



ORIGINAL ARTICLE

Flotillin1 promotes EMT of human small cell lung cancer via TGF- β signaling pathway

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ABSTRACT

Objective: Small cell lung carcinoma (SCLC) is considered one of the most aggressive types of lung cancer due to its rapid growth and early metastasis. No tumor markers or therapeutic targets have been demonstrated to be specific or effective in SCLC to date. This study aims to evaluate the potential of Flotillin1 (Flot1) as a target of SCLC treatment.

Methods: Flot1 expression level in the tissue of SCLC and other tissue of lung disease was detected using immunohistochemical staining. Transwell and Matrigel assays were employed to examine migration and invasion of cancer cells. Flow cytometry and xCELLigence system were used to evaluate cell apoptosis and cell viability, respectively. Expression levels of Flot1, epithelial-mesenchymal transition (EMT) marker E-cadherin, vimentin, cyclinD1, TGF- β -Smad2/3, and p-AKT were examined using Western blot. Furthermore, xenograft tumor in nude mice was used to evaluate the growth and metastasis of NCI-H446 cells *in vivo*.

Results: Our results demonstrated that Flot1 is highly expressed in SCLC samples and that its expression correlates strongly with clinical stage, distant metastasis, and poor survival. The knockdown of Flot1 decreased the growth, migration, and invasiveness of SCLC cells and reversed EMT phenotype *in vitro* and *in vivo*, while enhanced Flot1 expression exhibited the opposite behavior. Gene expression profile analysis demonstrated that Flot1-regulated genes frequently mapped to the AKT and TGF- β -Smad2/3 pathways. Our results further revealed that Flot1 affected the progression of SCLC via regulation of EMT progression.

Conclusions: These findings indicated an oncogenic role of Flot1 *via* promoting EMT in SCLC and suggested its potential as a tumor marker and prognostic indicator.

KEYWORDS

Flotillin1; small cell lung cancer; EMT; TGF- β -smad2/3

Introduction

It has been reported that lung cancer is one of the leading causes of cancer-related deaths worldwide. Small cell lung cancer (SCLC) is a highly aggressive disease among various histological types of lung cancer, representing 15%–20% of all lung cancers, with a median survival of < 2 years¹. Chemotherapy remains the primary method used to treat SCLC patients, because SCLC does not respond well to surgery or radiotherapy^{2,3}. However, the failure of

chemotherapy is primarily due to the emergence of drug resistance. Therefore, early diagnosis maybe the best means to improve the survival rate of patients with SCLC.

It has been reported that lipid rafts are associated with the progression of several malignancies^{4,5}, thus they may act as a potential therapeutic targets for malignant cancers. The flotillin family contains two homologous isoforms, flotillin1 and 2, which play essential physical roles in various cellular processes, such as adhesion, reorganization of the actin cytoskeleton, endocytosis, phagocytosis, and transduction of cellular signals^{6,7}. Flotillin1 (Flot1) has been reported to be involved in the progression of cancer including cervical carcinoma⁸, breast cancer⁹, and clear-cell renal cell carcinoma¹⁰. Moreover, a recent meta-analysis reported that a high expression level of Flot1 protein was correlated with poor clinical outcome in patients with various solid tumors¹¹.

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Li et al.¹² reported that the level of Flot1 protein was positively correlated with tumor size, tumor stage, and lymph node metastasis in non-small cell lung cancer (NSCLC). However, until now, the expression and function of Flot1 in human SCLC have remained largely unknown.

Our present study aimed to clarify the essential role of Flot1 in the tumorigenesis and progression of SCLC. In this study, the association of Flot1 with the survival rate of patients with various types of lung cancer was analyzed using a public database¹³, and the expression level of Flot1 in tissues derived from patients suffering from various lung diseases, especially SCLC, was investigated. In addition, with a combination of overexpression and knockdown approaches, we found that Flot1 overexpression promoted the proliferation and metastasis of SCLC, whereas its downregulation inhibited the proliferation and metastasis of SCLC both *in vitro* and *in vivo*, which suggested a tumorigenic role for Flot1. Our present study is the first to demonstrate that Flot1 is a critical and powerful regulatory protein and that it may be a potential molecular marker and therapeutic target for SCLC.

Patients and methods

Patients

Ninety one histologic sections of clinical tissue samples were obtained from 91 SCLC patients who underwent surgery or thoracic puncture surgery at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) from January 2012 to December 2013. The study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University (2016MEC110) and consent of all patients for tissues has obtained. None of these patients had received radiotherapy or chemotherapy prior to puncture surgery or surgery. Data of overall survival (OS) from 88/91 patients were analyzed. Considering the difference of clinical treatment guidelines, patients (27/91) who were in the extensive stage were treated with combination of chemotherapy and radiotherapy; and the patients (52/91) who were in the limited stage were treated with chemotherapy alone, so we divided these patients into two groups to analyze the progression-free survival (PFS). A lung disease tissue microarray (including squamous carcinoma, adenocarcinoma, SCLC, inflammatory pseudo tumor, tuberculosis, para-carcinoma, and normal lung tissues) was obtained from Shanghai Outdo Biotech Co., Ltd (Shanghai, China).

Ethical approval

All procedures were conducted according to guidelines defined by State Key Laboratory of Molecular Oncology, National Cancer Center/Cancer Hospital.

Materials

The SCLC cell lines NCI-H446 and NCI-H1688 were purchased from the Cellular Biology Institute of the Shanghai Academy of Sciences (Shanghai, China). Both cell lines were maintained in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL phytoerythrin, and 10% fetal calf serum (FCS), and were cultured in a humidified atmosphere in an incubator at 37°C and with 5% CO₂. RPMI-1640, PBS RPMI-1640, and FCS were purchased from Gibco-BRL (Life Technologies, Paisley, Scotland). Antibodies against total AKT, p-AKT, smad2/3, and p-smad2/3 were all obtained from Cell Signaling Technology, Inc. (CST, CA, USA). Antibodies against E-cadherin, cyclinD1, vimentin, caspase-3, PARP, TGF-β, MMP9, and Flot1 were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and transfection

Flot1 overexpression plasmid was obtained from Addgene (Cambridge, MA, USA) and pCDNA3.1 (+) was used as vector. siRNA oligonucleotide against Flot1 (Invitrogen, siFlot1-1: 5'-CAGAGAAGUCCCAACUAAUUA-3'; siFlot1-2: 5'-CUGCUUGGCUUUAGCUUCCCG-3') was prepared using RNAiMax and Lipofectamine® LTX with PlusTM Reagent (Life Technologies, Gaithersburg, MD, USA). Flot1-specific siRNAs and control siRNAs were transfected into SCLC cells using Lipofectamine 2000 (Invitrogen). Flot1 shRNA was synthesized by Genecopoeia (Guangzhou, China) with the target sequence: 5'-CAGAGAAGUCCCAACUAAUUA-3'. pCDH-CD511B-GFP (SBI) was used to as retroviral vectors. The packaging plasmids including pMD.MLV (1.5 µg), pVSV.G (0.5 µg), and the retroviral vectors mentioned above (2 µg) were transfected into HEK-293 T cells using effectene transfection reagent. The media containing the lentivirus were collected at 48 h and 72 h after transfection. The virus-containing pellet was dissolved in RPMI 1640 and stored at -80°C. Viral transduction was done using viral supernatant (25%) supplemented with Polybrene (8 µg/mL, Santa Cruz Biotechnology) by incubating the cells overnight at 37°C. Infected cells were confirmed by RT-PCR 96 h after infection. The primer sequences used for RT-qPCR were: β-actin, F: 5'-GTCAC

CAACTGGGACGACAT-3', R: 5'-GAGGCGTACAGGGATAGCAC-3'; Flot1, F: 5'-TGTCTTGAAGGGGGTTGCTC-3', R: 5'-TGAAAACCTCCAGCCCATCC-3'; twist2, F: 5'-GGCATTATGGTCAAACGGGC-3', R: 5'-TCCACCCTCTACCTC TCAGC-3'; sox11, F: 5'-CGGTCAAAGTGCCTGTTTCTG-3', R: 5'-CACTTTGGCGACGTTGTAGC-3'; cdc42, F: 5'-GTGTGTTGTTGTGGGCGATG-3', R: 5'-TGTGGATAACTCAGCGGTCG-3'.

