**ORIGINAL ARTICLE**

**FLOT1 promotes tumor development, induces epithelial–mesenchymal transition, and modulates the cell cycle by regulating the Erk/Akt signaling pathway in lung adenocarcinoma**

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**Keywords**
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**Abstract**

**Background:** FLOT1 is a scaffolding protein of lipid rafts that is believed to be involved in numerous cellular processes. However, few studies have explored the function of FLOT1 in the development of lung adenocarcinoma (LUAD) and the underlying mechanisms of FLOT1 activity.

**Methods:** FLOT1 knockdown and overexpression models were constructed via lentivirus. Cell growth, invasion, migration, and apoptosis were detected to evaluate the role of FLOT1 in LUAD development. Epithelial–mesenchymal transition (EMT) and cell cycle regulatory markers were then examined. Finally, the influence of FLOT1 on the Erk/Akt signaling pathway was investigated.

**Results:** FLOT1 promoted cell growth, invasion, and migration and inhibited cell apoptosis. In addition, FLOT1 induced EMT and modulated the cell cycle by activating the Erk/Akt signaling pathway.

**Conclusion:** The findings indicate a significant role of FLOT1 in LUAD development. Targeting FLOT1 may be a potential therapeutic strategy for LUAD.
Introduction

Lung cancer has become the leading cause of cancer-related death, both globally and domestically.\(^1,2\) The two major types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), according to the pathological features.\(^3\) Lung adenocarcinoma (LUAD) is the most common subtype of NSCLC, accounting for \(~80\%\) of all NSCLC cases, and is characterized by a high recurrence rate and a poor prognosis.\(^4,5\) Although a large number of therapeutic strategies have been developed in recent years, the overall survival rate of LUAD is <15\%.\(^6\) These conditions highlight the need to identify useful molecular biomarkers associated with LUAD malignant behavior, such as invasion, proliferation, and metastasis. More importantly, a detailed understanding of the mechanisms involved is necessary to further investigate the characteristics of new biomarkers to improve clinical treatment strategies.\(^7\)

It is well known that lipid rafts act as signaling and sorting platforms for a large number of molecules involved in various biological processes. Hence, the protein markers of lipid rafts are also reported to take part in the initiation and progression of human cancers.\(^8\) FLOT1 accelerates tumor growth in LUAD as NSCLC, and breast and cervical cancer.\(^15\) Several retrospective studies and meta-analyses have further indicated the unfavorable prognostic value of FLOT1 in solid tumors.\(^18–20\) However, the detailed characteristics and mechanisms of FLOT1 in LUAD have rarely been investigated.

In this study, we first determined the function of FLOT1 in the development of LUAD and then explored the underlying mechanisms of FLOT1 activity.

Methods

Cell line and reagents

The LUAD cell line A549 was obtained from the cell bank of the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10\% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin (100 IU/mL), and streptomycin (100 \(\mu\)g/mL), as described in our previous research.\(^21\)

FLOT1 knockdown and overexpression

Three small hairpin RNAs (shRNA1–shRNA3) targeting FLOT1 were designed, with shRNA sequences as follows: 5’-GGA AGA CGG AGG CTG AGA TTG-3’ for shRNA1; 5’-GCA TCA GTG TGT TTA GCT ACA-3’ for shRNA2; 5’-GCT GGG ATC CCG GAA GCT AAA-3’ for shRNA3; and 5’-GTA CGA AGC TGT CAC GT-3’ for scramble shRNA. shRNAs and scramble shRNA were cloned into pLKO.1 (Addgene, Cambridge, MA, USA). Full-length FLOT1 was inserted into pLVX-Puro (Addgene). For lentiviral particle preparation, targeted viral plasmids, psPAX2 and pMD2G, were employed to transfect 293 T cells with Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. The A549 cell line was infected with shRNAs or overexpressed viral supernatants, and FLOT1 expression was evaluated by quantitative PCR (qPCR) and Western blotting. FLOT1-knockdown (shFLOT1) and overexpression (OEFLOT1) of stable cell lines was then generated by puromycin (Sigma-Aldrich, St Louis, MO, USA) selection and used for further experiments.

Cell counting kit-8, Transwell, wound healing, and apoptosis assays

To explore the activity of FLOT1 in LUAD development, cell counting kit-8, Transwell, wound healing, and
apoptosis assays were performed according to the methods described in our previous study.22–24

One-step quantitative PCR analysis

Total messenger RNA was extracted from cells using Trizol following the manufacturer’s instructions (Takara Co., Ltd., Japan). The primers are listed in Table 1. The detailed qPCR protocol was described in our previous research.25

Western blot analysis

Total protein was extracted and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a membrane (nitrocellulose filter membrane [NC]). The membrane was then incubated with primary antibodies at 4°C, followed by incubation with the horse-radish peroxidase-conjugated secondary antibody at room temperature for one hour. The detailed protocol of Western blot analysis was described in our previous research.26 The antibodies used for Western blot analysis are listed in Table 2.

