPK1 and STAT3) and clinical pathological characteristics were used to determine whether the prognosis could be impacted by a pathological characteristic independently.

2.2 | Cell lines and NSCLC tissue samples

The cell lines used in this study, including A549, NCI-H292, NCI-H460, NCI-H1299 and human bronchial epithelial cells BEAS-2B, were all acquired from the Cell Bank of the Chinese Academy of Sciences. Cell culture was carried out in RPMI-1640 medium (Sangon Biotech) replenished with 10% fetal bovine serum (FBS; Sangon Biotech) under 5% CO_2 and 37°C conditions. The medium was refreshed 3 times per week.

A total of 51 pairs of NSCLC tissue and its corresponding adjacent tissues were acquired from patients diagnosed with NSCLC during surgery in Shenyang Tenth People's Hospital. After acquisition, these tissues were immediately frozen in liquid nitrogen for follow-up experiments. Each participant signed an informed consent form. This study had been performed according to the Declaration of Helsinki, and the procedures have been approved by the Ethics Committee of Shenyang Tenth People's Hospital.

2.3 | Cell transfection

Short hairpin RNAs (shRNAs), including FHL2 shRNA1, 2, 3 and a scrambled shRNA, were acquired from GenePharma. The empty pcDNA 3.1 plasmids and pcDNA3.1-FHL2 overexpression plasmids were purchased from Synbio Technologies. Cells were

precultured under 37°C and 5% CO_2 conditions overnight. Then, FHL2 knockdown were carried out in A549 and NCI-H460 cells by transfecting with the above mentioned shRNAs respectively. FHL2 overexpression was carried out in NCI-H292 and NCI-H1299 cells by transfecting with pcDNA3.1 and pcDNA3.1-FHL2 plasmids, respectively. Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used for cell transfection. The EMT characteristics of the transfected cells were observed 48 h post transfection, and then cell collection was carried out.

2.4 | Tumor xenograft formation

This part of the study was approved by the Animal Ethics Committee of Shenyang Tenth People's Hospital. Twenty male BALB/c nude mice (aged 5 weeks) were acquired from the Experimental Animal Center of China medical University and were divided into four groups. All animals were reared in a pathogen-free room under 45%–50% humidity and 20–25°C conditions for one week.

A549 cells transfected with sh-FHL2 or shRNA-negative control (NC) and H1299 cells transfected with pcDNA-FHL2 or pcDNA-NC were inoculated subcutaneously into BALB/c nude mice. After being maintained under standard conditions for the indicated times, the mice were anesthetized with intraperitoneal injection of 1% pentobarbital. The tumor size (width and length) in each group was measured every five days for the calculation of tumor volume according to the formula [tumor volume (V, mm^3) = 0.5 × length × width]. Finally, the tumor tissues were collected and the tumor weight was measured. The collected tissues were either fixed in 4% PFA or frozen in liquid nitrogen, respectively, for follow-up experiments.

2.5 | qRT-PCR

Expression of FHL2 in NSCLC tissues and cells were determined by qRT-PCR. Generally, isolation of total RNAs was performed using Trizol reagent (Sangon Biotech). A Nanodrop 2000 system (Thermo Scientific) was used for concentration determination. The first strand of cDNA was synthesized by First Strand cDNA Synthesis Kit (Thermo Scientific). Then, FHL2 expression was measured utilizing BeyoFast™ SYBR Green One-Step qRT-PCR Kit (Beyotime). GAPDH was set as internal control. $2^{-\Delta\Delta C_t}$ method was employed for calculation of FHL2 expression. The primer sequences used in this study are listed below:

FHL2: 5′-GTGGTGTGCTTTGAGACCCTGT-3′ (forward); 5′-GAGCAGTGGAAACAGGCTTCATG-3′ (reverse);

GAPDH: 5′-GTCTCCTCTGACTTCAACAGCG-3′ (forward); 5′-ACCACCCTGTTGCTGTAGCCAA-3′ (reverse).

2.6 | Cell proliferation

Cell proliferation was measured in a bromodeoxyuridine ($BrdU^+$) assay. Briefly, the transfected cells were plated into 6 well plates (2×10^5 per well) and cultured under 37°C and 5% CO_2 conditions for 72 h. Then, 10 μM of $BrdU^+$ solution (Sigma-Aldrich) was added and the cells incubated for another 4 h prior to paraformaldehyde (PFA) fixation. Afterwards, the $BrdU^+$ signal was visualized by successively incubating with the rabbit anti-BrdU primary antibody (ab152095, Abcam) and an Alexa Flour 594-labelled secondary antibody (Alexa Flour 594). In addition, for the determination of changes in nuclei, 4′,6-Diamidino-2-phenylindole (DAPI, Sigma) staining was included. Finally, pyknotic nuclei counting and image observation were completed under a fluorescence microscope (Olympus).