Cell proliferation and colony forming assay

Cell proliferation was measured using the xCELLigence system (Roche) and CellTiter 96 Aqueous non-radioactive cell viability assay [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetazolium]. xCELLigence is a real-time and label-free cell analysis system, the protocol for which has been previously described¹⁴. Briefly, 2000 cells in 50 μ L culture medium were added into each well for the impedance baseline measurement, and then, a final volume of 150 μ L was prepared by adding 100 μ L culture medium. The E-Plates were incubated at 37°C under 5% CO₂ and were monitored in the system at 15-minutes time intervals for up to 100 hours. For the MTS assay, the SCLC cells were seeded on 96-well plates at the density of 2000 cells/well for proliferation. The cells were incubated for the indicated time, and MTS solution was added (20 μ L/well) to the cells. After incubation for 2 h at 37°C, their absorbance at 492 nm was measured using a microplate reader. For colony forming assay, 1000 SCLC cells were seeded on 6-well plate in triplicates and were cultured in a 5% CO₂ humidified incubator for indicated number of days. Medium was changed after incubation for 2 days. Then the cell colonies were fixed with 4% paraformaldehyde for 15 min and air dried. The colonies were stained by adding 3 ml of 2% crystal violet for 5 minutes and counted.

Tumor cell migration and invasion assays

For the Transwell (Corning Costar, Cambridge, MA, USA) migration assays, 1×10^5 NCI-H446 or NCI-H1688 cells were seeded on the non-coated membrane of the upper chamber (24-well insert; 8- μ m pore size). The cells were seeded in serum-free medium, and 0.6 ml medium supplemented with 10% FBS was added to the lower chamber as a chemoattractant. The non-migrating cells on the upper surface of the membrane were removed with a cotton swab after 24 h incubation, and the cells that penetrated the lower surface of the membrane were stained with crystal violet. The number of cells that penetrated the membrane was counted

under a microscope from five randomly selected fields. Data was obtained from three independent experiments.

Western blot

Transfected cells or treated cells were lysed in lysis buffer (1% Triton X-100; 150 mM NaCl; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA, pH 8.0; 0.2 mM Na₃VO₄; 0.2 mM phenylmethylsulphonyl fluoride; and 0.5% NP-40), and the protein concentrations were measured using a BCA protein assay kit (Beyotime, Nanjing, China). Protein extracts were separated by 10%–15% SDS-PAGE, transferred to PVDF membranes, and incubated with antibodies, as shown in the results section. Endogenous β -actin was used as control to normalize the level of proteins. Peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technology) was used as a secondary antibody, and enhanced chemiluminescence (ECL) (EMD-Millipore, Billerica, MA, USA) was used to visualize the antigen-antibody reaction.

In vivo tumor growth assay

A xenograft mouse model used 4–6 week-old male nude mice that were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China); mice were maintained in an accredited animal facility according to standard institutional guidelines. Nude mice were subcutaneously inoculated with cells with stable downregulation of Flot1 in their left flanks and were inoculated with control cells in their right flanks. The tumors were continuously monitored for 4 weeks, and the volume of each tumor was measured using the formula as follows: $1/2 \times (\text{width})^2 \times (\text{length})$. Immunohistochemical staining was performed to detect the expression of E-cadherin, vimentin, p-AKT, and TGF- β in tumor tissues. For the metastasis model, the tail veins of 6 nude mice were injected with either 0.5×10^6 NCI-H446 cells, in which Flot1 was downregulated, or with control cells. Nine weeks later, tumor nodules in the lung were observed and examined histologically. The tumors that developed in these animals were imaged using micro-PET-CT (positron emission tomography-computed tomography) following injection of 18F-FDG [2-(18F)-fluoro-2-deoxy-D-glucose] into the tail vein.

Immunofluorescence method

NCI-H446 and NCI-H1688 cells were seeded on glasses and fixed with 4% paraformaldehyde for 15 min. All sections were in micrometers cryostat and fixed in methanol at -20°C

for 10 min, and then rehydrated in PBS. Non-specific binding in incubating sections was blocked by 1% of bovine serum albumin (BSA) in PBS for 30 min. Glasses were double-stained for pimonidazole in combination with Flot1 or DAPI. Glasses were rinsed in PBS and mounted with ProLong® Gold anti-fade reagent (P-36931, Invitrogen).

Immunohistochemistry (IHC) and pathological analysis

IHC of tumor tissues was performed according to the streptavidin-peroxidase (SP) method using the appropriate antibodies; the 3,3-diaminobenzidine (DAB) colorimetric reagent solution that was used to visualize the staining was purchased from Dako (Carpinteria, CA, USA). The results of the IHC were analyzed by two pathologists independently in a blinded manner and without prior information of the patients' clinical characteristics. We visualized and classified protein expression based on the percentage of positive cells and the intensity of staining. The percentage of positively stained cells was scored 0–3 (0 points for no cells stained, 1 point for < 25%, 2 points for 25%–75%, 3 points for > 75% of cells stained) and protein staining was scored 0 point for negative, 1 for (+), 2 (++) and 3 (+++++). The two scores were then multiplied to yield a total immune activity score, which demonstrated the protein expression in a sample. The intensity of immune activity was graded on a scale of 0–2 for low expression and scale of 3–6 for high expression.

Microarray for the detection of Flot1-target gene

Total RNA from human NCI-H446 cells, in which Flot1 was stably knocked down, and wild type NCI-H446 cells was isolated and quantified. The RNA integrity was assessed by standard denaturing agarose gel electrophoresis. The aberrant expression profiles were determined using RiboArray™ Custom Array (12 × 90K A10000-1-90) and with an Axon GenePix 4000B scanner. RMA (Robust Multi-array Average) method was performed to normalize samples and analyze subsequent data. The transcript profiling data were deposited in the Gene Expression Omnibus of NCBI and are accessible through the GEO series accession number GSE99337.

Statistical analysis

SPSS version 13.0 software were performed to analyze all

results. One-way analysis of variance, Fisher's exact test, Chi-square test, and Student's *t*-test were performed for comparisons, as described. The Mann-Whitney U test and the Kruskal-Wallis test were used to compare the clinical characteristics or the tumor volume. The survival rates of the mice were compared using a Kaplan-Meier analysis. Chi-square test was used to analyze the categorical variables. Correlation between proteins was analyzed by Spearman's rank correlation analysis. *P* values less than 0.01 or 0.05 were considered statistically significant, and all statistical tests were two-sided.

Results

The correlation between Flot1 expression in lung cancer and the clinical outcome

To evaluate the effect of the Flot1 expression level on the clinical prognosis of lung cancer, the correlation between Flot1 expression and clinical outcome of patients with either lung adenocarcinoma (LUAD, *n* = 500), lung squamous cell carcinoma (LUSC, *n* = 494), or both (LUSC + LUAD, *n* = 994) using the data from the Human Protein Atlas (www.proteinatlas.org) was explored. We found that high Flot1 expression was correlated with poor survival probability in LUSC patients, but not in patients LUAD (**Figure 1A** and **1B**). Moreover, the Flot1 expression level also had no significant correlation with the total lung cancer patient cohort (LUSC + LUAD) (**Figure 1C**), which suggests that Flot1 may act as a negative prognostic factor in patients with LUSC (*P* < 0.05), whereas its expression is not correlated with the prognosis of patients with LUAD. These results also demonstrated that the effect of Flot1 expression on the clinical prognosis of lung cancer might depend on the subtype of lung cancer. However, there is no any data for Flot1 in SCLC as indicated in the public database of Human Protein Atlas.

High level of Flot1 is correlated with progression and poor prognosis of patients with SCLC

To examine the expression level of Flot1, we first detected Flot1 expression in lung disease including various lung cancer tissues using a tissue microarray. As shown in **Figure 2** and **Supplementary Figure S1**, the protein levels of Flot1 were significantly high in SCLC samples compared with normal lung tissue and tissues from patients with other types of lung diseases including squamous carcinoma,

