



FLOT2 Promotes the Proliferation and Epithelial-mesenchymal Transition of Cervical Cancer by Activating the MEK/ERK1/2 Pathway

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Background: As prevalent cancer in women, approximately 569,847 cases of cervical cancer occur every year.

Aims: This study aimed to explore the role of FLOT2 and its related mechanism in the development of cervical cancer.

Study Design: Cell culture study and animal experimentation.

Methods: Quantitative reverse-transcription polymerase chain reaction PCR and Western blot analysis were performed to evaluate the expression of FLOT2. Flow cytometry was applied for the evaluation of cell apoptosis. Cell Counting Kit-8 and colony formation were utilized for proliferation measurement. Cervical cancer mice model was employed to measure the role of FLOT2 in vivo.

Results: FLOT2 mRNA and protein levels were dramatically elevated ($P < 0.001$) in cervical cancer cell line HcerEpic cells. The cell viability and proliferation of cervical cancer cells were enhanced ($P < 0.01$) by overexpression of FLOT2 and reduced ($P < 0.01$) by FLOT2 downregulation. In addition, FLOT2 overexpression elevated ($P <$

0.01) the cell migration abilities of cervical cancer cells, whereas its depletion inhibited ($P < 0.01$) the cell migration abilities. Moreover, the protein expression of epithelial-mesenchymal transition markers including Vimentin, N-cadherin, and E-cadherin were assessed, and the results showed enhanced Vimentin and N-cadherin levels ($P < 0.05$) by FLOT2 upregulation and declined ($P < 0.01$) by FLOT2 downregulation. FLOT2 upregulation reduced ($P < 0.05$) the level of E-cadherin protein, whereas FLOT2 suppression attenuated this effect ($P < 0.05$). Furthermore, FLOT2 increased ($P < 0.05$) p-MEK/MEK, p-ERK1/2/ERK1/2, and p-AKT/AKT levels to activate the MEK/ERK1/2 and AKT pathways in cervical cancer. Finally, our results indicated that FLOT2 inhibited ($P < 0.001$) cervical cancer growth in vivo.

Conclusion: FLOT2 aggravates the proliferation and epithelial-mesenchymal transition of cervical cancer by activating the MEK/ERK1/2 and AKT pathways.

INTRODUCTION

As prevalent cancer in women, approximately 569,847 cases of cervical cancer (CC) occur every year.¹ Moreover, 311,365 patients died from the disease, 85% of which occurred in developing countries.^{2,3} Previously, human papillomavirus (HPV) infection has been identified to be the main cause of CC, and early treatment such as surgery combined with chemotherapy has been widely implemented.^{4,5} Although patients with various CC received standardized treatments, the risk of recurrence and morbidity in these patients were still high⁶. In previous studies, numerous special biomarkers have been identified to be associated with the occurrence or treatment of CC⁷, and the prognosis of patients with CC is still poor. Exploring more effective and relevant biomarkers for CC remains of great importance.

As a specialized domain in cell membranes, lipid rafts are involved in different transductions of cell signals.⁸ As important indexes of lipid rafts, the flotillin family proteins including isoforms flotillin-1 (FLOT1) and flotillin-2 (FLOT2) are found to be involved in the cell process.⁹ In a previous study, flotillin proteins are found to participate in the vesicular invaginations of the plasma membrane and regulate signal transduction¹⁰. Evidence showed that flotillin proteins were ubiquitously expressed and were implicated in various biological processes, such as actin reorganization, signaling transduction, endocytosis, cell adhesion, actin cytoskeleton dynamics, and phagocytosis.⁹ FLOT2 is one of the flotillin protein, which was confirmed to directly interact with signaling molecules including kinases, G proteins, and adhesion molecules receptors¹¹. More importantly, FLOT2 was validated to be involved in cancer development. For instance, FLOT2 forms a positive feedback



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loop with TBL1X to participate in the nasopharyngeal carcinoma cell metastasis.¹² The increased FLOT2 is implicated in the development and prognosis of colorectal cancer.¹³ Despite the significant findings of FLOT2 in cancer development, its role in CC remains largely unknown.

In the present study, we aimed to explore the role of FLOT2 in the growth of CC. We found that FLOT2 aggravates the proliferation and epithelial-mesenchymal transition (EMT) of CC via the activation of the MEK/ERK1/2 and AKT pathways. The findings of this study might offer evidence on the role of FLOT2 in the prevention and treatment of CC in the future.