2.7 | Cell apoptosis

Cell apoptosis was evaluated by TUNEL assay employing a One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology). Briefly, the transfected cells were seeded on coverslips and cultured in RPMI-1640 medium replenished with 10% FBS under 37°C and 5% CO_2 conditions for 24 h in 24 well plates (5×10^3 per well). After rinsing with PBS, cells were fixed in 4% PFA for 30 min, and then permeabilized in 0.3% Triton X-100 for 5 min at room temperature. After washing, 50 μl of TUNEL reagent was provided to each coverslip and maintained under 37°C and dark conditions for 60 min. After washing, nuclei re-staining was performed utilizing DAPI reagent and the coverslips were sealed with the existence of anti-fade fluorescence mounting medium. Finally, a fluorescence microscope (Olympus) was used to obtain images.

2.8 | Western blot

Expression of proteins extracted from NSCLC cells and xenograft tumor tissues in nude mice was measured by western blot. Briefly, extraction of total proteins was completed employing Total Protein Extraction Kit (Solarbio) plus protease inhibitor cocktail (Beyotime). The determination of protein concentration was carried

out utilizing a BCA protein assay kit (Solarbio). Then, SDS-PAGE was conducted for the separation of interested proteins. After that, the separated proteins of interest were transferred on to PVDF membranes, and blocked in 5% bovine serum albumin (BSA, Beyotime) at 25°C for 2 h. Thereafter, primary antibodies, containing anti-FHL2 (MBS820438, MyBioSource), anti-caspase 9 (MBS8200853, MyBioSource), anti-cleaved-caspase 9 (MBS840456, MyBioSource), anti-caspase 3 (MBS820158, MyBioSource), anti-cleaved-caspase 3 (MBS9410752, MyBioSource), anti-E-cad (MBS822115, MyBioSource), anti-N-cad (MBS9412664, MyBioSource), anti-Ki67 (MBS854540, MyBioSource), anti-p21 (MBS821539, MyBioSource) and anti-GAPDH (MBS9407102, MyBioSource) were applied and incubated at 4°C for 16 h. After rinsing, secondary antibody (ab205718, Abcam, Cambridge, UK) incubation was added at 25°C for 1 h. Finally, the signals of proteins of interest were captured after enhanced chemiluminescence. The optimal dilutions of primary and secondary antibodies were 1:1000 and 1:5000, respectively.

2.9 | Immunohistochemistry

The collected tumor tissues were fixed in 4% PFA overnight, gradient-dehydrated, paraffin-embedded and sectioned. Afterwards, the sections were deparaffinized in xylene, rehydrated with gradient alcohol and heated in citrate buffer for 30 min to induce antigen retrieval. Thereafter, the sections were incubated with anti-N-cad (MBS175704, MyBioSource) and anti-Ki67 (MBS852597, MyBioSource) antibodies at 4°C overnight, and maintained with HRP-conjugated goat anti-rabbit antibody (MBS674746, MyBioSource) at 25°C for 1 h. After that, the diaminobenzidine reagent (DAB) staining was performed for 3 min. Finally, hematoxylin counterstaining was performed, and the results were visualized with a light microscope.

2.10 | Statistical analysis

Each experiment included three replicates. Data were analyzed with Graphpad Prism software 8 and were expressed as mean ±SEM. Comparisons between two groups and among multigroups were performed using student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, respectively. Unpaired t-test was used for analyzing statistical assessments. Pearson's chi-squared test or Fisher's exact test was used for the analysis of the association between FHL2 and clinical characteristic variables. The survival difference between high FHL2 expression and low FHL2 expression was compared with the Kaplan–Meier survival analysis with log-rank test. The Cox proportional hazards regression model was employed for multivariate and univariate analyses. $p < 0.05$ represents statistically significant values.

TABLE 1 Association between FHL2 expression and clinicopathological characteristics of NSCLC patients

Characteristics	High FHL2 expression	Low FHL2 expression	*p* value
Status			
Alive	294	317	0.158
Dead	213	190	
Age			
Mean (SD)	66.7 (9.5)	65.8 (9.3)	0.133
Median [Min, Max]	68 [38,90]	67 [33,88]	
Gender			
Female	207	199	0.654
Male	300	308	
Race			
American Indian	1		0.843
Asian	8	8	
Black	43	39	
White	361	375	
pT_stage			
T1	119	163	0.138
T2	298	271	
T3	68	50	
T4	22	23	
pN_stage			
N0	329	320	0.023*
N1	98	128	
N2	70	44	
N3	10	14	
pM_stage			
M0	372	383	0.218
M1	131	120	
pTNM_stage			
I	254	264	0.025*
II	135	148	
III	92	76	
IV	21	12	
Smoking			
Non-smoking	46	46	1
Smoking	446	450	

*$p < 0.05$.

