Int J Clin Exp Med 2020;13(6):4000-4010
www.ijcem.com /ISSN:1940-5901/IJCEM0110608

Original Article

Fumarate hydratase inhibits lung adenocarcinoma growth and metastasis through regulating ERK activity

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Received January 30, 2020; Accepted March 31, 2020; Epub June 15, 2020; Published June 30, 2020

Abstract: Objective: Fumarate hydratase (FH) is one of the enzymes in the tricarboxylic acid cycle which facilitates the production of L-malate from fumarate. FH mutations have been found in various malignancies. Our previous studies found that FH was down-regulated in lung cancer, however, its function in lung cancer is still unclear. The current study aims to explore the influence of FH on lung cancer development. Methods and Results: Using ELISA, we found reduced serum FH levels in lung adenocarcinoma (LUAD) patients. Up-regulation of FH using lentivirus vector in LUAD cell lines A549 and PC9 resulted in reduced growth, migration and invasion, while knockdown of FH by siRNA led to enhanced growth, migration and invasion. Western blot revealed that FH over-expression could inhibit ERK phosphorylation, whilst knockdown of FH increased ERK phosphorylation levels in both A549 and PC9 cell lines. Conclusion: Our data suggest that FH is involved in LUAD carcinogenesis through inhibiting LUAD growth and metastasis by suppressing ERK activation.

Keywords: FH, lung adenocarcinoma, growth, migration, invasion, ERK pathway

Introduction

Lung cancer has the highest incidence among all malignancies and is the worldwide number one culprit of cancer death [1]. Non-small cell lung cancer (NSCLC) takes up 85% of all lung cancer cases. The average 5-year survival rate is only 17.7% [2]. Such a high mortality rate results from the fact that most NSCLC patients are already at an advanced stage by the time of diagnosis. Therefore, it is of great importance to better understand the mechanisms involved in the development and progression of NSCLC to provide basis for new treatment strategies.

Fumarate Hydratase is an enzyme involved in the tricarboxylic acid cycle that facilitates the transformation from fumarate to L-malate. FH mutations have been reported in several tumor types including renal cancer [3], malignant paraganglioma [4], Leydig cell tumors [5] and ovarian mucinous cystadenoma [6].

FH affects tumor development in several ways. In renal cancer, FH deficiencies promote tumor growth through inducing glucose uptake and angiogenesis [7, 8]. Accumulation of fumarate caused by FH mutations can promote EMT and enhance migration property [9]. FH mutations also affect cancer development through up-regulation of antioxidant pathways [10] and impaired DNA damage repair [11].

Previously, our group observed lower FH levels in lung cancer tissue than benign lung tissue, as well as lower FH in lung cancer cell line compared to non-malignant lung epithelial cell line [12, 13], which indicates that FH might have a tumor inhibiting role in lung cancer. However, the specific functional role and possible mechanisms that FH has on lung cancer is not clear yet. In the current study, we explored the way FH functions in the pathogenesis lung adenocarcinoma (LUAD). First, we measured FH protein level in lung cancer patient serum using ELISA, and FH expression in LUAD cell lines using Western blot. Then we used a combination of overexpression and knockdown approaches to investigate FH function in tumor growth, migration and invasion. Finally, we used

Western blot to investigate the possible mechanisms involved in these functional changes.

Materials and methods

Participants

Patients are recruited using the following criteria. Inclusion criteria: patient should be 18 year-old and above; patient has symptoms suggestive of lung cancer; patient has radiological findings suggestive of lung cancer. Exclusion criteria: patient has other systemic diseases; patient is currently using medications for other clinical conditions; patient has received any previous chemotherapy or radiation therapy. Finally, 86 patients (24 with lung squamous cell carcinoma, 23 with lung adenocarcinoma, 17 with small cell lung cancer and 22 with benign lung diseases) were recruited between 2016 and 2017 from in-patients in Pulmonary and Critical Care Medicine, Second Affiliated Hospital of Xi'an Jiaotong University. Diagnosis was based on clinical, imaging and histological examinations of bronchoscopy or lung biopsy. Consent forms had been signed by each participant and all studies were conducted under the supervision of Ethics Committee of Second Affiliated Hospital of Xi'an Jiaotong University.

Blood collection and ELISA

Whole blood samples were collected from median cubital vein upon first admission. Samples were centrifuged (10 min, 2,000 rpm) and serum was collected, aliquoted and stored at -80°C for future analysis. Serum FH level was measured with a commercial ELISA kit (Yuanye Biotech, Shanghai, China) following the manufacturer's instructions.

