Knockdown of FLOT1 Impairs Cell Proliferation and Tumorigenicity in Breast Cancer through Upregulation of FOXO3a

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Abstract

**Purpose:** Lipid rafts, specialized domains in cell membranes, function as physical platforms for various molecules to coordinate a variety of signal transduction processes. Flotillin-1 (FLOT1), a marker of lipid rafts, is involved in the progression of cancer, but the precise mechanism remains unclear. The aim of the present study was to examine the role of FLOT1 on the tumorigenesis of breast cancer cells and its clinical significance in progression of the disease.

**Experimental Design:** FLOT1 expression was analyzed in 212 paraffin-embedded, archived clinical breast cancer samples by using immunohistochemistry (IHC). The effect of FLOT1 on cell proliferation and tumorigenesis was examined in vitro and in vivo. Western blotting and luciferase reporter analyses were carried out to identify the effects of downregulating FLOT1 on expression of cell cycle regulators and transcriptional activity of FOXO3a.

**Results:** IHC analysis revealed high expression of FLOT1 in 129 of the 212 (60.8%) paraffin-embedded archived breast cancer specimens. The overall expression level of FLOT1 significantly correlated with clinical staging and poor patient survival of breast cancer. Strikingly, we found that silencing FLOT1 inhibited proliferation and tumorigenicity of breast cancer cells both in vitro and in vivo, which was further shown to be mechanistically associated with suppression of Akt activity, enhanced transcriptional activity of FOXO3a, upregulation of cyclin-dependent kinase inhibitor p21Cip1 and p27Kip1, and downregulation of the CDK regulator cyclin D1.

**Conclusions:** FLOT1 plays an important role in promoting proliferation and tumorigenesis of human breast cancer and may represent a novel prognostic biomarker and therapeutic target for the disease. Clin Cancer Res; 17(10); 3089–99. ©2011 AACR.

Introduction

The dynamic structure of the cell membrane plays important roles in numerous biological processes, such as cell growth, survival, cell division, migration, and metastasis. Lipid rafts are specialized domains within cell membranes that function as physical platforms for various
adhesion, actin cytoskeleton reorganizations, endocytosis, phagocytosis, and transduction of cellular signals (11). It was shown that overexpression of active Fyn kinase results in the translocation of flotillins from the plasma membrane to intracellular organelles and are associated with the tyrosine kinase–regulated endocytic process (12). On the stimulation of growth factors, flotillins can initiate transduction signaling through binding to membrane resident receptor kinases. For example, insulin treatment induces the complex formation of Cbl-CAP and FLOT1 that generate a signal important for the uptake of glucose in adipocytes (13, 14). Besides the functions of flotillins in the cellular and organelle membranes, FLOT1 has been reported to be involved in tumorigenesis. Santamaria and colleagues found that overexpression of FLOT1 enhanced the expression and activity of Aurora B kinase that promotes the incorrect attachment of microtubules to kinetochores, implicating that FLOT1 may contribute to genomic instability (15). Moreover, Santamaria and colleagues reported that upregulation of FLOT1 induced proliferation of PC-3 prostate cancer cells, but the precise mechanism remains unclear (16).

In the present study, we found that the expression of FLOT1 was markedly upregulated in human breast cancer cell lines and tissues. Knockdown of FLOT1 in breast cancer cells not only obviously reduced cell proliferation in vitro but also inhibited tumor growth in vivo. Furthermore, we showed that silencing FLOT1 resulted in upregulation of p21\(^{Cip1}\) and p27\(^{kip1}\), downregulation of cyclin D1, as well as activation of FOXO3a transactivity. Taken together, our findings indicate that FLOT1 plays an important role in the proliferation and tumorigenesis of human breast cancer and suggest that FLOT1 may be a potential target for human breast cancer treatment.
GACCTCCAGCAT-CCA-3'; p27kip1, 5'-TGCAACCAGATTCTTCTTCTATA-3' and reverse 5'-CAACGAGTTGATGACATCTC-3' and reverse 5'-CCAAGGTCATGCCTAC-3'. Expression data were normalized to the geometric mean of housekeeping gene GAPDH (forward: 5'-ACCCACGATTCTTCTATA-3' and reverse: 5'-TCCACACCCTTGTGTCCTGA-3') to control the variability in expression levels and calculated as 2^&−[(Ct of gene)−(Ct of GAPDH)]]], where Ct represents the threshold cycle for each transcript.