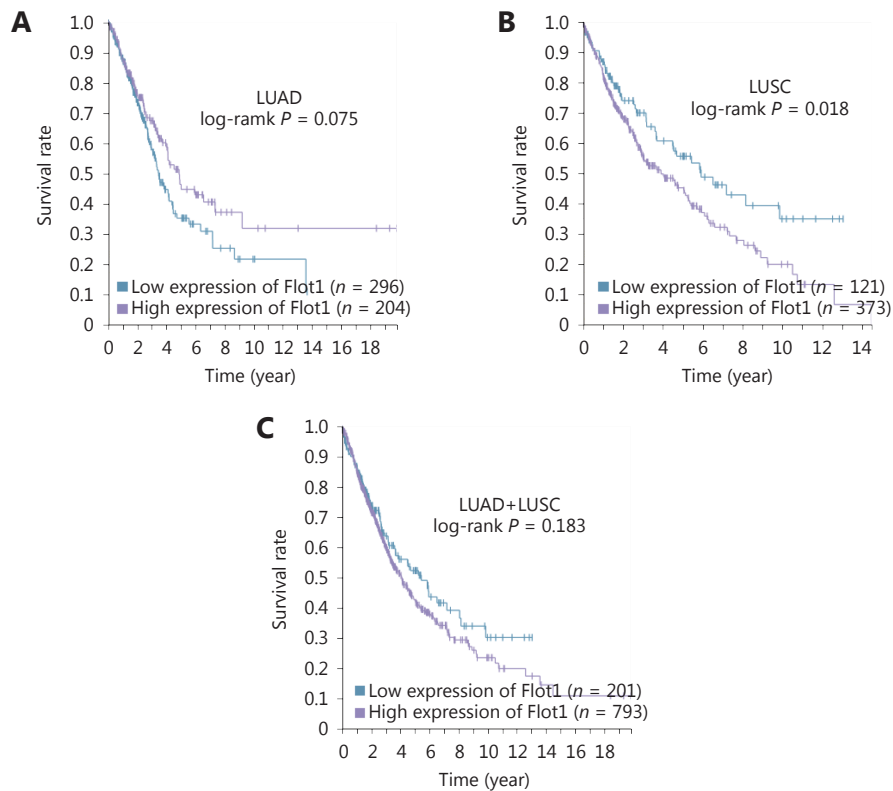


Figure 1 Kaplan-Meier overall survival curves for lung cancer patients according to the protein expression level of Flot1 from The Human Protein Atlas data. (A) The effect of the Flot1 expression level on the clinical prognosis of LUAD patients was analyzed by Kaplan-Meier survival analysis using the overall survival data of the patients (cohort 1, $n = 500$, $P = 0.075$, log-rank test). (B) The effect of the Flot1 expression level on the clinical prognosis of LUSC patients was analyzed by Kaplan-Meier survival analysis using the overall survival data of the patients (cohort 2, $n = 494$, $P = 0.018$, log-rank test). (C) Kaplan-Meier overall survival curves and recurrence-free survival curves for LUAD patients and LUSC patients according to the Flot1 expression level (cohort 1+ Cohort 2, $n = 994$, $P = 0.183$, log-rank test).

adenocarcinoma, inflammatory pseudotumor, tuberculosis, and para-cancerous tissue. This suggests that Flot1 might be a highly specific marker of SCLC. Furthermore, we also found that the Flot1 expression level was higher in LUSC carcinoma tissue than in LUAD tissue. This result is consistent with the result discussed above that Flot1 expression was closely correlated with the progression of LUSC, but not that of LUAD.

To further determine the Flot1 expression level and its clinical relevance in patients with SCLC, IHC was performed to investigate the expression of Flot1 in 91 SCLC samples. Immunohistochemical analysis showed that the Flot1 protein was highly expressed in 67.03% of SCLC samples (61/91) and that it was significantly overexpressed in cancer tissues compared with matched adjacent normal lung tissues from the same donor (Figure 3A and 3C). Moreover, Flot1 expression levels were higher in the tissues of stage III–IV compared with that in the stage I–II tissues of SCLC or normal lung tissue (Figure 3A and 3B). We further found

that Flot1 expression was positively correlated with clinical stage ($P = 0.029$) and distant metastasis of SCLC ($P = 0.008$) as indicated in Table 1. Furthermore, a Kaplan-Meier survival analysis demonstrated that higher Flot1 levels in patients were correlated with shorter overall survival (OS, $P = 0.04$, Figure 3D). The median survival of patients with SCLC with high Flot1 expression and low Flot1 expression was 13 and 18 months, respectively. Notably, high Flot1 expression was also significantly correlated with shorter progression-free survival in the limited stage of SCLC patients (PFS, $P = 0.002$, Figure 3E) and extensive stage of SCLC patients (PFS, $P < 0.001$, Figure 3E). Taken together, these results indicated that Flot1 protein was a marker of SCLC and it was positively correlated with poor prognosis of patients with SCLC.

The effect of Flot1 on SCLC cell proliferation and apoptosis

To examine the biological function of Flot1 in SCLC cells, we

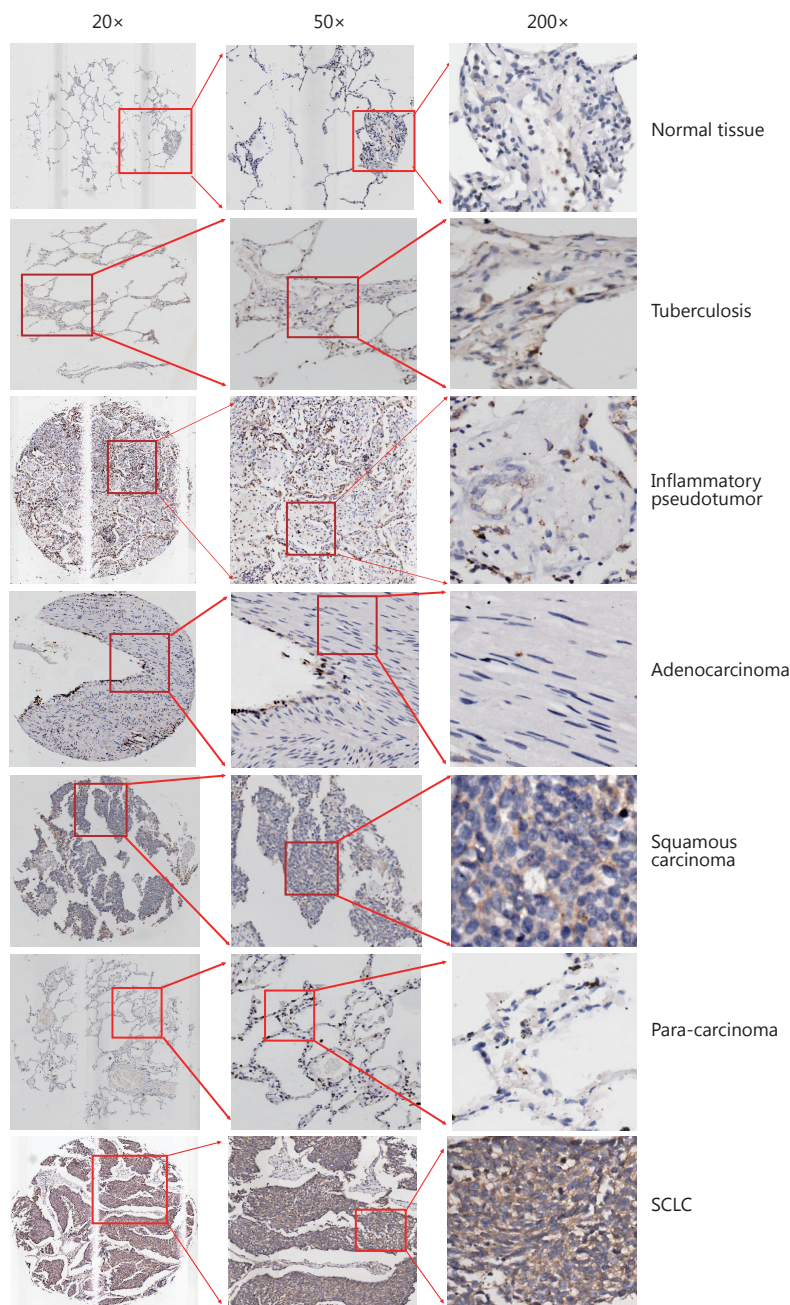


Figure 2 Flot1 is highly expressed in SCLC tissues Expression of Flot1 in tissues of various lung diseases was detected by IHC, and representative images are shown.

silenced Flot1 expression in the two SCLC cell lines NCI-H446 and NCI-H1688. The knockdown efficiency was confirmed by immunoblotting (**Figure 4A**). We examined the role of Flot1 in cell proliferation using the xCELLigence System and found that knockdown of Flot1 expression inhibited the proliferation of NCI-H446 and NCI-H1688 cells (**Figure 4B**), whereas overexpression of Flot1 promoted proliferation of NCI-H446 and NCI-H1688 cells

(**Supplementary Figure S2A and S2B**). In addition, we found that silencing of Flot1 expression led to significant decrease in colony formation (**Figure 4C and 4D**). In addition, cell cycle analysis by flow cytometry showed that the knockdown of Flot1 with siRNA resulted in an increase in the percentage of cells in G1 phase from 55.89% to 69.80% or 71.80% for two sequence of siRNA for Flot1 in NCI-H446, respectively (**Figure 4E and 4F**), suggesting that inhibiting Flot1 could