Cell culture

LUAD cell lines A549 and PC9 were cultured with RPMI 1640 cell culture medium (Hyclone, USA). 16HBE, a normal lung epithelial cell line, was cultured in DMEM cell culture medium (Hyclone, USA). All cells were purchased from Chinese Academy of Science. 10% fetal bovine serum (Biological Industries, USA) was added to keep cells nourished. Penicillin and streptomycin solutions (Hyclone, USA) were added to prevent bacterial growth. Cells were incubated at 37°C with 5% CO_2.

FH overexpression using lentivirus

FH overexpression and control lentivirus vectors were manufactured by GenePharma (Shanghai, China). Enhanced Infection Solution and Polybrene (5 μg/ml, Genechem, Shanghai, China) were added to enhance transduction efficiency. Add 40 μl (A549) or 160 μl (PC9) virus suspension (1×10^8 transducing units/ml) to 1×10^5 cells cultured in 6-well plates. After 24 hours, remove the virus suspension and wash twice with PBS. Cells were incubated with culture medium supplemented with 5 μg/ml puromycin for 2 weeks in order to select transfected cells.

FH knockdown using RNAi

SiRNA oligonucleotides were synthesized by GenePharma (Shanghai, China). 7.5 μl Lipofectamine RNAiMAX reagent (Invitrogen, CA, USA) was incubated with 25 pmol siRNA for 5 minutes, then the mixture was add to cells seeded in 6-well plates. The final incubation period was 48 hours. Sequences of the siRNA oligonucleotides are listed as follow: FH-siRNA1: sense chain 5'-GGUGCCAAAUGAUAAGUAUTT-3', anti-sense chain 5'-AUACUUAUCAUUUGGCACCTT-3'; FH-siRNA2: sense chain 5'-GCUGAAGUAAACCAGGAUUTT-3', anti-sense chain 5'-AAUCCUGGUUUACUUCAGCTT-3'; Ctrl-siRNA: sense chain 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense chain 5'-ACGUGACACGUUCGGAGAATT-3'.

Cell proliferation assay

MTT assay was adopted to assess cell proliferation. For measuring cell growth, culture media were removed, then 200 μl 0.5 mg/ml MTT was add to each well. After incubation for 4 hours at 37°C, remove MTT and add 200 μl dimethyl sulfoxide to show color. Afterwards, optical density was measured under an absorbance at 490 nm using a spectrophotometer. This assay was conducted every 24 hours for 3 consecutive days.

Wound healing assay

6-well plates were used for wound healing assay and each well was seeded with 1×10^5 cells. When confluence reached 70~80%, a "wound" was made by gently scratching a line across the center of each well with a pipette tip.

Wash each well twice with PBS and add culture medium supplemented with 1% FBS. Pictures of the gaps were taken under 10× field every 24 hours over a 72-hour period.

Transwell assay

The 24-well transwells (Millipore, MA, USA) were coated with 100 μl 10% Matrigel (BD Bioscience, NJ, USA). Upper chambers have 5×10^4 cells with medium containing no FBS, while lower chambers contain the same culture medium, but were supplemented with 10% FBS to create a nutrition gradient. After 24 hours, cells remained in the upper chamber were gently wiped away, while cells that migrated to the outer-surface of the membrane were stained with 0.1% crystal violet. Cells were observed under 10× field and average numbers of migrated cells were counted from five random fields.

Western Blot

Culture medium was discarded when cells reach logarithmic phase, then cells were gently washed with chilled PBS. Pre-chilled RIPA buffer (Sigma Aldrich, USA) containing proteinase inhibitors and phosphatase inhibitors was added to cells. After incubating at 4°C for an hour, the cell lysate and RIPA buffer mixture was collected and centrifuged (14,000×g, 4°C, 10 minutes), then the supernatants were pipetted to a clean, pre-chilled tube. Loading buffer (Beyotime, Shanghai, China) was added and thoroughly mixed with samples. Samples were heated at 95°C for 10 minutes for protein denature. Electrophoresis with SDS-PAGE was adopted to separate proteins. Separated protein samples were subsequently transferred to a PVDF membrane (Millipore, MA, USA), then the membrane was incubated in 5% skim milk-PBST solution for an hour and incubate with primary antibody overnight. Wash membrane 3 times with PBST after primary antibody and incubate with secondary antibody for 1 hour. After washing 3 times with PBST, add ECL mixture (Millipore, MA, USA) to membrane and capture signals with CCD camera image system (Millipore, MA, USA). Antibodies used in this study: Abcam (Cambridge, UK): FH (ab95947, 1:3000), MMP2 (ab97779, 1:1000) and MMP9 (ab73734, 1:1000); Cell Signaling (Danvers, MA, USA): β-catenin (D10A8, 1:1000), E-cadherin (24E10, 1:1000), ZO-1 (D7D12, 1:1000) and Vimentin (D21H3, 1:1000); Wanleibio (Shenyang, China): ERK (wl01864, 1:200), pERK (wlp1512, 1:200), AKT (wl0003b, 1:200), and pAKT (wlp001a, 1:200).