Immunohistochemistry

The immunohistochemistry (IHC) procedure and the scores of FLOT1 expression were carried out as previously reported (17). IHC staining was quantitatively analyzed with the AxioVision Rel.4.6 computerized image analysis system assisted with the automatic measurement program (Carl Zeiss, Oberkochen, Germany). Briefly, the stained sections were evaluated at 200× magnification, and 10 representative staining fields of each section were analyzed to verify the mean optical density (MOD), which represented the strength of staining signals as measured per positive pixels. The MOD data were statistically analyzed by using t-test to compare the average MOD difference between different groups of tissues, and P < 0.05 was considered significant.

Western blotting

Western blotting was carried out according to standard methods as described previously (17), by using anti-FLOT1 (Sigma), anti-Akt, anti-p-Akt, anti-GSK-3β, anti-p-GSK-3β, anti-total FOXO3a, anti-p-FOXO3a(Ser253), anti-cyclin D1, anti-p-Rb and anti-Rb antibodies (Cell Signaling), anti-total FOXO3a, anti-p-FOXO3a(Ser253), anti-cyclin D1, anti-p-Rb and anti-Rb antibodies (Cell Signaling), anti-p27kip1 and anti-p21Cip1 antibodies (BD). Blotting membranes were stripped and reprobed with anti-GAPDH antibody (Sigma) as a loading control.

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay

Cells were seeded in 96-well plates at initial density of (0.2 × 10^4)/well. At each time point, cells were stained with 100 μL sterile 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (0.5 mg/mL, Sigma) for 4 hours at 37°C, followed by removal of the culture medium and addition of 150 μL of dimethyl sulfoxide (Sigma). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were carried out in triplicates.

Anchorage-independent growth ability assay

Five hundred cells were trypsinized and suspended in 2 mL complete medium plus 0.3% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer with 1% agar completed medium mixture. At 10 days, viable colonies that contained more than 50 cells or were larger than 0.1 mm were counted. The experiment was carried out for 3 independently times for each cell line.

Colonies formation assays

Cells were plated in 6-well plates (5 × 10^2 cells per plate) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 seconds after fixation with 10% formaldehyde for 5 minutes.

Luciferase assay

Cells were transfected with p3x IRS-MLP-luc plasmid essentially as previously described (18). In brief, 50,000 cells were seeded in triplicate wells of 6-well plates and allowed to settle for 12 hours. One hundred nanogram of p3x IRS-MLP-luciferase plasmid or control-luciferase plasmid plus 10 ng pRL-TK renilla plasmid were transfected into breast cancer cells by using the Lipofectamine 2000 reagent (Invitrogen Co.). Media were replaced at 6 hours, and luciferase and renilla signals were measured 48 hours after transfection by using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

Flow cytometry

Cells were harvested, washed with cold PBS, and processed for cell cycle analysis by using flow cytometry. Briefly, the cells were fixed in 75% ethanol and stored at −20°C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed with cold PBS twice. RNase A (20 μg/mL final concentration) and propidium iodide staining solution (50 μg/mL final concentration) was added to the cells and incubated for 30 minutes at 37°C in the dark. Fifty thousand cells were analyzed by using a FACSCalibur cell sorting and analyser (BD Biosciences) equipped with CellQuest software. ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Bromodeoxyuridine labeling and immunofluorescence

Cells were plated on coverslips (Fisher). After 24 hours, cells were incubated with bromodeoxyuridine (BrdUrd) for 1 hour and stained with anti-BrdUrd antibody (Upstate) according to the manufacturer's instruction. Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus; Carl Zeiss Co. Ltd.).

Xenografted tumor model

Female BALB/c-nu mice (4–5 weeks of age, 18–20 g) were purchased from the Center of Experimental Animal of Guangzhou University of Chinese Medicine, and were housed in barrier facilities on a 12-hour light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The BALB/c nude mice were randomly divided into 3 groups (n = 7/group). A 0.72 mg E2 60-day release pellet (Innovative Research of America) was implanted subcutaneously on the dorsal side of each mouse 1 day before tumor cell implantation to support the growth of the estrogen-dependent MCF-7 cell derived tumors. For tumor cell implantation, MCF7/FLOT1 RNAi(s) or
control (MCF7/vector) cells (2 × 10⁶) in 0.25 mL of the mixture were injected into the mammary fat pads of female athymic mice. Tumors were examined twice weekly; length, width, and thickness measurements were obtained with calipers and tumor volumes were calculated. On day 35, animals were euthanized, and tumors were excised and weighed.