MATERIAL AND METHODS

Cell Culture and Transfection

CC cells (HeLa and C33A) and human normal cervical epithelial cell HcerEpic were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, MA, USA) at 37 °C in CO₂ (5%). Fetal bovine serum (FBS) (10%, Hyclone, South Logan, UT, USA), penicillin and streptomycin (100 µg/ml, Invitrogen, Carlsbad, CA, USA) were supplemented in the medium. The vectors were transfected into CC cells using Lipofectamine 2000 (Invitrogen). The vectors were obtained from OBiO Technology (Shanghai, China), including short-interfering (si)-FLOT2 (siFLOT2) and pc-FLOT2, as well as their respective negative control (NC).

RNA Extraction and Real-time Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (Thermo Fisher Scientific) was employed for the extraction of RNA from CC cells and mice tissues. RNA concentration was measured via a NanoDrop ND-1000 (Thermo Fisher Scientific). The qRT-PCR with Maxima SYBR Green on an ABI 7500 instrument (Applied Biosystems, MA, USA) was applied for testing FLOT2 level with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. The expression of FLOT2 was calculated via the 2^{-ΔΔCt} method. The primers for FLOT2 and GAPDH were as follows:

FLOT2:

F 5'-GGCTTGTGAGCAGTTTCTGG-3'

R 5'-TCGAAGGCTCGCTTAGAGTC-3'

GAPDH:

F 5'-CCAGCAAGAGCACAAAGAGGAA-3'

R 5'-ATGGTACATGACAAGGTGCGG-3'

Flow Cytometry

The cells were harvested 48 h posttransfection and subjected to trypsin digestion. After rinsing with phosphate-buffered saline, the Annexin V-APC and propidium iodide was used to label the CC cells and measure their apoptosis via a cell apoptosis assay

kit (BD Biosciences, USA). CC apoptoses were evaluated using a flow cytometer (Beckman Coulter, CA, USA) and analyzed by FlowJo v5.7.3 software (LA, USA). Cell apoptosis rate = (Q2+Q3)%.

Western Blot Analysis

Lysed in RIPA buffer, CC cells were subjected to protein quantification via a bicinchoninic acid assay. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was applied for the separation of proteins, followed by transferring to polyvinylidene difluoride membranes. After blocking, the membrane was incubated with primary antibodies. Further, the horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) were supplemented. The enhanced chemiluminescence system (Thermo Fisher Scientific) was employed for evaluating the protein bands, and the proteins were quantified via Image J software (National Institutes of Health, Bethesda, MD, USA). The primary antibodies used in this study included anti-FLOT2 (1:3000, ab96507, Abcam), Vimentin (1:1000, ab16700, Abcam), N-cadherin (1/1000, ab245117, Abcam), E-cadherin (1 µg/ml, ab231303, Abcam), MEK (1:10000, ab32576, Abcam), p-MEK (1 µg/ml, ab278716, Abcam), ERK1/2 (1/10000, ab184699, Abcam), and p-ERK1/2 (0.2 ng/ml, ab176660, Abcam).

Transwell Assay

The migration of CC cells was examined in a 24-well Transwell plate (Costar). Briefly, the CC cells (1 × 10³) were planted in the upper chamber with serum-free medium and incubated for 4 h at 37 °C. Moreover, 500 µL of DMEM containing 20% FBS were added to the lower chamber. After culturing for 1 day, the cells that migrated to the lower chamber were stained with 0.1% crystal violet, and those that remained in the upper chamber were removed. The microscope was applied to visualize the migrated cells. Images were obtained in five random fields.

Immunohistochemistry Analysis

Mice tissues were sliced into sections, followed by dewaxing by xylene. Primary alcohol and antigen water were employed for recycling the sections in turn. After boiling under microwave irradiation for 10 min at 95 °C, the samples were subjected to hydrogen peroxide (3%) for 0.5 h, followed by sealing with goat serum (20%) for 40 min. The sections were then incubated with primary antibodies against Ki-67 (0.1 µg/ml, ab15580, Abcam) and FLOT2 (1/1000, ab96507, Abcam) at 4 °C overnight. Then, the HRP-conjugated secondary antibody (Abcam) was cultured with the sections for 1 h, followed by staining with 2-aminobenzidine and hematoxylin.

Mice Model of CC

The animal assay was authorized by the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University. In total, the Vital River Laboratory (Beijing, China) provided us 6-week-old female Balb/c mice (total n = 10; each group, n = 5). HeLa cells transfected with siFLOT2 were injected into the right-side back

of mice subcutaneously. On day 35, all mice were euthanized, and tumor tissues were extracted. The size, volume, and weight of the tumor were measured. Volume = $(D \times d^2)/2$, where D was the longest diameter and d was the shortest diameter. The tissues were subjected to immunohistochemistry (IHC) staining to measure the expressions of Ki-67 and FLOT2. The levels of MEK/ERK1/2 and AKT pathway-related proteins were examined in the tissues via Western blot analysis.