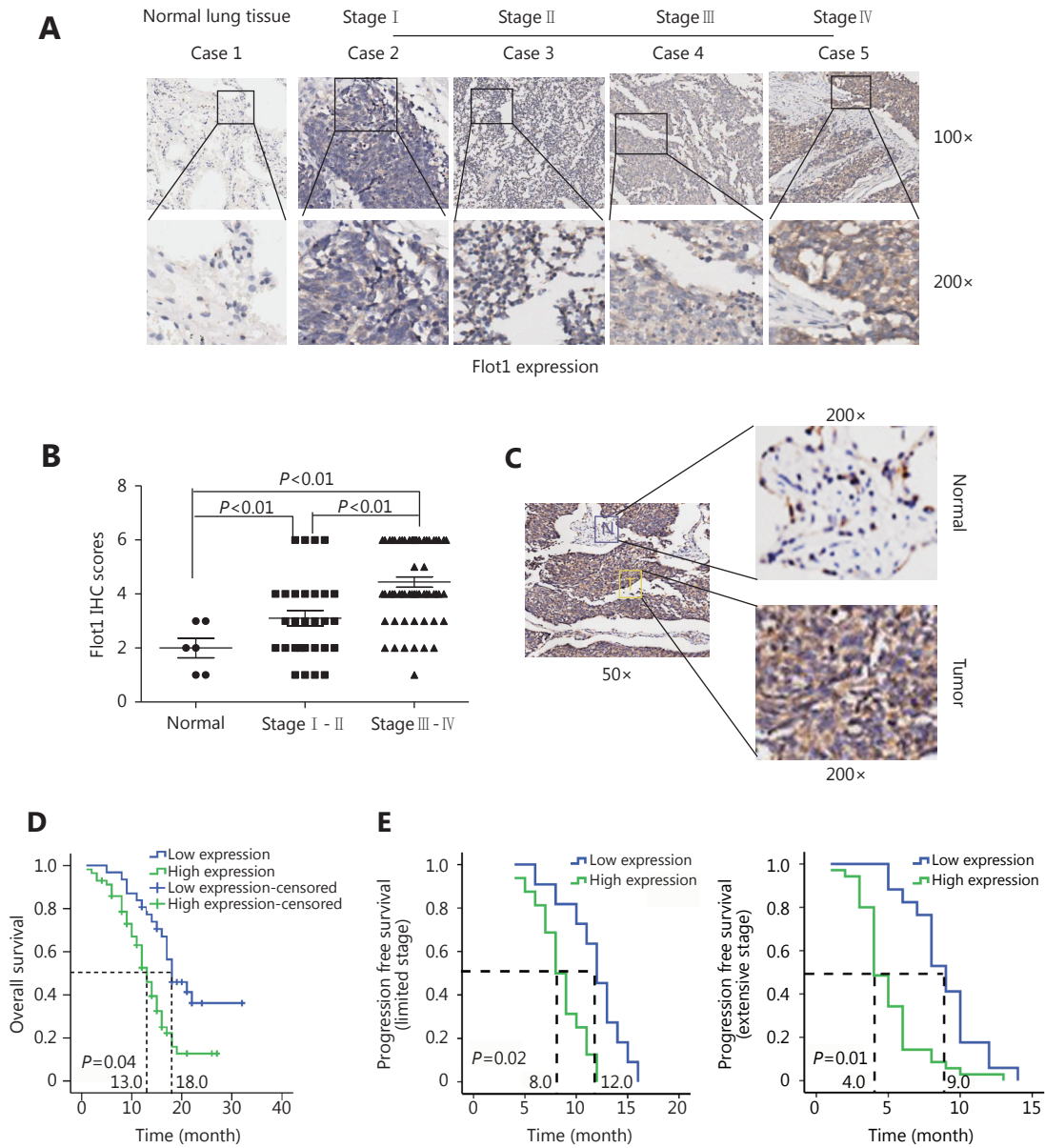


Figure 3 Flot1 is positively correlated with the progression and prognosis of SCLC. (A) Immunohistochemical staining indicated that Flot1 expression was upregulated in human stage III–IV SCLC tissue compared with stage I–II SCLC tissue and normal lung tissue. (B) The IHC scores of Flot1 expression were analyzed according to different clinical stages by two-tailed Pearson test. Flot1 expression was higher in human stage III–IV SCLC tissue compared with I–II stage SCLC tissue ($P < 0.01$) and normal lung tissue ($P < 0.01$). (C) Representative images of Flot1 in normal lung and lung primary tumors from the same donor are shown (IHC staining). (D, E) Kaplan-Meier overall survival curves and progression-free survival (PFS) of SCLC patients with low versus high expression of Flot1 (cohort 2, $n = 88$, 3/91 patients in cohort 1 were lost to follow up, log-rank test). The expression of Flot1 scores 0 to 2 was considered as low expression, and the expression of scores 3 and 4 was considered as high expression. All statistical tests were two-sided.

arrest the SCLC cells to G1 phase. We also treated cells with cisplatin, one of the most active cytotoxic agents used to treat SCLC³, under the knockdown of Flot1. As depicted in **Figure 4G** and **4H**, Flot1-depleted cells showed an increased sensitivity to cisplatin-induced apoptosis compared with

control cells. Together, our data suggested that Flot1 deficiency results in proliferation inhibition, cell cycle arrest, and increase in sensitivity of SCLC cells to cisplatin, suggesting that Flot1 could be a potential target of SCLC treatment.

Table 1 Correlation between Flot1 expression and clinicopathologic features in 91 SCLC patients

Characteristics	Flot1		Chi-square	P
	Low (n = 30)	High (n = 61)		
Age, years			0.139	0.710
> 60	14	31		
≤ 60	16	30		
Gender			0.08	0.778
Male	22	43		
Female	8	18		
Smoker			1.621	0.265
Yes	19	30		
No	11	31		
Pathologic type			4.753	0.029*
Stage I–II	17	20		
Stage III–IV	13	41		
Organ metastasis			7.560	0.008**
Yes	9	37		
No	21	24		

* $P < 0.05$ and ** $P < 0.01$, these values had statistically significant differences.

The effect of Flot1 on EMT of SCLC *in vitro*

EMT is the inceptive step of tumor invasion and metastasis, and contributes to the aggressive behavior of cancer cells. Proteins of the Flotillin family are mainly localized in the cell membrane, which may account for their role in EMT. It is reported that Flot2 promotes tumor growth and metastasis through modulation of the cell cycle and the induction of EMT in hepatocellular carcinoma¹⁵. As shown in **Figure 5A**, Flot1 was primarily expressed on the surface and in the cytoplasm of NCI-H446 and NCI-H1688 cells. Morphological changes were observed in the cells overexpressing Flot1, showing an elongated, mesenchymal morphology as compared to the parental NCI-H446 and NCI-H1688 cells (**Figure 5B**), which suggests that Flot1 may be involved in the progression of EMT in SCLC. We further observed that overexpression of Flot1 promoted migration and invasion of NCI-H446 and NCI-H1688 cells (**Figure 5C** and **5D**). In contrast, Flot1 knockdown impaired the migration of SCLC cells (**Figure 5E**). Furthermore, the silencing of Flot1 substantially increased the levels of the epithelial marker E-cadherin but decreased the levels of the mesenchymal marker vimentin in NCI-H446 and NCI-H1688 cells (**Figure 5F**). In order to confirm the association

of Flot1 with EMT marker in the clinical SCLC tissue, we further investigated the correlation between the Flot1 expression level and EMT markers in tumor tissues from 20 patients with SCLC. As shown in **Figure 5G** and **5H**, the Flot1 expression level in the primary tumor was positively correlated with the mesenchymal marker vimentin level ($r = 0.57$, $P < 0.01$) and was negatively correlated with the level of epithelial marker E-cadherin ($r = -0.25$, $P < 0.05$), suggesting that Flot1 was associated with EMT phenotype closely. Taken together, our results revealed that Flot1 was involved in EMT phenotype of SCLC and high expression of Flot1 was able to promote the progression of EMT.