3 | RESULTS

3.1 | The association between FHL2 expression and clinical characteristic variables

To determine the relationship between FHL2 expression and NSCLC, we explored the association between FHL2 expression and clinical characteristic variables, which include status, age, gender, race, pT stage, pN stage, pM stage, pTNM stage, new tumor event type, smoking, radiation therapy and history of neoadjuvant treatment. The data were collected from the acbi database. Results revealed that a significant association between FHL2 expression and pN, pTNM stages (both p <0.05), while no correlation waes observed between FHL2 and other clinical characteristic variables (Table 1, both p <0.05). Besides, FHL2 expression in NSCLC tissues and normal tissues was analyzed on the basis of the data obtained from the TCGA database, and results demonstrated that FHL2 was highly expressed in NSCLC tissues compared with normal tissues (Figure 1A, p <0.001). Furthermore, the association between FHL2 level and the overall survival probability of patients with NSCLC was evaluated by conducting Kaplan–Meier analysis. Results showed that NSCLC patients with high FHL2 expression had poor prognosis (Figure 1B, p <0.01). In addition, univariate Cox regression analysis revealed that FHL2level, age, pT, pN and pTNM stages were factors related to prognosis, while multivariate Cox regression analysis revealed that FHL2level, age, pT and pTNM stages were independent prognostic factors (Table 2, p <0.05 or p <0.01 or p <0.001). Considering that there was no significant correlation between these four pathway-related genes and prognosis, the follow-up research did not detect pathways, but focused on disclosing the influence(s) of FHL2 expression on NSCLC cells.

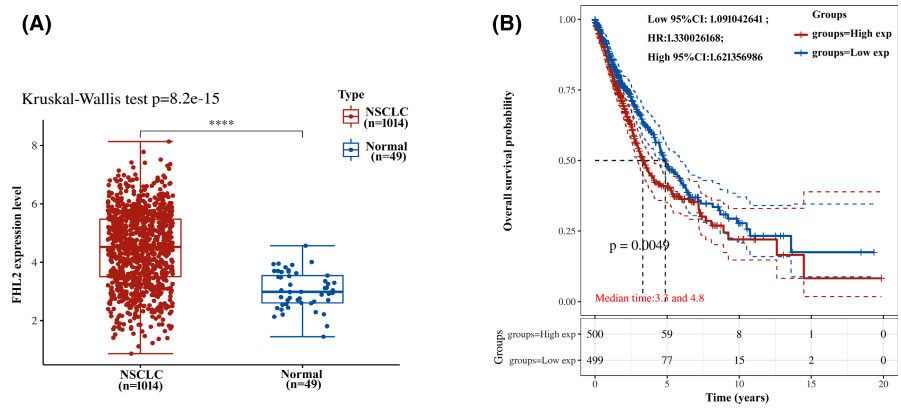


FIGURE 1 FHL2 was highly expressed in NSCLC tissues and was correlated with poor prognosis. (A) FHL2 expression in NSCLC tissues and normal tissues was analyzed with data obtained from the TCGA database. (B) The correlation between FHL2 expression level and the overall survival probability of NSCLC patients was evaluated by Kaplan–Meier analysis with log rank test. ****p <0.001

TABLE 2 Univariate and multivariate Cox regression analysis of FHL2, four pathway-related gene expression and clinical characteristics

	Uni_cox		Mult_cox	
	***p* value**	**Hazard Ratio (95% CI)**	***p* value**	**Hazard Ratio (95% CI)**
AKT1	0.62778	1.04495 (0.8748, 1.24819)	0.94214	1.00719 (0.83012, 1.22202)
FHL2	**0.01115***	1.10403 (1.02279, 1.19171)	0.03390*	1.08637 (1.00632, 1.17279)
MAPK1	0.68305	1.0313 (0.88947, 1.19574)	0.94667	1.00633 (0.8364, 1.2108)
TCF4	0.51142	0.96512 (0.86808, 1.07301)	0.15781	0.91288 (0.80443, 1.03596)
STAT3	0.91678	1.00913 (0.85092, 1.19677)	0.94591	1.00646 (0.83552, 1.21239)
Age	0.03159*	1.01213 (1.00106, 1.02332)	0.00807**	1.01552 (1.00401, 1.02715)
Gender	0.19453	1.14558 (0.93293, 1.40668)	0.30487	1.11792 (0.90352, 1.3832)
pT_stage	<0.0001***	1.43442 (1.26443, 1.62727)	0.02065*	1.19842 (1.0281, 1.39695)
pN_stage	<0.0001***	1.41787 (1.24792, 1.61096)	0.15013	1.14723 (0.9515, 1.38323)
pTNM_stage	<0.0001***	1.47137 (1.32522, 1.63365)	0.02389*	1.22709 (1.02746, 1.46552)

*p < 0.05; **p < 0.01; ***p < 0.001.

3.2 | FHL2 was markedly upregulated in NSCLC tissues and cells

To further explore the potential role(s) of FHL2 in NSCLC, we first tested FHL2 levels in NSCLC tissues and cells. The outcome was that FHL2 was highly expressed in NSCLC tissues (Figure 2A, p <0.01) and cells (A549, NCI-H292, NCI-H460 and H1299). More importantly, the results showed that FHL2 had higher expression in A549 and NCI-H460 cells, and lower expression in NCI-H292 and H1299 cells (Figure 2B,C, p <0.05 or p <0.01). In order to better reflect the effects of FHL2 interference and overexpression, A549 and NCI-H460 cells were selected to perform FHL2 knockdown, while NCI-H292 and H1299 cells were used to carry out FHL2 overexpression referring to earlier studies.[19,20] FHL2 knockdown was carried out by transfecting with the sequences of shRNA-NC, FHL2-shRNA1, 2, 3 into A549 and NCI-H460 cells, respectively. Considering that FHL2-shRNA1 has the best interference effect, it was selected for further follow-up research. Data displayed in Figure 2D and E verified that FHL2 expression was remarkably lowered both at mRNA and protein