Cell Counting Kit-8 (CCK-8) Assay

To investigate the proliferation of CC cells, the transfected CC cells were grown on 96-well plates at 37 °C and CO₂ (5%). After culturing for 24, 48, and 72 h, 10 μL of CCK-8 (Beyotime, Shanghai, China) was added into each well and grown for 2 h. Subsequently, a microplate reader (Thermo Fisher Scientific) was applied to measure the 450-nm absorbance. The growth curves were made based on the absorbance of every 24 h.

Colony-forming Assay

The harvested CC cells posttransfection was resuspended in DMEM with FBS (10%) and plated into six-well plates. After maintaining for 2 weeks, colonies were fixed with methanol, followed by crystal violet staining. Images were captured in five random fields. We counted the colonies with a diameter of > 2 mm.

Statistical Analysis

Data were displayed as the mean ± SD, and differences were compared via Student's t-test between two groups, and one-way analysis of variance, followed by Tukey's post hoc test, was used to compare the difference among more than two groups. The statistical difference was set as $P < 0.05$. All tests were repeated at least thrice. The calculations were made by IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

FLOT2 Expression was Upregulated in CC Cells

To assess the role of FLOT2 in CC, RT-qPCR was performed to measure its mRNA in CC cells (HeLa and C33A). The results showed that the FLOT2 mRNA level was dramatically elevated in CC cells compared with that in HcerEpic cells (Figure 1a). Similarly, the protein level of FLOT2 was also enhanced in CC

cells (Figure 1b). Overall, FLOT2 expression was upregulated in CC cells.

FLOT2 Aggravated CC Cell Proliferation

Subsequently, the function of FLOT2 in CC was explored. FLOT2 was overexpressed or knocked down in CC cells via respectively transfecting of pc-FLOT2 or siFLOT2 plasmids. Data depicted that FLOT2 mRNA and protein expressions were evidently increased as a result of pc-FLOT2 transfection, whereas decreased as a result of siFLOT2 transfection in CC cells (Figure 2a-c). These results indicated that pc-FLOT2 and siFLOT2 could be employed for the following functional assays. The CCK-8 assay unveiled that the viability of CC cells was enhanced by FLOT2 overexpression and reduced by FLOT2 downregulation (Figure 2d). In addition, FLOT2 upregulation increased the colony formation of CC cells, and its downregulation decreased the colony formation of CC cells (Figure 2e). Moreover, the suppressed apoptosis of CC cells was observed in the FLOT2 overexpression group, and the aggravation of apoptosis of CC cells was found in the FLOT2 knockdown group (Figure 2f). These results revealed that FLOT2 aggravated CC cell proliferation.

FLOT2 Increased CC Cell Metastasis and EMT

Moreover, the effects of FLOT2 on CC cell metastasis and EMT were explored. As exhibited in Figure 3a, FLOT2 overexpression elevated the migration abilities of CC cells, whereas its downregulation inhibited the migration. Meanwhile, the protein expressions of EMT markers including Vimentin, N-cadherin, and E-cadherin proteins were assessed, and the results showed that the Vimentin and N-cadherin levels were enhanced by FLOT2 overexpression and declined by FLOT2 knockdown. FLOT2 overexpression reduced the level of E-cadherin, while its knockdown inhibited E-cadherin levels (Figure 3b). Taken together, FLOT2 increased CC cell metastasis and EMT progression.

FLOT2 Activated the MEK/ERK1/2 and AKT Pathways in CC

Furthermore, the mechanisms of FLOT2 in CC were probed. Western blot analysis revealed the protein levels of MEK/ERK1/2 pathway-related proteins, p-MEK and p-ERK1/2 levels as well as p-MEK/MEK, and p-ERK1/2/ERK1/2 were heightened by FLOT2