Flot1 activates the EMT phenotype via the TGF- β and AKT signaling pathways

To gain insights into the molecular mechanism that underlies EMT promotion by Flot1, we conducted a gene expression microarray analysis (GEO series accession number GSE99337) of NCIH-446 cells, with stable depletion of Flot1, and control cells. Subsequently, we identified 401 genes with significant changes in expression (384 annotated genes, fold change > 2 , $P < 0.01$) (**Figure 6A**). Three representative genes were confirmed by qRT-PCR and the result indicated that the

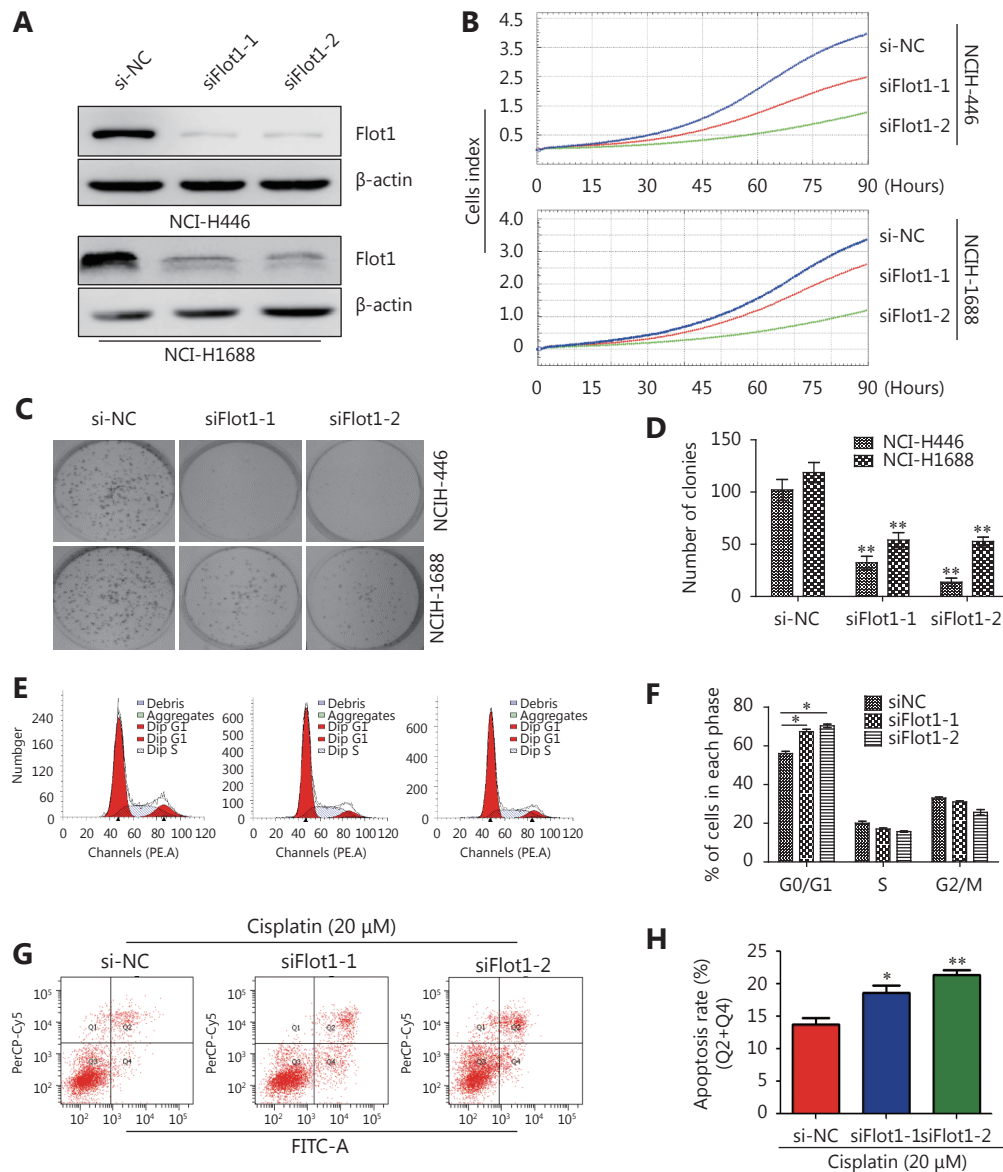


Figure 4 Effects of Flot1 on cell proliferation and apoptosis. (A) Transfection efficiency of Flot1 siRNAs in the NCI-H446 and NCI-H1688 cell lines after transfection for 48 h was analyzed by Western blot. The preventative image is shown. β -actin was used as a loading control. Three independent experiments were performed. (B) NCI-H446 and NCI-H1688 cells were transfected with two specific Flot1 siRNAs, si1 and si2 or non-sense control siRNA. After transfection for 24 h, the xCELLigence System was used to examine cell proliferation of SCLC cells and representative images from one of the experiments are shown. Three independent experiments were performed. (C, D) NCI-H446 and NCI-H1688 cells were transfected with Flot1 siRNA (si1 and si2 or non-sense control siRNA) for 24 h, and then, 2,000 cells from each group were plated in six-well plates where they were cultured for 7 days. Three independent experiments were performed. The number of colonies was calculated ($n = 5$) and plotted on a histogram. $**P < 0.01$. (E, F) NCI-H446 cells were transfected with Flot1 siRNA for 48 h and treated with nocodazole (Noc) for 0 h or 10 h. Cell-cycle distribution was measured by PI staining followed by flow cytometry. Representative images from one of the experiments are shown. Data are represented as the mean \pm SD from three independent experiments. $*P < 0.05$. (G, H) NCI-H446 cells were transfected with the indicated siRNAs for 48 h, and after cisplatin (20 μ M) treatment for 12 h, the cells were collected, and apoptosis was detected using flow cytometry. A histogram was plotted according to the apoptosis rate from three separate experiments. $*P < 0.05$ and $**P < 0.01$.

genes *twist2*, *CDC42*, and *Sox11*, which are all related to tumor progression, were downregulated in cells in the shFlot1 group (Figure 6B). A Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis demonstrated the “top 20” significantly upregulated pathways; the TGF- β signaling pathway, which is involved in the EMT phenotype, was found to be upregulated (Figure 6C). Specifically, we further found that the knockdown of Flot1 led to a pronounced decrease in the activity of the TGF- β -smad2/3 and AKT signaling pathways, which has been reported to be responsible for Flot1 function^{9,15}. We also

found that targets of AKT signaling pathway, cyclin D1 and MMP9, were also involved in the function of Flot1 (Figure 6D). Taken together, these results suggested that TGF- β -smad2/3 and AKT signaling pathways were critical for Flot1-mediated EMT and tumor-promoting phenotype.

Downregulation of Flot1 inhibits the growth and metastasis of SCLC cells *in vivo*

We further extended our observations to investigate whether Flot1 could regulate the capacity of tumor growth and