Immunofluorescence assay

The expression of cell cycle regulatory markers, including CDK2, CyclinE, CyclinD1, and P16, were detected by immunofluorescence assay as previously described.27 The antibodies used for immunofluorescence assay are listed in Table 2.

Results

FLOT1 knockdown and overexpression in the lung adenocarcinoma (LUAD) cell line

As shown in Figure 1a-c, all three shRNAs demonstrated effective knockdown of FLOT1. shRNA1–FLOT1 showed the optimal efficacy and thus was chosen for the subsequent experiments. The efficacy of OEFLOT1 was also confirmed.
FLOT1 influences malignant LUAD behavior

Cell counting kit-8, Transwell, and wound healing assays showed that shFLOT1 significantly inhibited A549 cell proliferation (Fig 2a), invasion (Fig 2b,c), and migration (Fig 2d,e), respectively. In comparison, OEFLOT1 dramatically enhanced the malignant behavior of LUAD. Annexin V/propidium iodide staining and flow cytometry assay revealed that shFLOT1 caused a significant increase in apoptosis, while OEFLOT1 led to a critical decrease in apoptosis.
apoptosis (shFLOT1 25.87% vs. sh-scramble 10.60% vs. OEFLOT1 3.47% vs. OEFLOT1-NC 8.23% vs. control 9.79%) (Fig 2f,g).

**FLOT1 regulates the epithelial–mesenchymal transition (EMT) of LUAD**

We detected the expression of epithelial–mesenchymal transition (EMT)-related markers to explore the function of FLOT1 in EMT. The results demonstrated that shFLOT1 significantly increased the expression of the epithelial marker E-cadherin and decreased the expression levels of mesenchymal markers β-catenin and matrix metalloproteinase 2 (MMP-2). In comparison, OEFLOT1 downregulated the expression of the epithelial marker E-cadherin and upregulated the expression of the mesenchymal markers β-catenin and MMP-2 (Fig 3a-c).

**FLOT1 modulates cell cycle LUAD**

As FLOT1 exerts diverse roles in cellular activity, the expression of several cell cycle regulatory markers was further examined by Western blot analysis and immunofluorescence assay. Western blot analysis showed that shFLOT1 inhibited the expression of CDK2, Cyclin E, and Cyclin D1 and elevated the expression of P16 (Fig 4a,b). In comparison, OEFLOT1 significantly increased the expression of CDK2, Cyclin E, and Cyclin D1 and decreased the expression of P16. Immunofluorescence assay further confirmed the results of Western blot analysis (Fig 4c).

**FLOT1 affects the Erk and Akt signaling pathways**

In the Erk signaling pathway, shFLOT1 decreased the phosphorylation of Erk and p-90RSK expression. As an Erk inhibitor, SCH772984 could intensify the effectiveness of Erk phosphorylation and p-90RSK expression when induced by shFLOT1. In comparison, OEFLOT1 increased the phosphorylation of Erk and p-90RSK expression, while SCH772984 antagonized the promotion of Erk phosphorylation and p-90RSK expression from OEFLOT1 (Fig 5a). In the Akt signaling pathway, shFLOT1 decreased the phosphorylation of Akt and elevated FOXO3a expression. As an Akt inhibitor, GDC-0068 increased Akt phosphorylation and alleviated the FOXO3a expression induced by shFLOT1. In comparison, OEFLOT1 upregulated the phosphorylation of Akt and downregulated FOXO3a expression, while GDC-0068 ameliorated the role of OEFLOT1 in Akt phosphorylation and strengthened the downregulation of FOXO3a induced by OEFLOT1 (Fig 5b).