Statistical analysis

One-way ANOVA with LSD post test was applied to compare serum FH levels in non-cancer patients and various types of lung cancer patients. For comparing serum FH level among different clinical characteristics, student's t-test was used when comparing between two groups and one-way ANOVA with LSD post test was used when there were three or more groups. For MTT, wound healing and transwell assays, the comparison between treatment and control group was performed using student's t-test. For data processing, we used SPSS 21 statistical package and GraphPad Prism 7.0. Data are presented as mean ± SEM (standard error of mean). $P < 0.05$ was considered statistically significant.

Results

Serum fumarate hydratase expression is low in LUAD

86 patients (24 with lung squamous cell carcinoma, 23 with lung adenocarcinoma, 17 with small cell lung cancer and 22 with benign diseases) were recruited. In 22 benign cases, there were 10 cases of pneumonia, 9 cases of tuberculosis and 3 cases of interstitial lung disease. We used ELISA to measure serum FH levels in lung cancer patients, and patients with benign disease were used as control. Comparing with control group, lung cancer patients had lower FH levels in their serum (43.79±2.13 vs 60.10±7.36, **Figure 1A** and **Table 1**). When comparing FH level in different cancer types, LUAD was the lowest and the only one with statistical significance. We also looked at the correlation of FH level with age, sex, smoking index and clinical stage, however, no significant difference was found (**Figure 1B** and **Table 2**). Then we further investigated FH protein levels in LUAD cell lines. We found that FH protein expression was lower in LUAD cell lines A549 and PC9 compared to 16HBE, a bronchial epithelial cell line (**Figure 1C**). These data suggest that FH might function as a tumor-suppressor in LUAD.

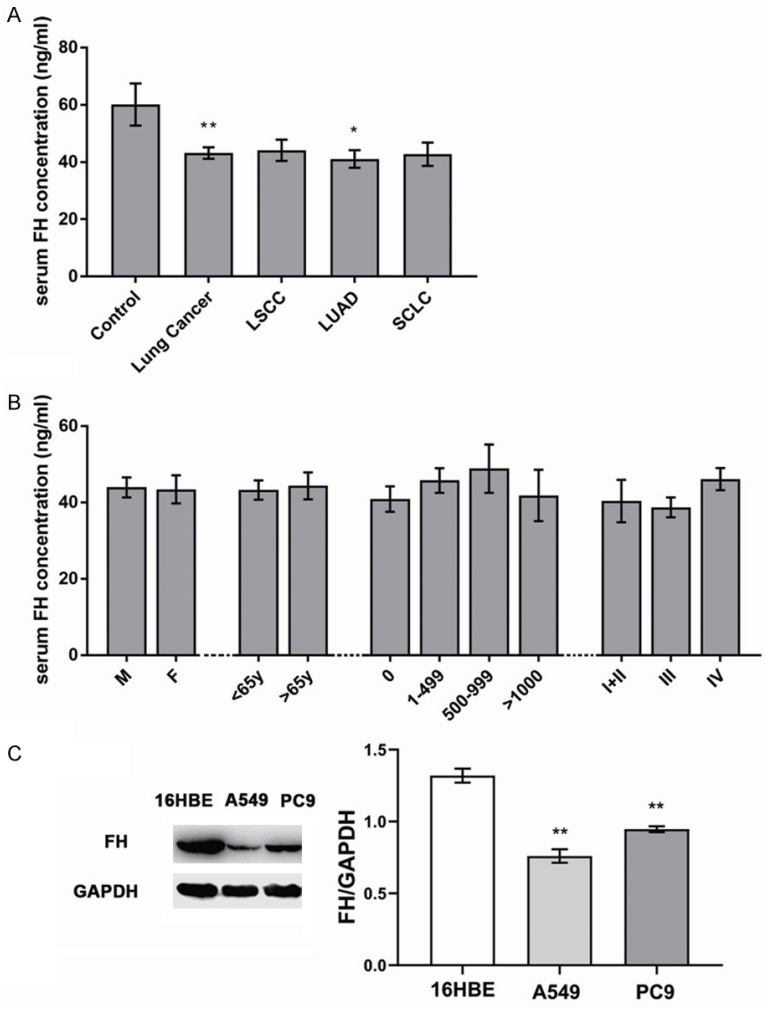

Figure 1. FH level in patient serum and LUAD cell lines. A. Serum level of FH in different patient groups. B. FH serum level in different clinical characteristics. C. Western blot of FH protein level in normal lung epithelial cell line 16HBE and LUAD cancer cell lines A549 and PC9. Data represent mean ± SEM; **, $P < 0.01$, *, $P < 0.05$.