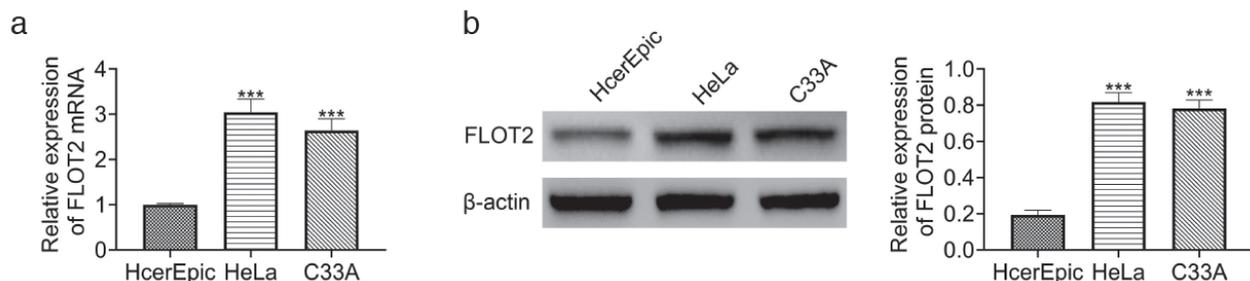


FIG. 1. FLOT2 expression was upregulated in CC cells. (a) RT-qPCR measured the expression of FLOT2. FLOT2 mRNA level was dramatically elevated in CC cells relative to HcerEpic cells. (b) Western blot analysis was employed to evaluate the protein level of FLOT2. FLOT2 protein level was dramatically elevated in CC cells relative to HcerEpic cells. *** $P < 0.001$ compared with the HcerEpic group.

overexpression and decreased by FLOT2 knockdown (Figure 4a). As displayed in Figure 4B, the p-AKT/AKT level was upregulated after overexpressing FLOT2, but downregulated after silencing FLOT2 (Figure 4b). To sum up, FLOT2 activated the MEK/ERK1/2 pathway in CC.

FLOT2 Attenuated CC Growth In Vivo

Finally, the role of FLOT2 in CC was investigated in vivo. After the construction of the in vivo mice model of CC, we evaluated the size, volume, and weight of the tumor. The tumor size, volume, and weight were decreased by FLOT2 downregulation (Figure