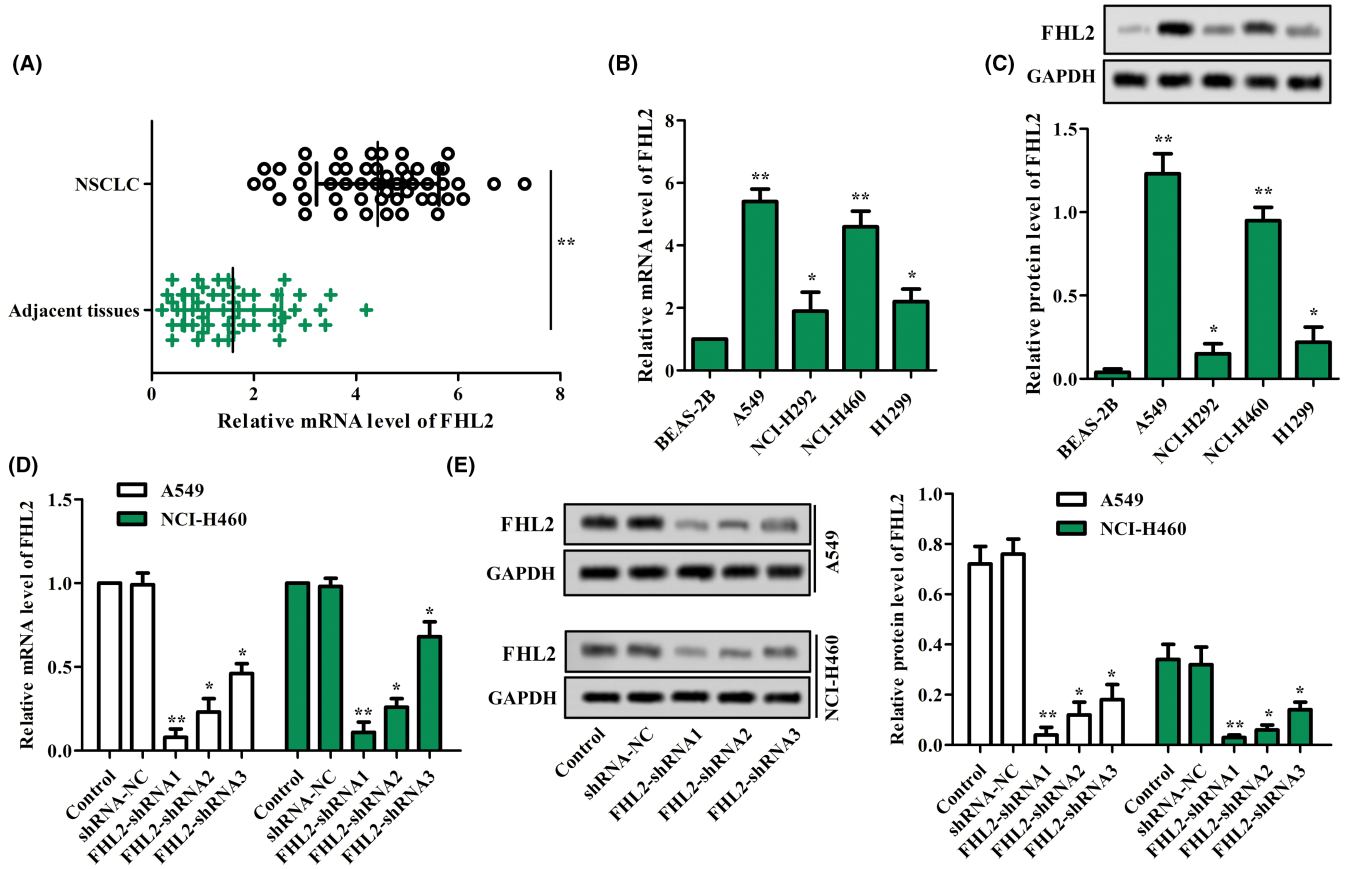
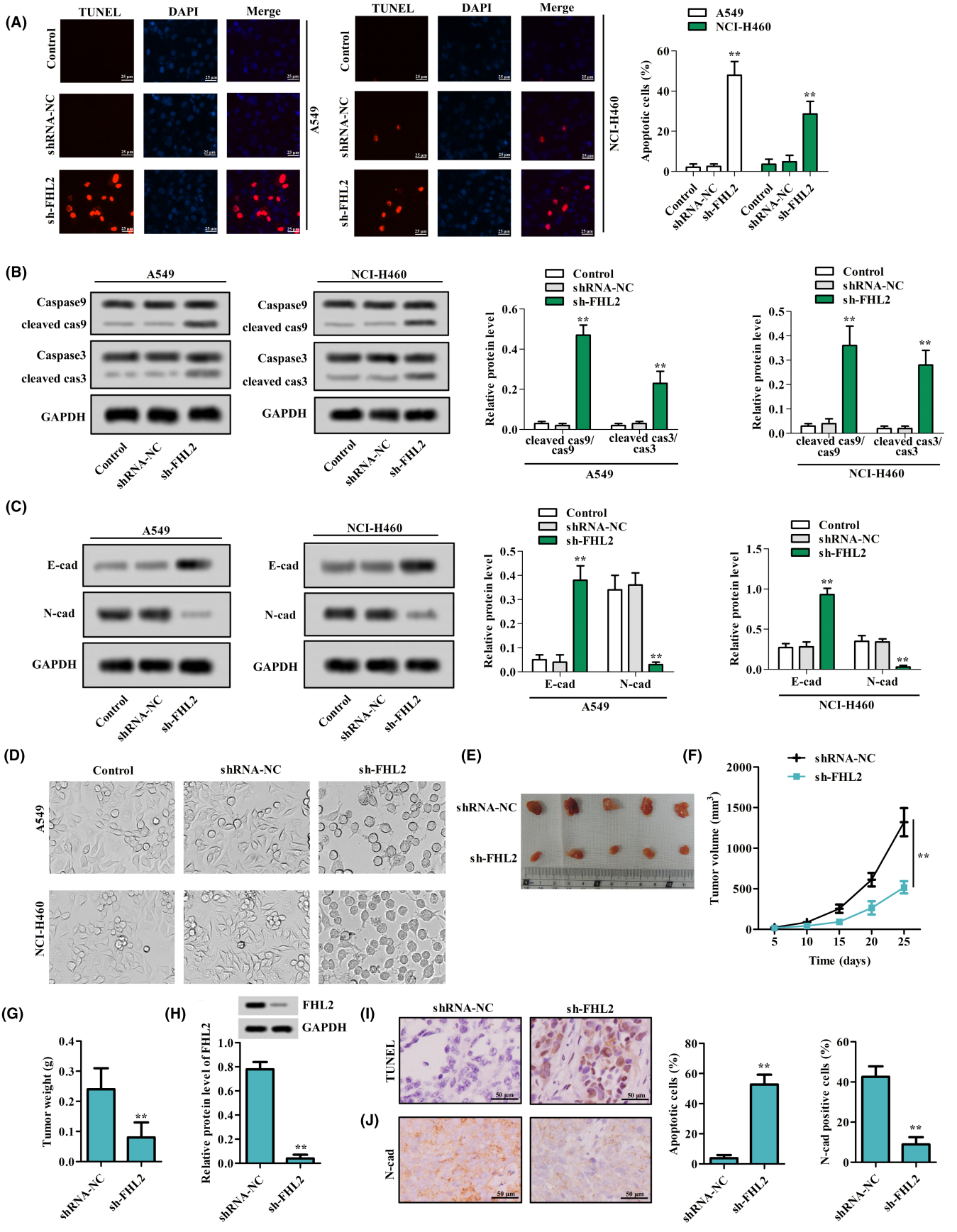