Table 1. Serum FH concentration in different patient types

Pathological subtypes	N	FH (ng/ml)	*P* value
Control	22	60.10±7.36	
Lung Cancer	64	43.79±2.13	0.005
LSCC	24	47.05±3.94	0.444
LUAD	23	41.12±3.06	0.020
SCLC	17	42.79±4.06	0.066

Note: Data shown as mean ± SEM. LSCC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; SCLC, small cell lung cancer.

FH inhibits LUAD cell growth

In order to test our hypothesis that FH might inhibit LUAD development, we first overexpressed FH using lentivirus vector in A549 and PC9 cells (**Figure 2A**). We adopted MTT assay to evaluate the proliferative abilities of cells. We found that in both A549 and PC9 cells, overexpression of FH suppressed proliferation compared to vector group in a time-dependent manner (**Figure 2B**), which suggested that FH could inhibit LUAD cancer cells growth. To confirm, we knocked down FH via small-interfering RNA (siRNAs) in A549 and PC9 cells (**Figure 2C**). In line with what we found in overexpression model, knocking down of FH promoted cell growth in both cell lines (**Figure 2D**). Our findings in both overexpression and knockdown experiments showed that FH inhibits growth of LUAD cells.

FH inhibits LUAD cell migration

In vitro wound healing assay were adopted to evaluate cancer cell migration ability. As demonstrated in **Figure 3A**, **3B**, overexpression of FH significantly inhibited the "healing" ability of A549 cells at 48 hours, and PC9 cells at 24 hours and 48 hours, suggesting that FH inhibits LUAD cell migration. Then we used siRNA to knockdown FH to see if we could reverse this change. As expected, the wound healing speed increased with FH knockdown in both LUAD cell lines (**Figure 3C**, **3D**).

FH inhibits LUAD cell invasion

We used transwell assay to explore the effect of FH on LUAD cell invasion property. Overexpression of FH inhibited invasion of both LUAD cells lines as the cell number migrated through the matrigel and membrane were significantly reduced (**Figure 4A**, **4B**). On the other hand, as we expected, invasion ability of both cell lines were enhanced after FH knockdown as numbers of cells went through the transwell

Table 2. Serum FH concentration and different clinical characteristics

Clinical Characteristics	N	FH (ng/ml)	*P* value
Gender			
Male	42	43.97±2.63	
Female	22	43.45±3.70	0.9089
Age			
< 65 y	33	43.25±2.53	
> 65 y	31	44.36±3.50	0.7980
Smoking Index			
0	28	40.88±3.33	
1-499	19	45.75±3.20	0.3193
500-999	11	48.85±6.306	0.2355
> 1000	6	41.84±6.719	0.9033
Clinical Stage			
I+II	4	40.36±5.557	
III	17	38.71±2.605	0.7863
IV	43	46.12±2.912	0.5582

Note: Data shown as mean ± SEM.

increased significantly (**Figure 4C**, **4D**). These data suggest that FH inhibits LUAD cell invasion.

FH does not regulate EMT in LUAD cells

Epithelial-mesenchymal transition (EMT) is a crucial mechanism that facilitates tumor invasion and metastasis; therefore, we further analyzed protein levels of several EMT molecules. In A549 cells, we observed decreased epithelial markers ZO-1 and E-cadherin in FH knockdown groups, however, no significant changes have been found in mesenchymal markers Vimentin and β-catenin (**Figure 5A**). In PC9 cells, there were decreased expression of E-cadherin after FH knocking down; however, no significant changes were observed in other EMT markers (**Figure 5B**). Therefore, although there were reduced epithelial markers in FH knock down groups in both cells, since there were no change in mesenchymal markers, FH might not affect LUAD cell migration through regulating EMT.

FH suppresses LUAD through ERK pathway

To dissect mechanisms in the cancer suppression of FH, we measured two major signaling pathways regulating cell growth and invasion: the PI3K/AKT pathway and ERK pathway. Overexpression of FH reduced both AKT and ERK phosphorylation in A549 and PC9 cells, whilst no changes in total AKT and ERK were observed (**Figure 5C**). Conversely, knockdown of FH significantly increased levels of p-ERK with no alteration in total ERK in both A549 and PC9 cells. However, p-AKT levels were increased in PC9 cells but remained unaltered in A549 cells with FH knockdown (**Figure 5D**), suggesting that there might be other signals affecting AKT activity. Taken together, FH inhibits LUAD development through regulating ERK pathway.

Discussion

Fumarate hydratase is a highly conserved protein which acts as part of the TCA cycle by hydrolyzing fumarate to generate malate, and it is a tumor suppressor as well. FH mutations have been found in several human cancers and can affect tumor initiation, growth and metastasis [14]. Previous studies of our group showed that FH level was decreased in lung cancer [12, 13]. Another group found that single-nucleotide polymorphisms (SNPs) in FH gene was the primary risk factor contributing to overall survival of NSCLC, indicating that SNPs in FH could be a good biomarker for patient outcome [15]. These data suggest that FH serves as a tumor suppressor in lung cancer. In the current study, we aimed at investigating the role that FH plays in lung cancer, especially LUAD.