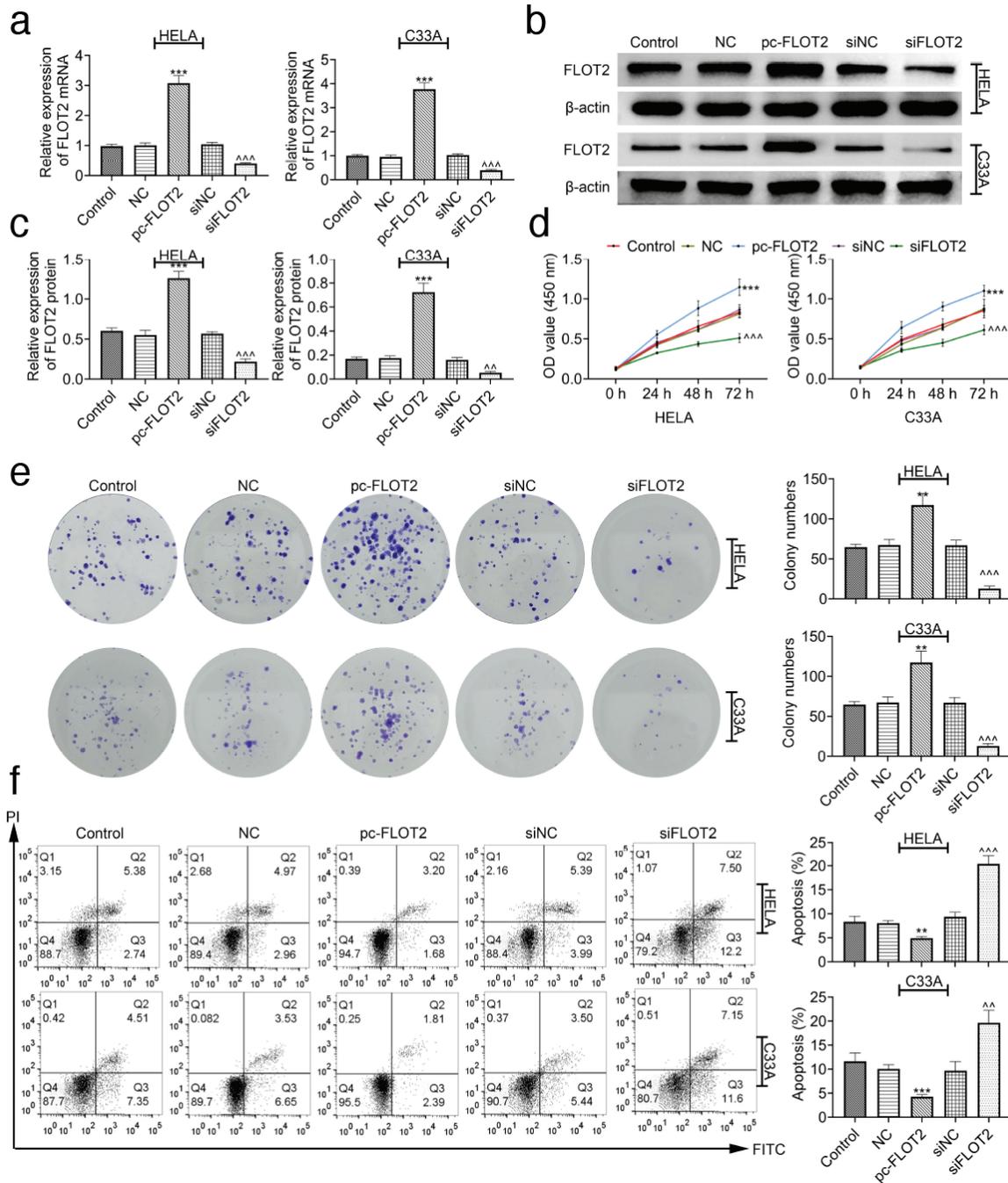


FIG. 2. FLOT2 aggravated CC cell proliferation. (a-c) The level of FLOT2 was tested via RT-qPCR and Western blot analysis. The overexpression and knockdown efficiencies were confirmed. (d-e) CCK-8 was used to examine the viability and proliferation of CC. Cell proliferation was enhanced after overexpressing FLOT2 and reduced after silencing FLOT2. (f) The apoptosis of CC cells was detected by flow cytometry. Cell apoptosis decreased after overexpressing FLOT2 and increased after silencing FLOT2. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the NC group. ^*P* < 0.05, ^^*P* < 0.01, and ^^*P* < 0.001 compared with the siNC group.

5a). Additionally, IHC staining identified that the protein levels of FLOT2 and Ki-67 (cell proliferation index) in the tumor tissues were restrained by FLOT2 knockdown (Figure 5b). Besides, the levels of p-MEK/MEK, p-ERK1/2/ERK1/2, and p-AKT/AKT were all decreased in the tumor tissues of the siFLOT2 group (Figure 5c-d). Altogether, FLOT2 inhibited CC growth in vivo.

DISCUSSION

As one of the most prevalent cancer in women, CC seriously affects the health of women every year.¹⁴ Despite the wide application of the HPV vaccine, CC was still one of the fatality cancers in women¹⁵. The prognosis and survival of patients with CC who were at an