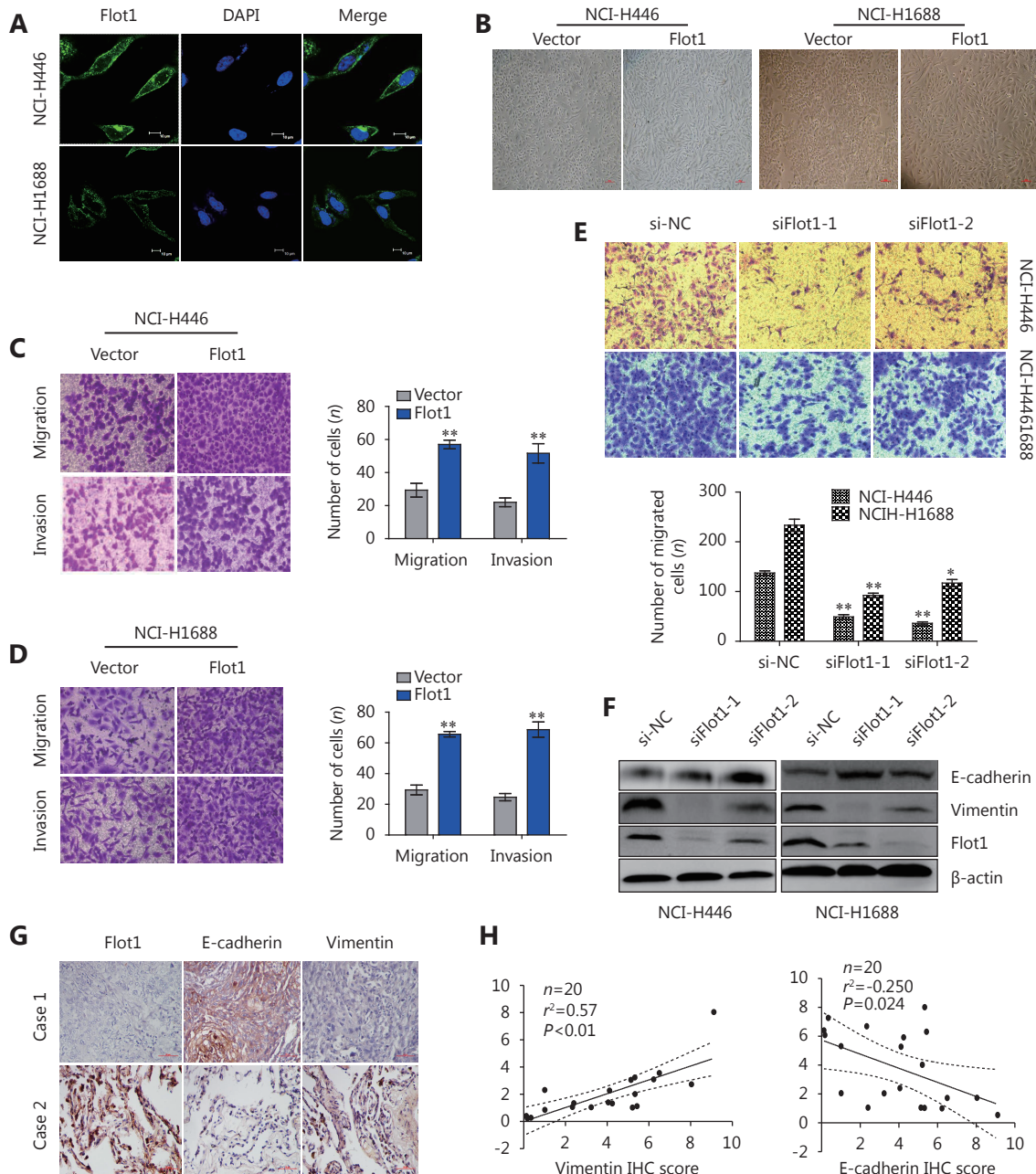


Figure 5 Effects of Flot1 on the progression of EMT. (A) Flot1 was localized in the cell membrane of NCI-H446 and NCI-H1688 cells. Cells were seeded on glass coverslips in 6-well plates and then fixed in 4% paraformaldehyde. A fluorescence-conjugated antibody against Flot1 (green) was used for immunofluorescence. The cell nucleus was stained with DAPI (blue). Imaging of the cells was performed using an LSM510 META confocal laser scanning microscope. Representative images are shown and three independent experiments were performed. Magnification: 1000 ×, as indicated. Scale bars: 10 μm. (B) Morphological changes in NCI-H446 and NCI-H1688 cells in the presence of the Flot1 plasmid were evaluated by phase contrast microscopy. Representative images are shown. Magnification: 200 ×, as indicated. Scale bars: 100 μm. (C, D) The effect of Flot1 overexpression on the migration and invasion of NCI-H446 (upper panel) or NCI-H1688 (lower panel) cells; average counts were obtained from five random microscopic fields. Representative images of cells stained with crystal violet are shown and three independent experiments were performed. Statistical analysis was performed using one-way analysis of variance or Mann-Whitney U-tests, as appropriate. All statistical tests were two-sided. * $P < 0.05$ and ** $P < 0.01$. Magnification: 200 ×, as indicated. (E) The effect of Flot1 knockdown on the migration of SCLC cells was investigated using Transwell assay, and average counts were obtained from five random microscopic fields. Statistical analysis was performed using one-way analysis of variance or Mann-Whitney U-tests, as appropriate. ** $P < 0.01$. Magnification: 200 ×, as indicated. (F) Western blot analysis for the effect of Flot1 silencing on E-cadherin and vimentin protein expression in NCI-H446 and NCI-H1688 cells. Three independent experiments were performed and representative images are shown. (G) Immunostaining for Flot1 and the EMT markers E-cadherin and vimentin in SCLC tissues from 20 patients with SCLC; representative images are shown. Magnification: 200 ×, as indicated. Scale bars: 100 μm. (H) The correlations between the Flot1 expression level and the expression levels of EMT marker E-cadherin and vimentin in the primary tumors of 20 SCLC patients were analyzed. χ^2 -test was used to analyze the categorical variables. Correlation between Flot1 and levels of E-cadherin or vimentin was analyzed by Spearman's rank correlation analysis.

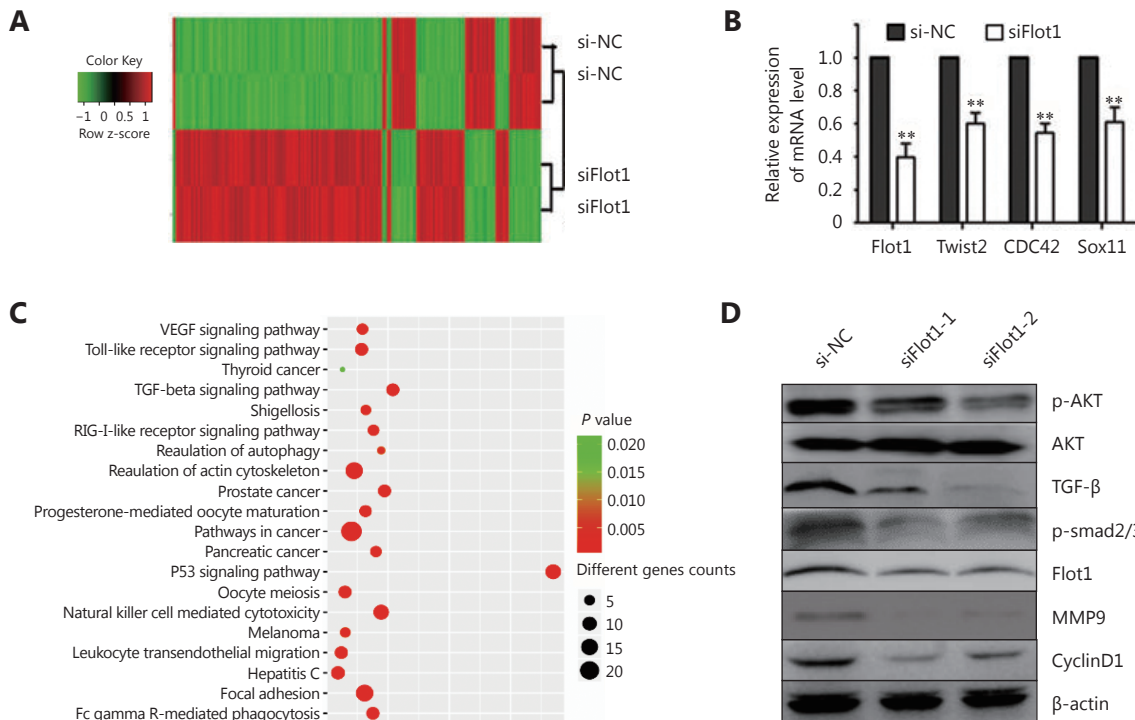


Figure 6 Flot1 activates the EMT phenotype via the TGF- β and AKT signaling pathways. (A) Heatmap representation of the fold change in gene expression as determined by transcriptome analysis in Flot1-silenced cells and control cells (NCI-H446). (B) RT-qPCR confirmed that twist2, cdc42, and sox11 mRNA expression was downregulated in Flot1-knockdown versus control cells. The data represent the mean \pm SD ($n = 3$). ** $P < 0.01$. (C) KEGG pathway analysis of Flot1-regulated genes. Pathways showing enrichment ($P < 0.01$) are presented. (D) Western blot showed that p-AKT, TGF- β , p-smad2/3, MMP9, and cyclin D1 were regulated in Flot1-knockdown versus control cells.

metastasis in SCLC cells *in vivo*. We used a lentivirus to establish the stably downregulated Flot1 cells and selected the shFlot1-1 cell group for further experimentation due to its greater efficiency (Figure 7A and 7B). The corresponding control cells and NCI-H446-shFlot1-1 cells were subcutaneously injected into nude mice. Our results showed that xenografts formed by Flot1-depleted cells revealed slower growth than tumors formed by control cells. In

addition, there is no tumor formed in the three mice of Flot1-depleted cells group, nevertheless all of mice ($n = 6$) in the control group formed tumors, as shown in Figure 7C and 7D. Furthermore, the staining of tumor tissues for ki-67 showed a significantly decreased percentage of ki-67-positive cells in the Flot1-1-depleted group (Figure 7E). To further investigate the effect of knockdown Flot1 on SCLC cells metastasis *in vivo*, cell lines transfected with Flot1 shRNA