For starters, we performed ELISA to measure the serum levels of FH in different patient groups. In consistency with our previous findings [12, 13], lung cancer patients, especially LUAD patients, have significantly lower serum FH levels than patients with benign lung diseases. However, FH levels do not correlate with age, sex, smoking index or clinical stage. These data suggest that FH deficiency promotes LUAD development, and FH level can be used as a diagnostic, but not a prognostic marker. We then compared FH protein levels in two LUAD cell lines A549 and PC9 to that of normal lung epithelial cell line, 16HBE. Both of the two LUAD cell lines showed lower FH protein levels comparing to 16HBE. Our data from patient samples and human LUAD cell lines revealed that FH might serve as a tumor suppressor gene in lung cancer, especially LUAD. Then we proceed to dissect the specific tumor suppressing functions that FH might have on LUAD.

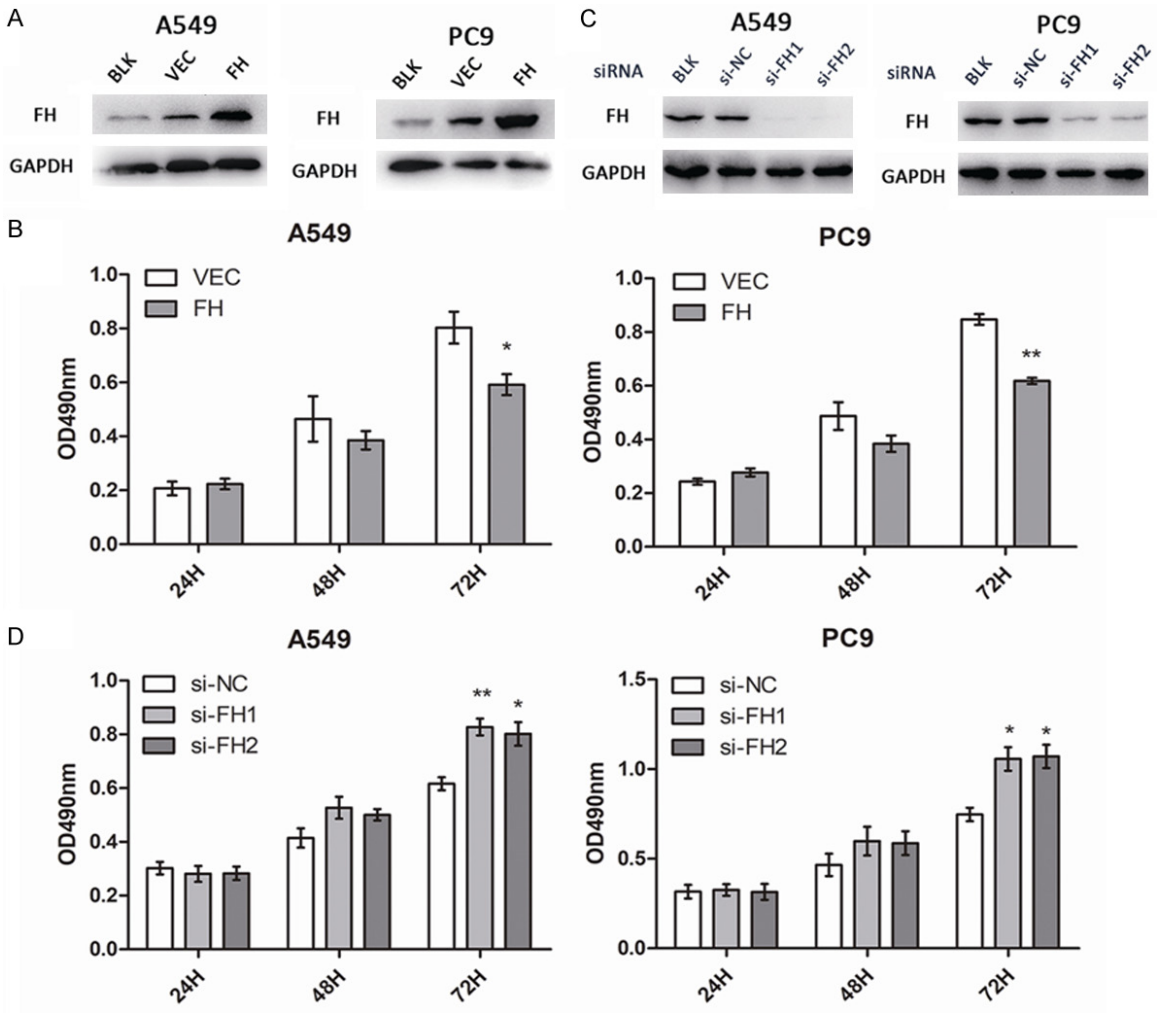

Figure 2. Effect of FH overexpression and down-regulation on cell proliferation. (A and C) FH protein expression was measured by western blot after lentivirus transfection (A) and siRNA interference (C). (B and D) MTT assay was performed with A549 and PC9 cells at 24, 48 and 72 hours after FH overexpression and knockdown. Data represent mean ± SEM; **, $P < 0.01$, *, $P < 0.05$. BLK, blank transfected group; H, hours; OD, optical density; si, small interfering RNA; si-NC, negative control siRNA; VEC, vector transfected group.