FIGURE 2 FHL2 was highly expressed in NSCLC tissues and cells. (A) The mRNA level of FHL2 in the collected NSCLC tissues and normal adjacent tissues was analyzed by qRT-PCR. (B) The mRNA and protein levels of FHL2 in BEAS-2B and series of NSCLC cell lines (A549, NCI-H292, NCI-H460 and H1299) were analyzed by qRT-PCR and western blot respectively. (D,E) FHL2 knockdown was carried out in A549 and NCI-H460 cells by transfecting with the sequences of different FHL2-shRNAs (FHL2-NC, FHL2-shRNA1, FHL2-shRNA2 and FHL2-shRNA1) respectively. FHL2 level after transfection was evaluated by qRT-PCR and western blot, respectively. *p <0.05, **p <0.01

FIGURE 3 FHL2 knockdown notably enhanced apoptosis, but restrained EMT of NSCLC cells both in vitro and in vivo. (A) Cell apoptosis in the transfected A549 and NCI-H460 cells was evaluated by TUNEL assay. (B,C) The levels of apoptosis-related (Caspase 9, Cleaved-caspase 9, Caspase 3 and Cleaved-caspase 3) and EMT-associated (E-cadherin and N-cadherin) factors in the transfected A549 and NCI-H460 cells were evaluated by western blot. (D) EMT characteristics of the transfected A549 and NCI-H460 cells were determined by microscope observation. Tumor xenograft formation was completed by inoculating sh-FHL2 transfected cells into BALB/c nude mice. (E–G): Tumor size was measured every 5 days and tumor weight was measured finally. Tumor volume was calculated employing the formula listed below: tumor volume (V, mm^3) = 0.5 × length × width. (H) FHL2 expression in the generated tumor in BALB/c nude mice was tested by western blot. (I,J) Cell apoptosis and N-cadherin expression in the generated tumor in BALB/c nude mice were determined by TUNEL assay and immunohistochemistry respectively. **p <0.01


(A)
TUNEL
DAPI
Merge
Control
shRNA-NC
sh-FHL2
A549
NCI-H460
Apoptotic cells (%)
(B)
Caspase9
cleaved cas9
Caspase3
cleaved cas3
GAPDH
Relative protein level
cleaved cas9/ cas9
cleaved cas3/ cas3
(C)
E-cad
N-cad
(D)
(E)
(F)
Tumor volume (mm3)
Time (days)
(G)
Tumor weight (g)
(H)
FHL2
Relative protein level of FHL2
(I)
(J)
N-cad positive cells (%)

levels after transfection (p <0.05 or p <0.01). Based on these findings, we conjectured that FHL2 might work as an oncoprotein in NSCLC.