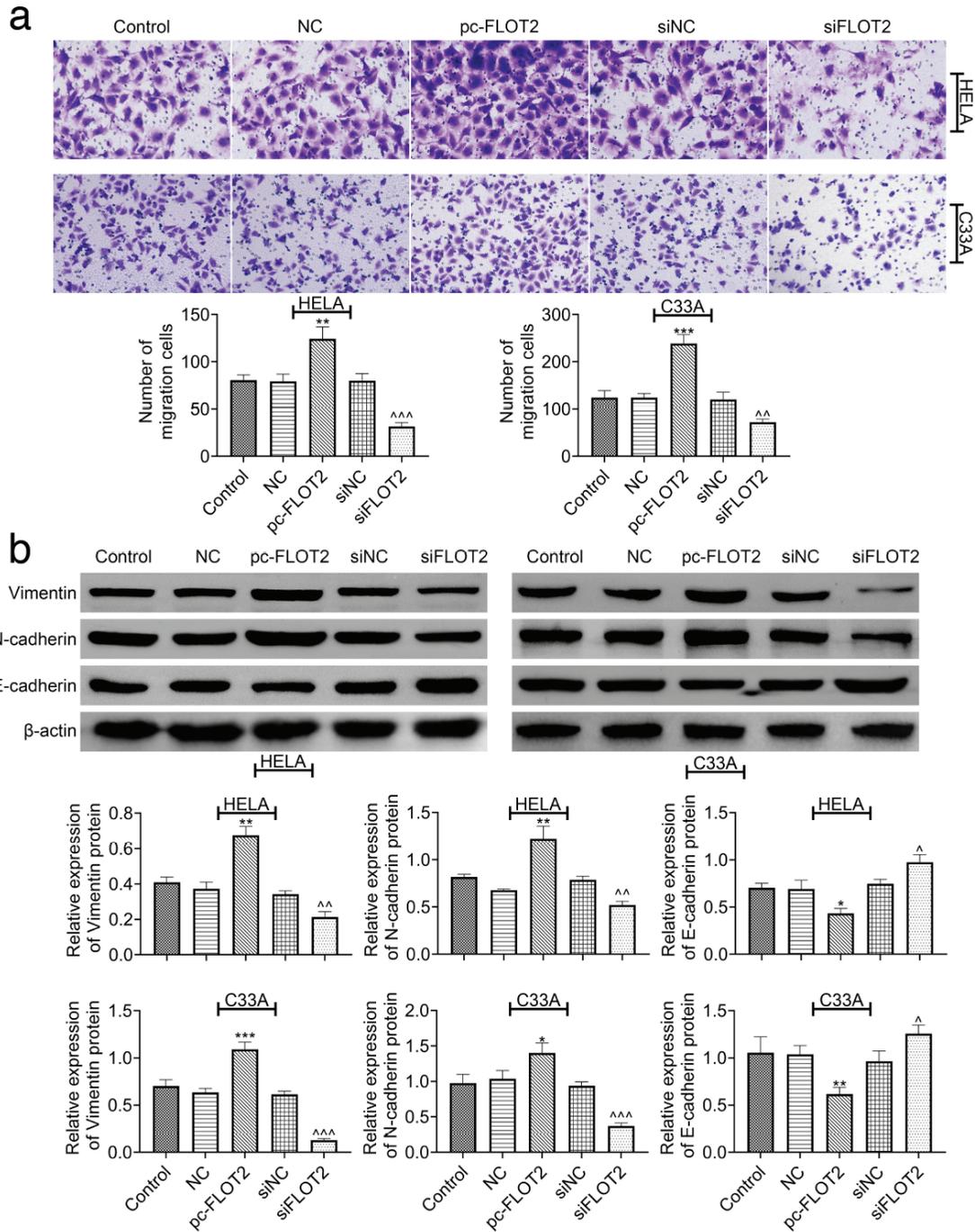


FIG. 3. FLOT2 increased CC cell metastasis and EMT. (a) Transwell assay was used to measure the migration of CC cells. Cell migration was strengthened after overexpressing FLOT2 and weakened after silencing FLOT2. (b) Levels of EMT-related proteins were measured by Western blot analysis. EMT progress was facilitated after overexpressing FLOT2 and retarded after silencing FLOT2. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the NC group. ^ $P < 0.05$, ^^ $P < 0.01$, and ^^ $P < 0.001$ compared with the siNC group.

advanced stage were even poor. Thus, it is of great value to explore novel biomarkers for preventing CC occurrence or improving the outcomes of patients with CC. In previous studies, messenger mRNAs (mRNAs) were frequently reported to be associated with CC development. For instance, sponged by miR-1236-3p, tripartite motif-containing 37 mediates the cell proliferation and cell cycle in CC development.¹⁶ MDM2 expression is enhanced in CC tissues and cells to aggravate the viability and inhibit the apoptosis of

CC cells.¹⁷ KIF20A is boosted by long non-coding RNA UCA1 to modulate the growth and metastasis of CC cells.¹⁸ Although FLOT2 was identified to be implicated in nasopharyngeal carcinoma¹², colorectal cancer¹³, and glioma¹⁹, whether FLOT2 participated in CC growth remains to be elucidated. In the present study, FLOT2 mRNA and protein levels were increased in CC cells. FLOT2 upregulation evidently increased the proliferation and suppressed the apoptosis of CC cells. In addition, FLOT2 overexpression