Two of the most fundamental traits of cancer cells are their ability to sustain proliferation and to migrate to distant locations. Our data from both overexpression and knockdown of FH revealed that FH had suppressive function on LUAD cell growth, migration and invasion. Dissecting the mechanisms, we found changes in two major signaling pathways: AKT and ERK signaling.

The PI3K/AKT signal transduction cascade is well-known for its roles in oncogenic formation, suppression of apoptosis [16], cell-cycle regulation [17, 18] and metastasis [19]. Studies on renal cancer found that UOK262 cells, which were established from an HLRCC patient [20], had FH mutation and displayed nearly absent mitochondrial complex I activity [21], and loss of complex I activity has been proven to increase ROS level, leading to AKT phosphorylation [22]. In our study, we detected decreased AKT phosphorylation in both cell lines after FH overexpression, however, we did not find increased AKT activation after FH knockdown in A549 cells, indicating that AKT might not play a major role under this circumstance.

The RAS/RAF/ERK pathway is an evolutionary conserved signaling cascade that relays signals from cell surface receptors to mediate proliferation and survival [23, 24]. Abnormal activities in ERK pathways has been found in various

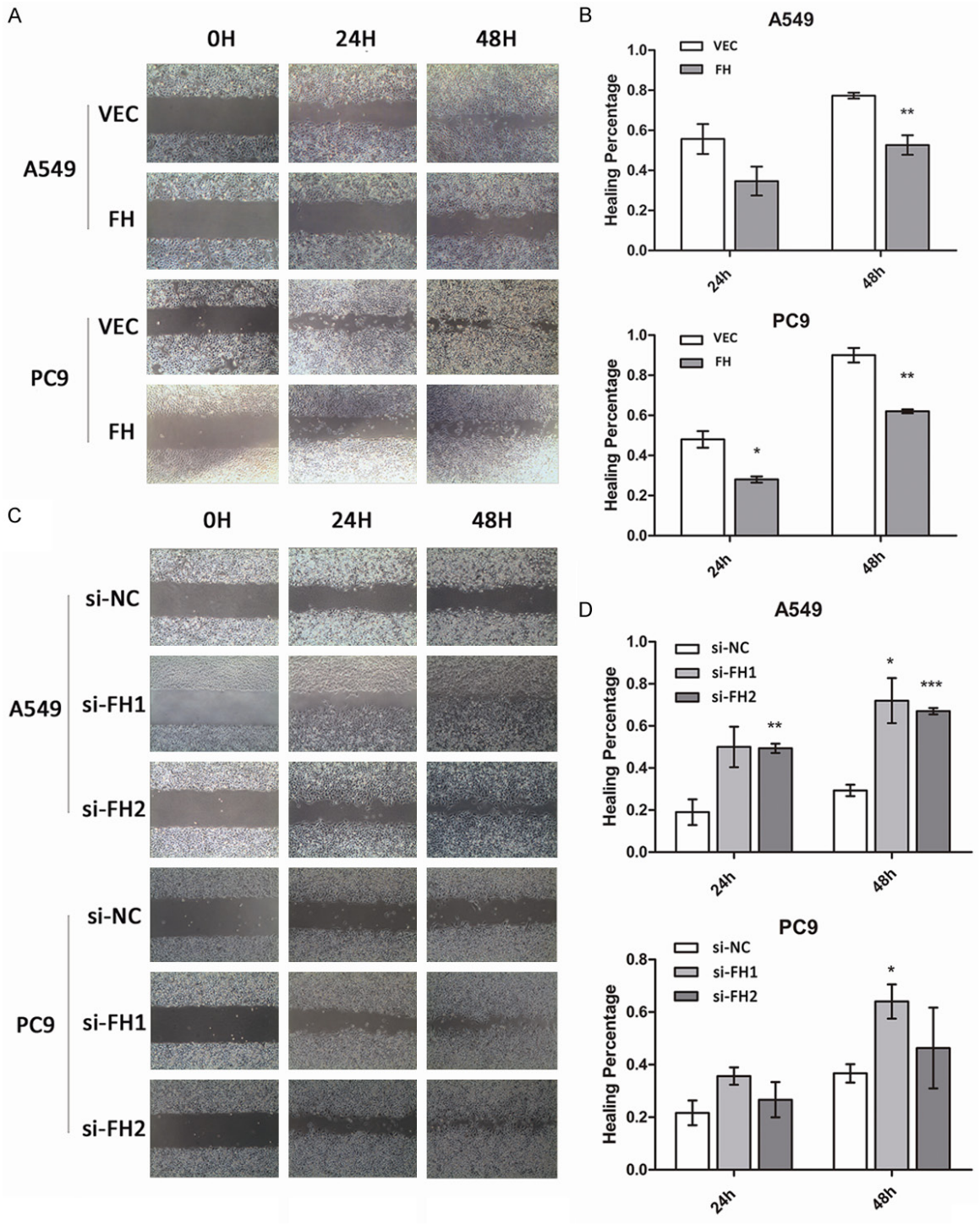

Figure 3. Effect of FH on cell migration ability by wound healing assay. A and C. Wound healing assay was used to investigate the influence of FH on cell migration property. B and D. Quantification of healing rate. Pictures were acquired under 10× field 24 and 48 hours after seeding. Data represent mean ± SEM; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$.

malignancies, including lung cancer [25]. There has been no report about the effect of FH on ERK signaling yet. In the present study, we found reduced activation of ERK after FH overexpression and increased ERK activity with FH knockdown, which explained reduced tumor