**Discussion**

Flotillins are the scaffolding proteins of lipid rafts, and accumulating studies have reported that flotillins participate in a variety of signaling pathways, membrane trafficking, cell adhesion, and EMT.\textsuperscript{17,28,29} Several studies have revealed that FLOT1 plays an important role in the...
Figure 4  (a-b) Western blot analysis (a) Control, (b) OE-FLOT1-NC, (c) OE-FLOT1, (d) small hairpin (sh)-scramble, and (e) shFLOT1 and (f) immunofluorescence assay showed that shFLOT1 inhibited the expression of CDK2, Cyclin E, and Cyclin D1 and elevated the expression of P16. In comparison, FLOT1 overexpression (OE-FLOT1) significantly increased the expression of CDK2, Cyclin E, and Cyclin D1 and decreased the expression of P16. *P < 0.05 compared to the control. DAPI, 4′,6-diamidino-2-phenylindole.
development of various human cancers, such as hepato-cellular carcinoma, and breast, cervical, and bladder cancers. Li et al. proved that FLOT1 was upregulated in NSCLC tumor samples and that a high expression of FLOT1 correlated with tumor progression and poor survival. Zhang et al. reported that the upregulation of FLOT1 was significantly correlated with advanced clinical stage, increased lymph node metastasis, increased postoperative relapse, and decreased overall survival in LUAD. In a recent report, Guo et al. employed small interfering RNA to knockdown FLOT1 expression and showed that FLOT1 downregulation markedly reduced the malignant behavior of LUAD cells in vitro and in vivo. In the present study, we constructed an shFLOT1 and OEFLOT1 model in a LUAD cell line and detected proliferation, apoptosis, invasion, and migration capacities. We also evaluated the expression of EMT markers. Consistent with the results of previous reports, our data demonstrated that FLOT1 downregulation significantly inhibits the malignant behavior of LUAD and suspends EMT. In comparison, FLOT1 upregulation markedly accelerates the malignant behavior of LUAD and boosts EMT.
Moreover, as they play diverse roles with FLOT1 to regulate cellular behavior, the expression of cell cycle regulatory markers were examined by Western blot analysis and immunofluorescence assay. The data showed that FLOT1 could inhibit the expression of CDK2, Cyclin E, and Cyclin D1 while elevating the expression of P16. Similarly, Lin et al. also showed that silencing FLOT1 upregulated the expression of cyclin-dependent kinase inhibitor p21\(^{cip1}\) and p27\(^{kip1}\), and downregulated the expression of the CDK regulator cyclin D1. 33

In mechanism research, FLOT1 has been described as a regulatory signaling molecule that involves a large number of signal transduction processes. 34 For example, FLOT1 strengthens cell motility and invasion in cervical cancer through the Wnt/β-catenin and NF-κB pathways. 17 FLOT1 overexpression increases cell proliferation, anchorage-independent growth, and invasive ability by activating NF-κB signaling in esophageal cancer. 35 FLOT1 promotes the secretion of TGF-β1 and affects EMT by facilitating the activation of TGF-β/Smad3 signaling in nasopharyngeal carcinoma. 36 In the present study, we explored the underlying mechanism of FLOT1 activity in LUAD.

Erk signaling is activated and correlated with metastasis in several human cancers, including LUAD. 37 Amaddii et al. confirmed that FLOT1 knockdown led to the inactivation of Erk1/2 and that FLOT1 played a direct role in both the early phase (activation of the receptor) and late phase (activation of MAP kinases) of the growth factor signaling pathway. 29 The p-90RSKs, as the downstream mediators of the Erk pathway, play crucial roles in the proliferation and survival of cancer cells via the inhibition of apoptosis, suggesting the significance of RSks in tumorigenesis. 38,39 In our study, we showed that OEFLOT1 boosted the phosphorylation of Erk and its downstream p-90RSK expression in LUAD cells. In comparison, shFLOT1 or an ERK inhibitor significantly ameliorated the phosphorylation of Erk and p-90RSK expression.

Akt signaling is another important intracellular signal transduction pathway. It exerts a tremendous impact on cell behavior by affecting the activity of downstream molecules and is significantly associated with LUAD development. 40 Liang et al. reported that the downregulation of FLOT1 could critically inhibit cancer cell proliferation by activating the Akt signaling pathway. 41 FOXO3a is an important target of the Akt signaling pathway and has been shown to modulate cell apoptosis through the activation of transcriptional targets. 42,43 Lin et al. reported that FLOT1 knockdown impaired cell proliferation and tumorigenicity by upregulating FOXO3a expression in breast cancer, which was further shown to be mechanistically associated with suppression of Akt activity and enhanced transcriptional activity of FOXO3a. 33 In the present study, we proved that OEFLOT1 expression upregulated Akt phosphorylation and downregulated FOXO3a expression in LUAD cells. By using shFLOT1 or Akt inhibitors, Akt phosphorylation was suspended, and FOXO3a expression could be augmented.

In summary, our results show that FLOT1 promotes tumor development, induces EMT, and modulates the cell cycle in LUAD by simultaneously affecting the Erk and Akt signaling pathways. Exploring the role played by FLOT1 in the progression of LUAD will not only widen our understanding of the carcinogenesis of LUAD but also provide a therapeutic strategy for LUAD.

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Disclosure

No authors report any conflict of interest.

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