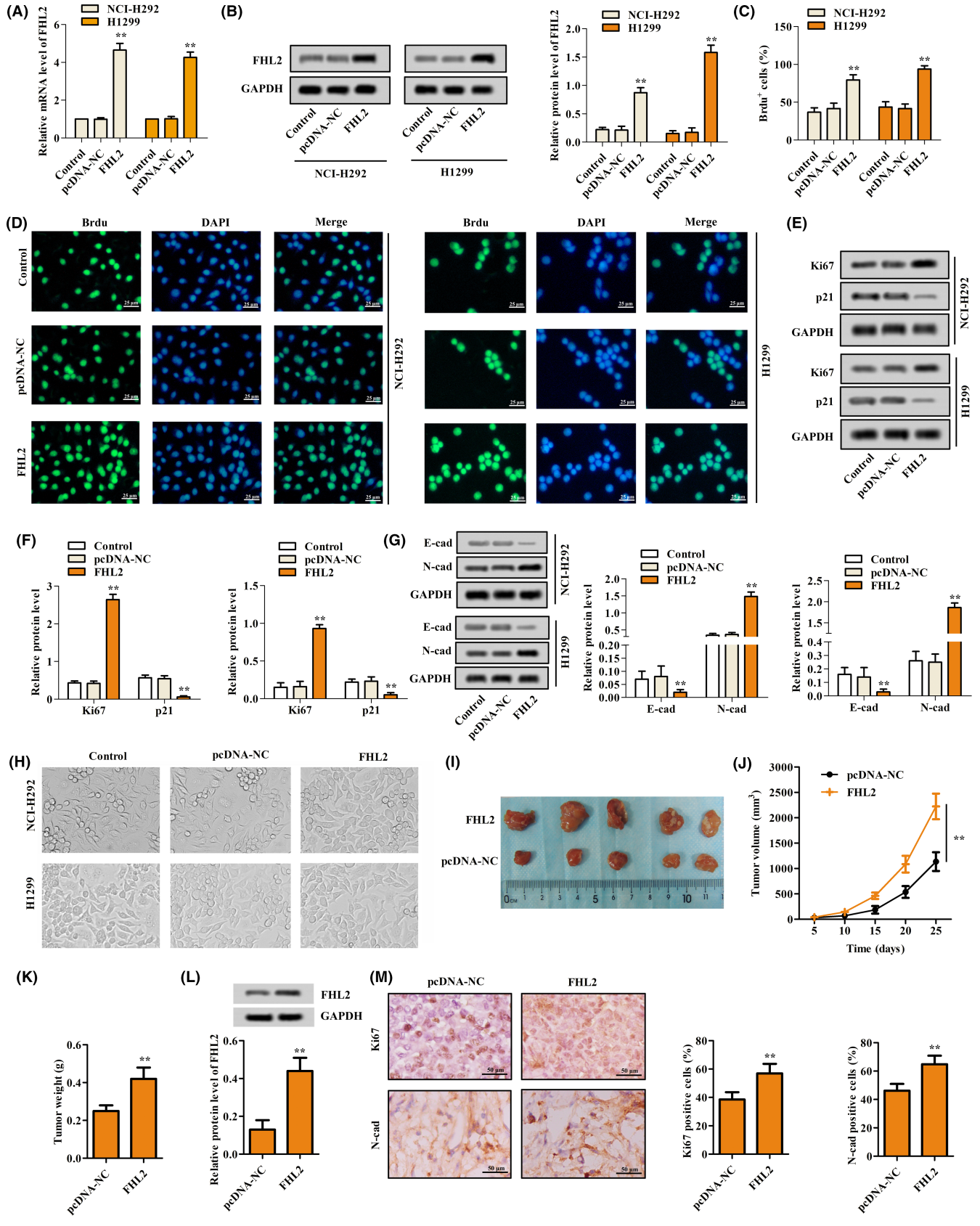
3.3 | FHL2 knockdown notably enhanced apoptosis, but restrained EMT of NSCLC cells both in vitro and in vivo