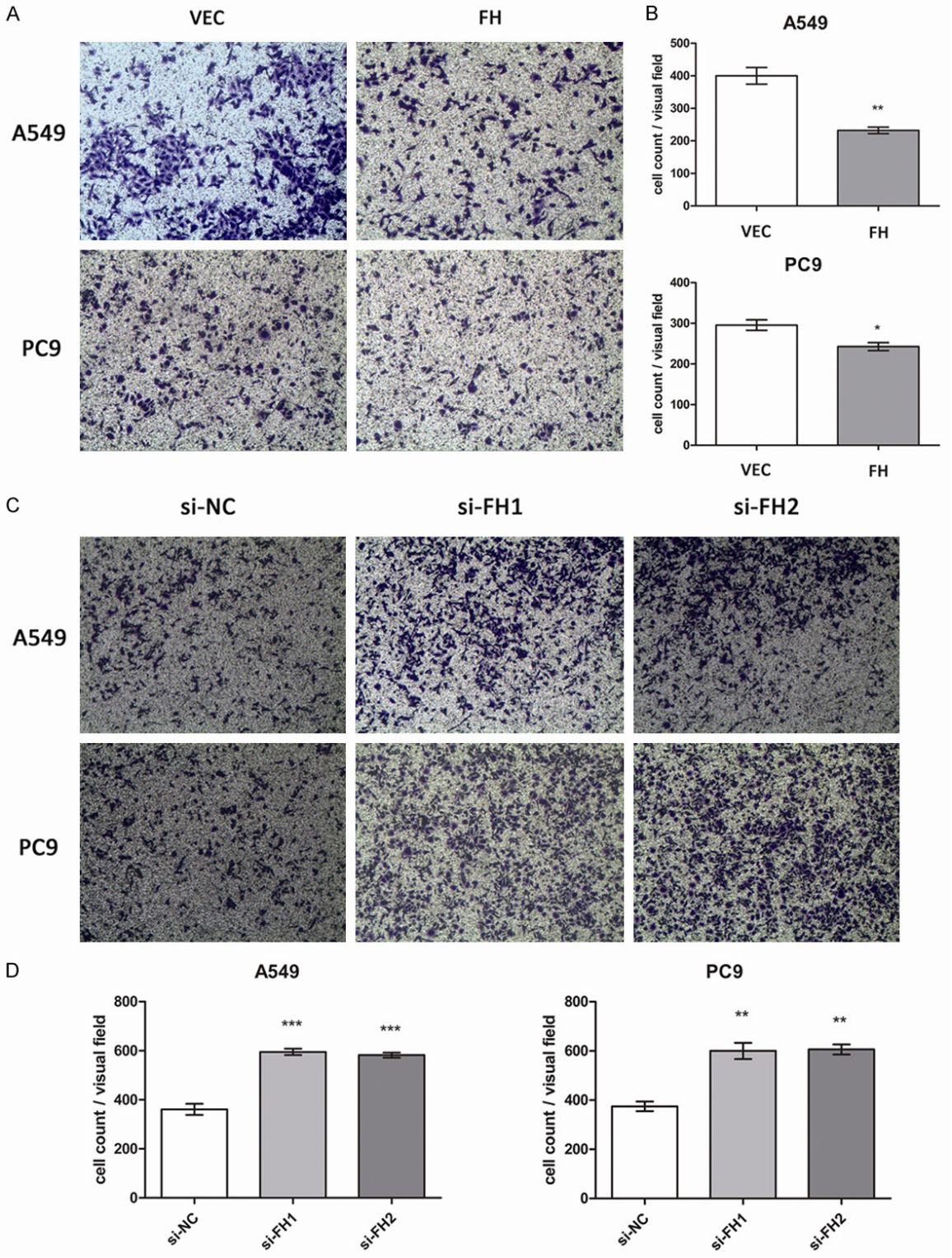

Figure 4. Effect of FH on cell invasion ability by transwell assay. A and C. Transwell assay was used to investigate the effect of FH on cell invasion property. B and D. Quantitative analysis of migrated cell number. Pictures were acquired under 10× field 24 hours after seeding. Data represent mean ± SEM; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$.

cell proliferation after overexpression and enhanced growth after FH knockdown. Our findings suggest that FH mutation regulates LUAD growth via ERK signaling pathway.

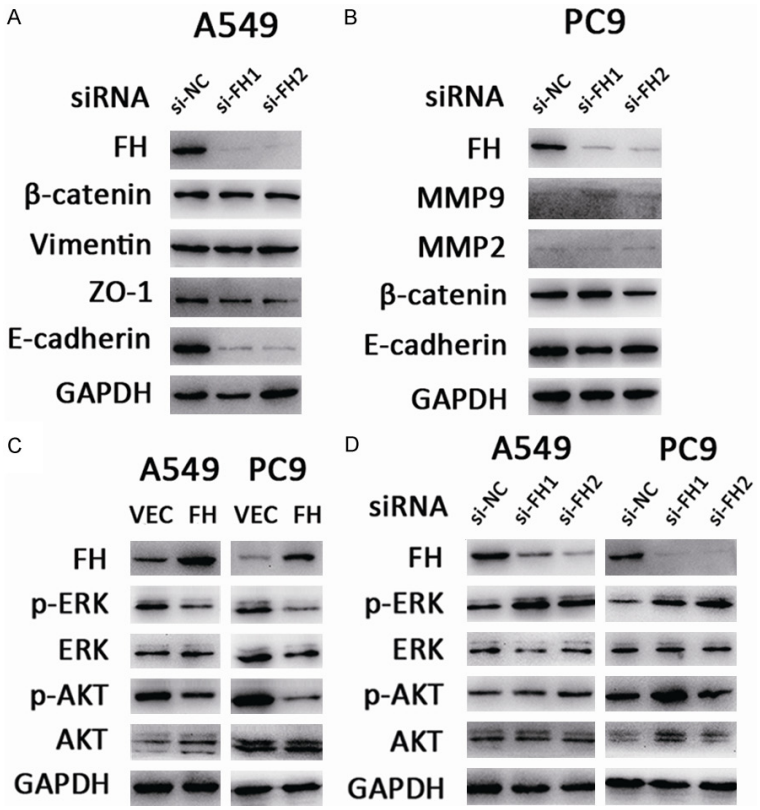

Figure 5. Western blot of EMT markers, AKT and ERK pathways after FH overexpression and knockdown. A and B. EMT markers in A549 and PC9 cells after FH knockdown. C. Protein levels of AKT, p-AKT, ERK, p-ERK after FH overexpression. D. Protein levels of AKT, p-AKT, ERK, p-ERK after FH knockdown.

One of the mechanisms involved in tumor cell proliferation is the loss of contact inhibition [26]. Curto M found that stabilizing adhesion molecules (e.g. E-cadherin) inhibits cancer cell growth by strengthening contact growth inhibition [27]. When analyzing EMT markers, we observed no significant change in FH knockdown cells except for decreased E-cadherin and ZO-1, which are important epithelial cell surface adhesion molecules that keep cells in tight junction, and the loss of them might explain increased proliferation after FH knockdown. Thus, our findings revealed that FH deficiency might not cause EMT in LUAD, but decrease cell-to-cell adhesion to evade contact growth inhibition and thus promote cell growth.

Taken together, our study found that FH level is reduced in LUAD patients and FH functions as a tumor suppressor that inhibits LUAD growth, migration and invasion through regulating ERK pathway. However, our study has its limitations and further investigation is needed to support our findings. FH plasma levels suggest that FH expression is lower in LUAD patients, however, FH expression in LUAD tissue samples may provide stronger evidence. Changes in p-ERK levels suggest possible interplay between FH and ERK pathway; however, further experiment involving changes of other signaling molecules in ERK pathway, such as RAS and RAF, as well as direct molecular interactions are needed to elucidate the underlying mechanisms. In all, FH might be a potential biomarker and tumor suppressor for LUAD, which provides basis for new diagnostic and therapeutic strategies in LUAD.

Acknowledgements

This study was supported by National Natural Science Foundation of China (NSFC) (grant number: 81672300).

Disclosure of conflict of interest

None.

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