**Statistical analysis**

All statistical analyses were carried out by using the SPSS version 13.0 statistical software packages. Comparisons between groups for statistical significance were carried out with a 2-tailed paired Student’s t-test. The relationship between FLOT1 expression and clinicopathologic characteristics was analyzed by the χ² test. Survival curves were plotted by the Kaplan-Meier method and compared by using the log-rank test. Survival data were evaluated by using univariate and multivariate Cox regression analyses. A P-value of less than 0.05 was considered statistically significant in all cases.

**Results**

**FLOT1 is overexpressed in breast cancer cell lines and breast cancer tissues**

Western blotting and real-time reverse transcriptase (RT)-PCR analyses revealed that expression of FLOT1, at both the protein and mRNA levels, was markedly higher in all 13 breast cancer cell lines tested than in NBECs (Fig. 1A and B). Furthermore, comparative analysis showed that the mRNA and protein levels of FLOT1 were differentially upregulated in all 6 breast cancer samples as compared with matched adjacent nontumor tissues (Fig. 1C and D and Supplementary Fig. S1), suggesting that FLOT1 expression is upregulated in breast cancer.

**Association between FLOT1 expression and clinical features of breast cancer**

To determine whether FLOT1 is clinically correlated with breast cancer progression, the expression of FLOT1 was examined by IHC in 212 paraffin-embedded, archived breast cancer tissues, including 23 cases of clinical stage
I (10.8%), 97 cases of stage II (45.8%), 62 cases of stage III (29.2%), and 30 cases of stage IV breast cancers (14.2%).

The IHC analysis summarized in Supplementary Table S2 showed that FLOT1 was drastically upregulated in breast cancer lesions, but it was only marginally detectable or not at all in normal breast tissue and in adjacent noncancerous tissues (Fig. 2A). Quantitative analysis of the IHC staining indicated that FLOT1 expression in clinical stage I–IV primary tumors was statistically higher than in normal breast tissues ($P < 0.05$, Fig. 2B). Furthermore, the survival time was significantly different between patient groups with low and high FLOT1 expression as determined by the Kaplan–Meier and log-rank tests for survival analysis ($P < 0.001$; Fig. 2C). Moreover, univariate and multivariate analyses revealed that the T classification, N classification, PR expression, and FLOT1 expression was each recognized as an independent prognostic factor (Supplementary Table S3), suggesting that FLOT1 expression may be utilized as a predictor of patient survival with breast cancer.

Interestingly, consistent with our result, the assessment from a publicly available breast cancer microarray data KM plotter (http://kmplot.com/breast/), which further supported the notion that FLOT1 expression may represent a novel prognostic biomarker for the disease.

Downregulation of FLOT1 inhibits proliferation of breast cancer cells

The impact of FLOT1 on proliferation of breast cancer cells was evaluated by knockdown of FLOT1 transcripts (Fig. 3A). An MTT assay showed that depletion of FLOT1 expression caused a significant reduction in viability of both MCF-7 and MDA-MB-231 breast cancer cell lines (Fig. 3B), and these results were further confirmed by the colony formation assay (Fig. 3C). Furthermore, areas of IHC stained sections that presented a high level of FLOT1 also strongly stained for Ki67, whereas those with low FLOT1 expression had only a marginally detectable level of Ki67 expression. Indeed, statistical analyses confirmed that FLOT1 expression was significantly correlated with Ki67 expression ($P < 0.001$; Supplementary Table S4). These results show that FLOT1 is overexpressed in highly proliferative breast cancer cells.