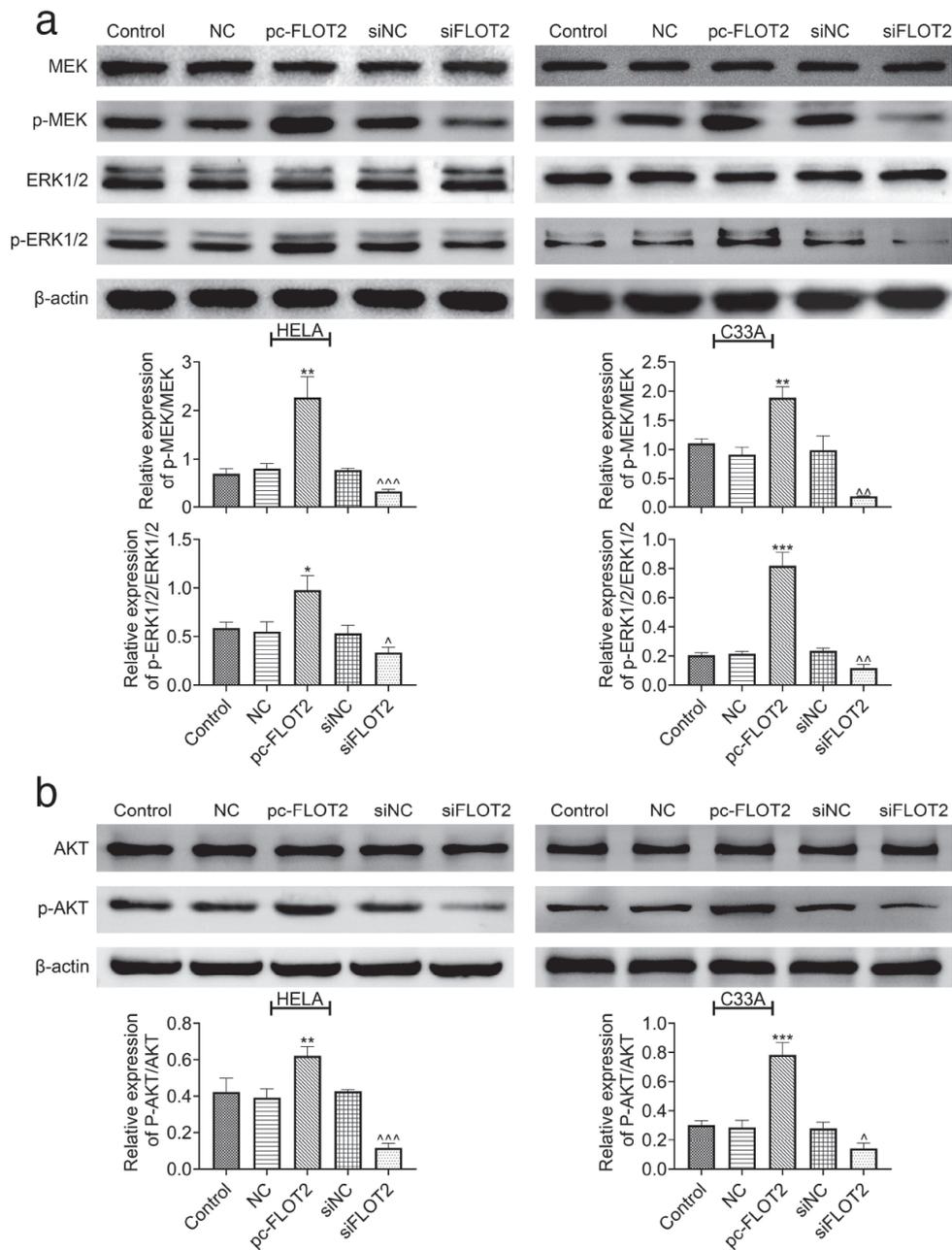


FIG. 4. FLOT2 activated the MEK/ERK1/2 and AKT pathways in CC.

(a) Levels of MEK/ERK1/2 pathway-related proteins (MEK, p-MER, ERK1/2, and p-ERK1/2) were measured by Western blot analysis. The MEK/ERK1/2 pathway was activated after overexpressing FLOT2 and retarded after silencing FLOT2. (b) The levels of AKT pathway-related proteins (AKT and p-AKT) were examined by Western blot analysis. The AKT pathway was activated after overexpressing FLOT2 and retarded after silencing FLOT2. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the NC group. ^ $P < 0.05$, ^^ $P < 0.01$, and ^^ $P < 0.001$ compared with the siNC group.