After transfection, the influence(s) of FHL2 knockdown on NSCLC were examined further. The TUNEL assay was used for measurement of the apoptosis of A549 and NCI-H460 cells. Results shown in Figure 3A validated that transfection with FHL2-shRNA (sh-FHL2) notably augmented the proportion of apoptotic A549 and NCI-H460 cells (both p <0.01), which was further verified by the increased ratios of cleaved-caspase 3/caspase 3 and cleaved-caspase 9/caspase 9 (Figure 3B, all p <0.01). Besides, the expression of EMT-related E-cadherin and N-cadherin were also assessed. Outcomes displayed that transfection with sh-FHL2 distinctly augmented the expression of E-cadherin, but lowered that of N-cadherin both in A549 and NCI-H460 cells (Figure 3C, all p <0.01). Meanwhile, morphologic changes in sh-FHL2 transfected A549 and NCI-H460 cells illustrated that the EMT process was markedly inhibited (Figure 3D), which was consistent with the changes of E-cadherin and N-cadherin expression. In addition, we also determined the influences of FHL2 knockdown on the apoptosis and EMT of NSCLC cells in *vivo*. The results showed that the tumor volume and weight generated in the inoculated nude mice were notably less compared with the control group (Figure 3E–G, both p <0.01). Besides, FHL2 expression in the NSCLC tissues generated in the inoculated nude mice was also remarkably lower compared to the control group (Figure 3H, p <0.01). Furthermore, the apoptosis and EMT of the generated NSCLC tissues were also determined. The outcome was that the ratio of apoptotic cells in the generated NSCLC tissues was augmented, while the number of N-cadherin positive cells was markedly decreased (Figure 3I,J, both p <0.01). The combination of these outcomes illustrate that FHL2 knockdown facilitated apoptosis rematkably, but notably restrained EMT and tumorigenesis of NSCLC cells.

3.4 | FHL2 upregulation notably facilitated the proliferation and EMT of NSCLC cells both in vitro and in vivo

Additionally, the influences of FHL2 upregulation on the phenotype of NSCLC cells were also evaluated. Results revealed that FHL2 expression in pcDNA-FHL2 transfected NCI-H292 and H1299 cells was augmented both at mRNA and protein levels, which meant that FHL2 was successfully overexpressed (Figure 4A,B, all p <0.01). Thereafter, the $BrdU^+$ assay was employed for the determination of cell proliferation in the transfected NCI-H292 and H1299 cells. The outcome was that numbers of BrdU positive NCI-H292 and H1299 cells were notably higher in pcDNA-FHL2 transfected group (Figure 4C,D, both p <0.01). Meanwhile, the determination of expression of proliferation-associated Ki67 and p21 validated that transfection with pcDNA-FHL2 remarkably elevated the expression of Ki67, but lessened that of p21 (Figure 4E,F, all p <0.01), which was in line with the results of $BrdU^+$ assay. Furthermore, data displayed in Figure 4G illustrated that transfection with pcDNA-FHL2 lessened the expression of E-cadherin, but increased that of N-cadherin in both NCI-H292 and H1299 cells (all p <0.01). Morphologic changes demonstrated that the EMT process of pcDNA-FHL2-transfected NCI-H292 and H1299 cells were facilitated (Figure 4H), which was consistent with the changes observed in E-cadherin and N-cadherin expression. Alongside these evaluations, determination of the influence of FHL2 upregulation on NSCLC were carried out in *vivo*. The results validated that the tissues generated by inoculation of pcDNA-FHL2 transfected cells were larger and heavier than the tissues generated by the inoculation of pcDNA-NC transfected cells (Figure 4I–K, both p <0.01). The detection of FHL2 expression revealed that the tumor tissues generated by inoculating pcDNA-FHL2 transfected cells had remarkably higher

FIGURE 4 FHL2 upregulation notably facilitated the proliferation and EMT of NSCLC cells both in vitro and in vivo. FHL2 upregulation was carried out in NCI-H292 and H1299 cells by transfecting with pcDNA-NC and pcDNA-FHL2 respectively. (A,B) The mRNA and protein levels of FHL2 in the transfected NCI-H292 and H1299 cells were evaluated by qRT-PCR and western blot respectively. (C,D) Cell proliferation of the transfected NCI-H292 and H1299 cells was tested by $BrdU^+$ assay. (E–G) The proliferation-associated (Ki67 and p21) and EMT-related (E-cadherin and N-cadherin) levels in the transfected NCI-H292 and H1299 cells were tested by western blot. (H): EMT characteristics of the transfected NCI-H292 and H1299 cells were determined by microscope observation. Tumor xenograft formation was completed by inoculating pcDNA-FHL2 transfected cells into BALB/c nude mice. (I–K): Tumor size was measured every 5 days, and tumor weight was measured finally. Tumor volume was calculated employing the formula listed below: tumor volume (V, mm^3) = 0.5 × length × width. (L) FHL2 expression in the generated tumor in BALB/c nude mice was tested by western blot. (M) Ki67 and N-cadherin expression in the generated tumor in BALB/c nude mice were determined by immunohistochemistry. $^{**}p$ <0.01



FHL2 expression (Figure 4L, p <0.01). Additionally, outcomes of IHC assay validated that the expression of Ki67 and N-cadherin in the NSCLC tissues generated by inoculating pcDNA-FHL2 transfected cells was both markedly augmented (Figure 4M, both p <0.01). Thus we have conclusively shown that FHL2 upregulation facilitated proliferation, EMT process and tumorigenesis of NSCLC cells.