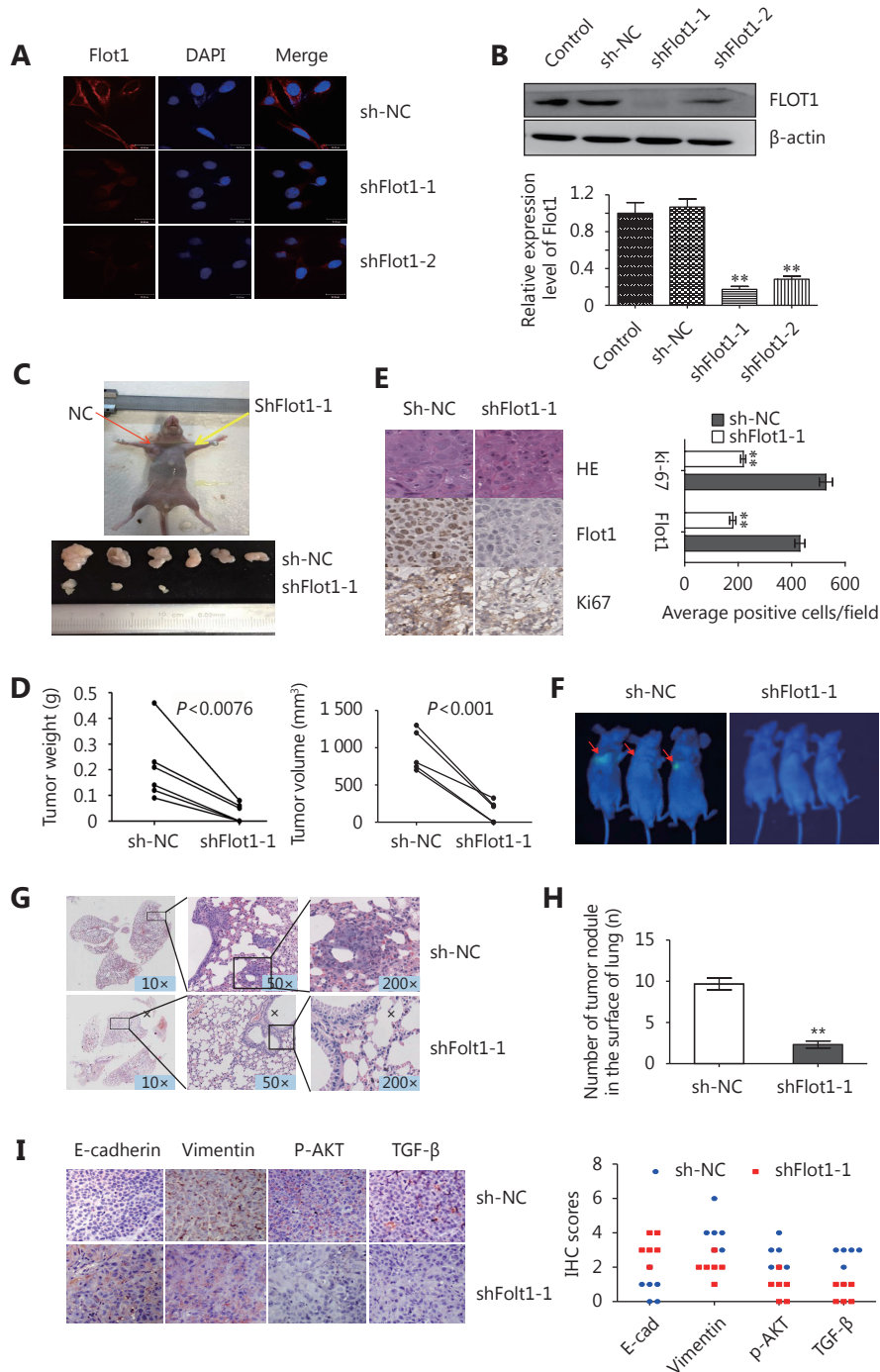


Figure 7 Downregulation of Flot1 inhibits the growth and metastasis of SCLC cells *in vivo*. (A, B) Lentivirus was used to establish the stable Flot1-knockdown NCI-H446 cell line. Immunofluorescence and immunoblotting assays were performed to verify the efficiency of the Flot1 knockdown. The data represent the mean \pm SD ($n = 3$). $**P < 0.01$. (C, D) The growth of Flot1-silenced NCI-H446 cells was measured by subcutaneous injection of these cells into nude mice ($n = 6$). Above: Representative images of nude mice with loaded tumors; tumors isolated from nude mice are shown. Below: Tumor weights and volume were measured. (E) Representative immunohistochemical images and quantification showed Flot1 and ki-67 expression in NCI-H446 xenograft tumors (400 \times). The data represent the mean \pm SD ($n = 6$ for the shNC group and $n = 3$ for the shFlot1 group). $**P < 0.01$. (F) The metastatic ability of Flot1-silenced NCI-H446 cells was measured by injection of these cells in the tail veins of mice; images of the animal models were obtained using a micro-PET-CT scanner after 9 weeks. (G, H) Images of tumor nodules found in the lungs of the mice with HE staining; Representative histological images and quantitative analysis of the lung-tumor nodules were shown. The data represent the mean \pm SD ($n = 6$ for the shNC group and $n = 6$ for the shFlot1 group). $**P < 0.01$. (I) Representative immunohistochemical images of E-cadherin, vimentin, p-AKT, and TGF- β expression in NCI-H446 xenograft tumors are shown (200 \times). IHC scores of E-cadherin, vimentin, p-AKT, and TGF- β in tumor tissues of mice from shNC and shFlot1 group were analyzed.

were injected stably into the lateral veins of nu/nu mice and their metastatic growth was detected in the liver, lymph glands, and lungs. After 9 weeks, the Flot1 shRNA-injected mice displayed significantly lower number of lung metastases than those injected with control shRNA cells, as shown in **Figure 7F**. Fewer lung metastatic nodes were observed in the mice intravenously injected with Flot1-silenced cells compared with mice injected with control cells (**Figure 7G and 7H**), while no tumor nodules formed in the liver and lymph glands in either of the two groups, as shown as **Supplementary Figure S3**. Taken together, these results indicated that Flot1 was required for the growth and highly metastatic phenotype of SCLC cells *in vivo*. Moreover, tumors formed in mice from stably sh-Flot1-transfected NCI-H446 cells exhibited decreased TGF- β and AKT activity, enhanced expression of E-cadherin, and decreased vimentin expression compared to the tumors formed from control cells (**Figure 7I**).

Discussion

In the present study, the clinical significance of Flot1 in SCLC progression and biological function was defined. Flot1 expression was positively correlated with malignant phenotypes and the poor outcome of patients. Our results also showed that cell growth and metastasis were markedly suppressed by downregulation of Flot1 while increasing SCLC cell apoptosis and chemo sensitivity. We further demonstrated that Flot1 knockdown affected the expression of EMT biomarkers in cultured SCLC cells and xenograft mouse models.

A previously conducted meta-analysis has reported that a high expression level of Flot1 protein is correlated with poorer clinical outcomes and that this protein might serve as a potential predictive factor and a prognostic biomarker of

clinicopathology of many solid tumors¹¹. However, the results concerning Flot1 in lung cancer cells have been inconsistent. Li et al.¹² reported that patients with higher Flot1 expression have a shorter overall survival time, whereas those with lower Flot1 expression have a longer survival time. We analyzed the correlation between the Flot1 expression level and the clinical prognosis of patients with LUAD and LUSC using a public database. The result showed that Flot1 acted as a negative prognostic factor in patients with LUSC, while its correlation was not significant with the prognosis of patients with LUAD. These results demonstrated that the effect of Flot1 expression on the clinical prognosis of lung cancer might depend on the subtype of lung cancer. The present study showed that Flot1 was highly expressed in SCLC compared to other types of lung cancer, and that high Flot1 expression was positively correlated with tumor progression and tumor stage in SCLC. Moreover, patients with higher Flot1 expression had a shorter overall survival time and progression-free survival.

Our study further showed that downregulation of Flot1 decreased the growth and migration of SCLC cells *in vitro* and *in vivo*, and the underlying mechanisms of Flot1 in terms of tumor progression were evaluated. It has been reported that Flot1 sustains activation of NF- κ B and activates tumor necrosis factor- α receptor signaling in ESCC cells¹⁶. Lin et al.⁹ found that downregulation of Flot1 impairs cell growth and tumorigenicity via upregulation of FOXO3a in breast cancer. EMT is the initial step of tumor invasion and metastasis. Cancer cells that undergo EMT acquiring aggressive phenotypes will detach from the primary tumor mass and migrate to distant sites^{17,18}. A great deal of evidence from clinical and experimental studies has supported the role for EMT in SCLC progression^{19,20}. Many studies have reported that Flot2 promotes progression of EMT in prostate cancer and hepatocellular carcinoma^{21,22}. However, whether Flot1

can affect the EMT phenotype of SCLC has remained unclear. In our present study, it was observed that Flot1 knockdown downregulated the genes that mapped to TGF- β signaling pathway, which is involved in the EMT process²³⁻²⁵. The introduction of exogenous Flot1 enhanced the EMT phenotype and enhanced the migratory capacity of SCLC cells, whereas the siRNA-mediated decrease of Flot1 reversed the process described above, which suggests that EMT progression is one of the most important factors for Flot1 in the promotion of aggressive malignant SCLC. Furthermore, expression analysis of a set of EMT markers (E-cadherin and vimentin) was performed to further explore whether Flot1 is involved in the EMT process. Our results showed that the silencing of Flot1 substantially enhanced the levels of the epithelial marker E-cadherin and decreased the levels of vimentin. These data demonstrated that the main role of Flot1 in the promotion of SCLC tumors could be ascribed, at least partly, to epithelial-mesenchymal transition.