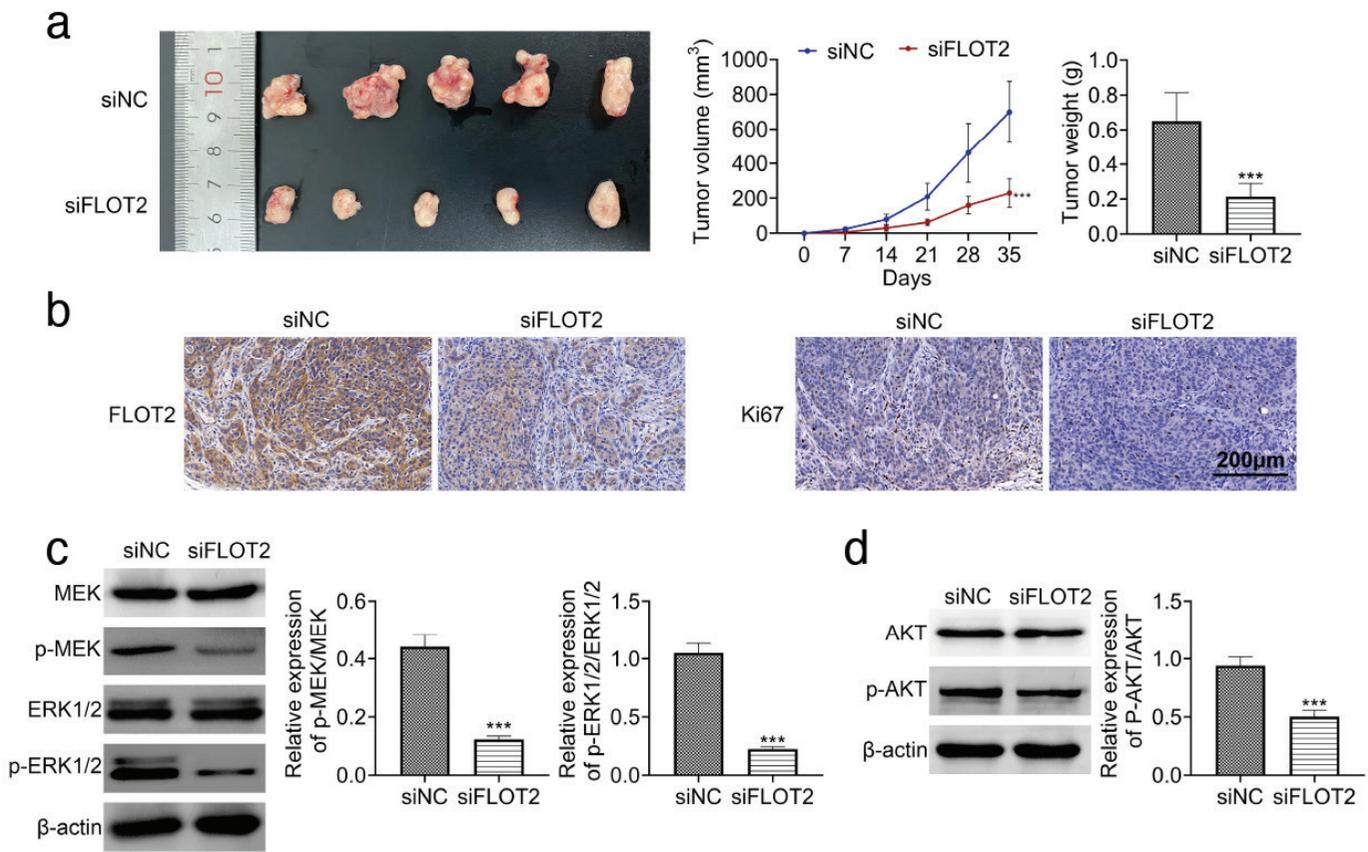


FIG. 5. FLOT2 attenuated CC growth in vivo. (a) The tumor size, volume, and weight were suppressed after FLOT2 knockdown. (b) IHC staining was applied to measure the expressions of FLOT2 and Ki-67. The protein levels of FLOT2 and Ki-67 were reduced after FLOT2 knockdown. (c) Western blot analysis evaluated the expression of MEK/ERK1/2 pathway-related proteins. The MEK/ERK1/2 pathway was retarded after FLOT2 knockdown. (d) Western blot analysis detected the expressions of AKT pathway-related proteins. The AKT pathway was retarded after FLOT2 knockdown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the siNC group

elevated the migration of CC cells. EMT progression was also aggravated because of FLOT2 overexpression. More importantly, we also identified that FLOT2 downregulation suppressed the tumor growth in vivo. In summary, FLOT2 was involved in the tumorigenesis of CC in vitro and in vivo.

The MEK/ERK signaling pathway has been extensively accepted to play a vital part in the progression of various cancers. For instance, the MEK/ERK1/2 pathway is modulated by DGCR5/miR-3619-5p and involved in the development of gallbladder cancer.²⁰ The MEK/ERK1/2 signaling pathway participates in the CCL21/CCR7 interaction-mediated urinary bladder cancer cell migration and invasion and lymphatic metastatic spread.²¹ The MEK/ERK1/2 pathway regulates the migration and invasion of glioblastoma by enhancing the mesenchymal phenotypes.²² The MEK/ERK signaling pathway is also involved in the insulin-like growth factor-1 receptor-induced immunosuppression in lung cancer.²³ FLOT2 has been shown to modulate MEK/ERK pathway activation in cancers.^{24,25} FLOT2 has also been found to modulate the AKT pathway in glioma.¹⁹ Nonetheless, the regulatory effects of FLOT2 on the MEK/ERK and AKT pathways in CC are unclear.

Herein, the p-MEK/MEK, p-ERK1/2/ERK1/2, and p-AKT/AKT levels were enhanced by FLOT2 overexpression and decreased by FLOT2 knockdown, indicating that FLOT2 activated the MEK/ERK1/2 pathway in CC.

In conclusion, we evaluated the role of FLOT2 in CC and found that FLOT2 promoted the proliferation and EMT of CC by activating the MEK/ERK1/2 pathway. The findings of this study might highlight the role of FLOT2 in the prevention and treatment of CC.

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Concept- Y.L., S.D.; Design- Y.L., S.D.; Data Collection or Processing- Y.L., S.D.; Analysis or Interpretation- Y.L., S.D.; Literature Search- Y.L., S.D.; Writing- Y.L., S.D.

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