Knockdown of FLOT1 reduces tumorigenicity of breast cancer cells

Because FLOT1 expression was correlated with the clinical staging and T classification of breast cancer (Supplementary Table S2), we further evaluated the effect of FLOT
on the tumorigenic activity of breast cancer cells. Knockdown of FLOT1 significantly decreased the anchorage-independent growth of both breast cancer cell lines, as indicated by the reduction in colony number and colony size on soft agar (Fig. 4A). Moreover, to determine whether knockdown of FLOT1 could inhibit the tumorigenicity of breast cancer cells in vivo, MCF-7/vector and 2 MCF-7/FLOT1-RNAi cells were inoculated into the fat-pad of the nude mouse (n = 7/group). As shown in Figures 4B and C and Supplementary Fig. S3, the tumors formed from MCF-7/FLOT1-RNAi(s) cells in nude mice grew more slowly than those from MCF-7/vector cells, consistent with the cell proliferation results in vitro. After 5 weeks, the weights of tumors induced by FLOT1 silenced cells were drastically reduced compared with those from control cells (Fig. 4D). Collectively, our results indicated that FLOT1 plays an important role in enhancing the tumorigenicity of breast cancer cells both in vitro and in vivo.

**Silencing FLOT1 results in G1-S-phase arrest of breast cancer cells**

A BrdUrI incorporation assay was employed to investigate the mechanism underlying the promotion of cellular proliferation by FLOT1. As shown in Figure 5A, silencing of FLOT1 in MCF-7 and MDA-MB-231 cells dramatically decreased the percentages of cells with incorporated BrdUrI. Furthermore, knockdown of FLOT1 resulted in a significant increase in the percentages of cells in the G1/G0 peak and a decrease in the percentages of cells in the S peak (Fig. 5B and supplementary Fig. S4). These results suggest that silencing FLOT1 induces G1-S-phase arrest of breast cancer cells. Moreover, real-time PCR and Western blotting analyses revealed that the expression of CDK inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were dramatically upregulated, whereas the expression of the CDK regulator cyclin D1 was downregulated in FLOT1 knockdown cells compared with those in control cells at both the mRNA and protein levels (Fig. 5C and Fig. 5D). As expected, the phosphorylation level of Rb, the downstream target protein of CDK, was shown to be suppressed in the FLOT1 silenced cells, further supporting the notion that FLOT1 is involved in the regulation of the proliferation of breast cancer cells.

**Downregulation of FLOT1 enhances transcriptional activity of FOXO3a**

It has been reported that the transcriptional factor FOXO3a can upregulate p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and downregulate cyclin D1 at the transcriptional level (21). As shown in Figure 6A, silencing FLOT1 increased the transcriptional activity of FOXO3a in both breast cancer cell lines in a luciferase reporter assay. Furthermore, we found that knockdown of FLOT1 decreased phosphorylation of FOXO3a (Fig. 6B), as well as that of kinase Akt that functions upstream of FOXO3a (Fig. 6B). These results suggest that the effect of FLOT1 on FOXO3a transactivity may be through inhibition of Akt kinase activity.

To determine the role of FOXO3a in FLOT1 mediated-proliferation, we further knocked down the expression of FOXO3a in FLOT1 RNAi(s)-transduced cells. As expected, the luciferase activity of FOXO3a reporter was dramatically reduced in FLOT1 silenced cells on FOXO3a siRNA(s) transfection (Supplementary Fig. S5A). Meanwhile, additional knockdown of FOXO3a in FLOT1 silenced cells decreased expressions of p27<sup>Kip1</sup> and p21<sup>Cip1</sup> but increased that of cyclin D1 (Fig. 6C and Supplementary Fig. S5B). Moreover, the MTT and colony formation assays revealed that silencing FOXO3a restored the growth rate of both cell lines with silenced FLOT1 (Fig. 6D and
Supplementary Fig. S6), suggesting that FOXO3a plays an important role in the antiproliferative effect of FLOT1 depletion.

Discussion

This study presented the pivotal finding that knockdown of endogenous FLOT1, a lipid raft specific protein, inhibited the proliferation and tumorigenicity of breast cancer cells in vitro and in vivo. We showed that silencing FLOT1 by using RNAi resulted in activation of FOXO3a transactivity, which lead to upregulation of cyclin-dependent kinase inhibitors p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) and downregulation of CDK regulator cyclin D1. These findings provide strong evidence that upregulation of FLOT1 plays important roles in promoting tumorigenesis and progression of breast cancer.