4 | DISCUSSION

FHL2 was identified initially as a biomarker of lung adenocarcinoma and has been confirmed to function as an oncoprotein in human LC.[17,18] However, until now, there is still no report that revealed the detailed role of FHL2 in NSCLC. In this research, we preliminarily explored the efficacies of FHL2 on NSCLC both in vitro and in vivo. We found that FHL2 was highly expressed in NSCLC tissues and high FHL2 level is correlated with poor prognosis, which is in line with the results obtained in other diseases.[21-23] Besides, FHL2level, age, pT and pTNM stages were independent factors related to prognosis. These findings illustrate that high levels of FHL2 exacerbated the serious outcome for NSCLC patients. They also indicate that FHL2 might be a new biomarker for NSCLC that could be used for prognostic purposes.

Recently there have been several reports of investigations recording the effects of FHL2 on tumor cell growth. For instance, it was discovered that FHL2 function as oncoprotein in epithelial ovarian cancer (EOC) and FHL2 knockdown distinctly decreased the growth of EOC cells.[24] Another study revealed that FHL2 silencing notably induced apoptosis by increasing the ratio of Bax-to-Bcl2, and repressed cell proliferation in cervical cancer.[21] Furthermore, FHL2 knockdown was reported to enhance apoptosis and block cell proliferation in ovarian cancer.[25] Our research reached similar conclusions, demonstrating that FHL2 knockdown enhanced cell apoptosis as indicated by the increased ratios of cleaved-caspase/caspase 3/9. Besides, FHL2 upregulation markedly augmented proliferation, which was validated by the increased Ki67 and decreased expression of the tumor suppressor gene p21. Taking into account the previous investigations that have shown that miR-340 could repress cancer cell proliferation by targeting various oncogenes including FHL2,[25,26] we deduced that miR-340 might be involved in the increase of NSCLC cell proliferation by increasing FHL2 expression in this study. These studies therefore demonstrated conclusively that FHL2 can function as an oncoprotein in NSCLC, and high level of FHL2, which might be regulated by miR-340, remarkably facilitated the growth of NSCLC cells.

In recent years, EMT has been widely recognized as a critical event during cancer metastasis.[27,28] An accumulating number of research studies have explored the influence of FHL2 expression on cancer metastasis. For example, a previous report demonstrated that FHL2 induced invasion and EMT of colorectal cancer cells by inhibiting the transcriptional activity of E-cadherin.[29] Another study disclosed that FHL2 knockdown markedly abolished Krüppel-like factor 8 (KLF8)-induced invasive and EMT phenotypes in pancreatic cancer.[30] Furthermore, FHL2 was confirmed previously to be a potent EMT inducer in colon cancer. This research displayed that FHL2 overexpression promoted EMT, which was validated by the repressed expression of E-cadherin, and elevated expression of vimentin, snail, twist and MMP-9.[31] In our research, we found that FHL2 knockdown repressed EMT, elevated E-cadherin and lowered N-cadherin expression, which was in high accordance with the aforementioned literatures. More importantly, previous research proved that KLF8, a transcriptional factor, could activate cancer cell invasion and EMT via binding to the promotor of FHL2.[30,32] A combination of these findings support strongly the hypothesis that high level of FHL2 could promote the EMT process of NSCLC, which might be regulated by KLF8.

In addition, to further evaluate the influences of FHL2 expression changes in biological organism, we constructed an in vivo animal model by inoculating the FHL2 silenced/overexpressed cells into BALB/c nude mice. Results showed that the generated tumor volume and weight were both notably decreased in FHL2 silenced cells transfected group. Besides, FHL2 knockdown efficiently enhanced cell apoptosis, but repressed EMT in the generated tumor tissues, which was validated by the increased ratio of apoptotic cells and decreased ratio of EMT-related N-cadherin positive cells. These findings were highly consistent with an increasing range of previous investigations in other cancers: for example, in osteosarcoma, FHL2 silencing reduced tumor growth and the occurrence of lung metastasis in a xenograft experiment[33]; and another study validated that FHL2 knockdown repressed tumor growth using an in *vivo* animal model.[21] In human tongue squamous cell carcinoma (TSCC), the tumorigenicity of FHL2 overexpression group was augmented in in vivo nude mice experiments.[34] Overall taken together these outcomes add further weight to the suggestion that enhanced FHL2 expression could effectively promote tumorigenesis of NSCLC.

The present research validated that FHL2 was highly expressed in NSCLC, that FHL2 knockdown induced apoptosis and repressed EMT, and that FHL2 upregulation aggravated the malignant proliferation and EMT characteristics of NSCLC cells. The novelty of this study is that it reports for the first time that FHL2 expression influences the phenotypes of NSCLC. The outcomes indicated that FHL2 could be a key factor for NSCLC progression and might be used as a diagnosis indicator in future treatment of NSCLC. However, the underlying regulating mechanisms of FHL2 were not uncovered in this research, which will be carried out in our follow-up research.

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CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work.

AUTHOR CONTRIBUTIONS

Ji Zhang and Yongyu Liu contributed to conception and design of the experiments. Na Li and Ling Xu performed the experiments, analyzed the data. Na Li drafted the manuscript. Yongyu Liu finally revised the manuscript.

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