Niu et al.²⁶ identified Flot1 and histone H1 as downstream factors for the functional pathway of S100A11, which is required for LASP1-S100A11 axis-mediated EMT progression and CRC development. Cao et al.¹⁵ reported that Flot1 promotes migration by activating TGF- β signaling pathway and its targets in nasopharyngeal carcinoma. To clarify the Flot1-driven gene change in SCLC, we performed the transcriptome analysis in Flot1 knockdown cells. We found that 401 genes were significantly altered and that many of these genes are involved in the TGF- β signaling pathway including *twist2* and *sox11*. In addition, the alterations in other cancer-associated signaling pathway components showed the importance of Flot1 in SCLC progression. We also found that the knockdown of the Flot1 decreased the activity of the TGF- β -smad2/3 and AKT signaling pathways *in vitro* and *in vivo*. However, further research should be performed to understand the precise regulatory mechanism of the Flot1-TGF- β axis in SCLC progression to advance our knowledge of role of Flot1 in SCLC.

In addition, it has been reported that Curcumin could regulate the expression of Flot1²⁷ and some microRNA such as miR-485-5p, miR-506, microRNA-34a, and microRNA-124 could inhibit the expression of Flot1²⁸⁻³⁰, so some small molecular compounds or microRNAs could target Flot1, and could be used as a therapeutic strategy for targeting Flot1 in SCLC treatment, which need further research.

Conclusions

In conclusion, our study presents the vital finding that Flot1 promotes proliferation, invasion, and metastasis of human

SCLC cells through the regulation of EMT, which is mainly dependent on the TGF- β -smad2/3 and AKT pathways. This study provides insights into the underlying molecular mechanism of the Flot1-mediated malignant phenotype and progression of SCLC. Flot1 is highly expressed in SCLC, which is one of the most common malignant tumors in humans with early and distant metastasis. The present study suggests a promising therapeutic target for treatment of SCLC.

Acknowledgements

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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Supplementary materials

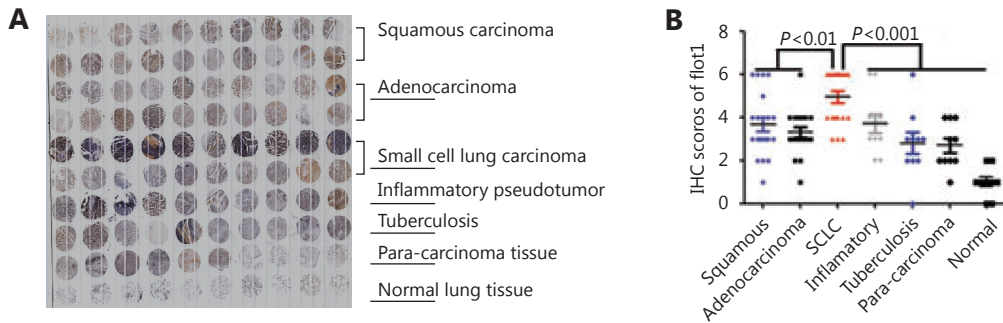


Figure S1 Expression of Flot1 in tissues of various lung diseases . (A) Immunohistochemistry of a tissue microarray was used to investigate the expression of Flot1 in tissues of various lung diseases. (B) The IHC scores of Flot1 expression in tissues of different lung disease were investigated and non-parametric Mann-Whitney test was used to analyze the scores of various groups.

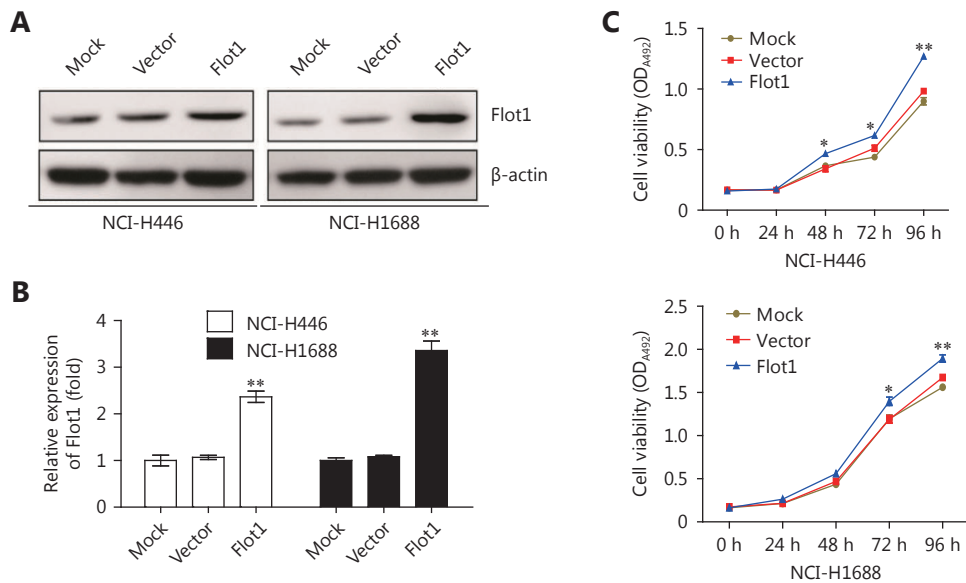


Figure S2 Overexpression of Flot1 promotes the growth of SCLC cells. (A) (B) Overexpression of Flot1 was detected in NCI-H446 and NCI-H1688 cells by western blot after transfection of a plasmid that overexpresses the full-length human Flot1 sequence (NM_005803.3, Mock: negative control) and the transfection efficiency was observed. $**P < 0.01$. (C) Proliferation of SCLC cells overexpressing Flot1 was assessed by MTS assay. NCI-H446 and NCI-H1688 cells were transfected with a Flot1-overexpressing plasmid or empty vector for 12 h, and then 2000 cells from each group were plated in 96-well plates, and MTS assay was used to detect the proliferation of cells in the indicated time.

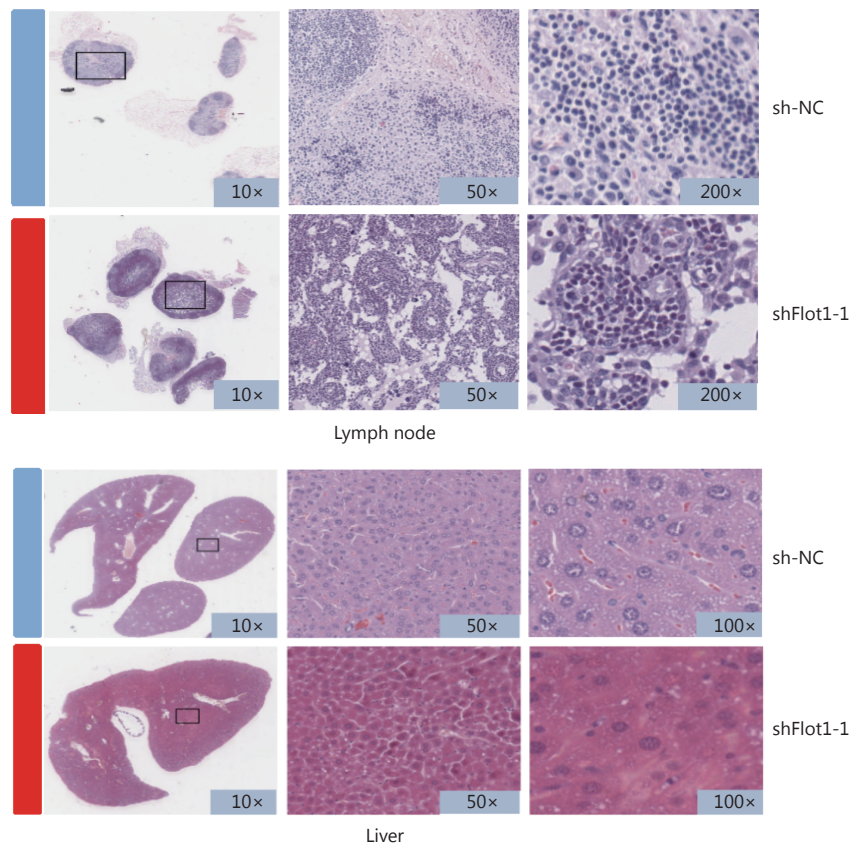


Figure S3 Effect of Flot1 on pathological morphology changes of liver and lymph gland tissue representative images of hematoxylin-eosin H&E staining of the liver and lymph gland tissue in the animal models are shown.