Tumorigenesis, characterized by uncontrolled cell growth and tumor formation, is associated with various alterations in genes or proteins related to regulation of proliferation, cell death, and genomic instability (22). Thus, identification of genes and their products involved in the molecular events leading to tumorigenesis is critical to developing effective therapeutic strategies. Here, we reported that FLOT1 was markedly upregulated in breast cancer cells and tissues. Statistical analysis of IHC staining revealed that expression of FLOT1 was significantly correlated with clinical characteristics of patients, including clinical stage, tumor-nodule-metastasis classification, histologic differentiation, ER expression, and PR expression. Moreover, silencing FLOT1 reduced the tumorigenicity of breast cancer cells both in vitro and in vivo, implicating that FLOT1 may function as an oncogenic protein in the development and progression of breast cancer.

Although the potential oncogenic function of FLOT1 has been implicated in prostate cancer cells, the precise mechanism remains unclear (15). In the present study, we found that FLOT1 was strongly expressed in the highly proliferative lesions of human breast cancer, as indicated by a significant correlation between FLOT1 and Ki67 expressions \((P<0.001)\). Further experiments on the impact of FLOT1 depletion on cell viability and colony formation showed that FLOT1 promotes proliferation, as silencing FLOT1 in breast cancer cells enhanced G1-S arrest. Such a connection between FLOT1 and G1/S transition was showed to be mechanistically mediated by the cell cycle

![Figure 4](https://example.com/figure4.png)

Figure 4. Knockdown of FLOT1 inhibits tumorigenicity of breast cancer cells in vitro and in vivo. A, silencing endogenous FLOT1 inhibited cell growth as determined by an assay of anchorage-independent growth. Soft agar colony formation by the indicated cells after 10 days of culture (left panel). Colonies that contained more than 50 cells (middle panel) or were larger than 0.1 mm in diameter (right panel) were scored. Each bar represents the mean \(\pm\) SD of 3 independent experiments. B–D, xenograft model in nude mice. MCF-7/vector cells and MCF-7/FLOT1 RNAi(s) cells were injected into the mammary fat pads of female athymic mice given supplementary 17\(\beta\)-estradiol (E2). B, tumor volumes were measured on the indicated days. Representative graph of tumor growth (C) and mean tumor weights (D) 5 weeks after inoculation. All data are shown as mean \(\pm\) SD.
Figure 5. Depletion of FLOT1-induced G1-S arrest of breast cancer cells. A, representative micrographs (left panel) and quantification (right panel) of BrdU incorporation in cells transduced with vector or each of the 2 FLOT1 shRNAs. B, flow cytometric analysis of indicated breast cancer cells. C, relative mRNA expressions of p21Cip1 (left), p27Kip1 (middle), and cyclin D1 (right) in indicated breast cancer cells were determined by real time RT-PCR. Expression levels were normalized with GAPDH. D, Western blotting analysis of phosphorylated Rb (p-Rb), total Rb, p21Cip1, p27Kip1, and cyclin D1 proteins in indicated breast cancer cells. GAPDH was used as a loading control. Error bars represent SD from 3 independent experiments. *, P < 0.05.
inhibitors p21Cip1 and p27Kip1 and by the CDK regulator cyclin D1, suggesting that the effect of FLOT1 on proliferation in breast cancer is tightly linked with the regulation of cell cycle regulators.

FOXO3a, 1 member of the FOXO family, has been showed to be a critical tumor suppressor in breast cancer through transcriptional regulation of multiple proteins, including p21Cip1, p27Kip1, and cyclin D1 (21). FOXO3a can also physically interact with the p53 protein and activate transcription via p53 sites, suggesting that FOXO3a may cooperate with p53 in suppression of tumorigenesis (23). Overexpression of FOXO3a can suppress the proliferation and tumorigenesis in athymic mice (24). Enhancement of FOXO3a transactivity on treatment with MAPKK inhibitor, EGFR antibody (cetuximab)/HER2 antibody (trastuzumab), or paclitaxel induces apoptosis of breast cancer cells, indicating that FOXO3a plays an important role in inhibiting development and progression of breast cancer (25–27). Meanwhile, inhibition of FOXO3a activity mediated by IκB kinase increases resistance to apoptosis and promotes tumor growth in nude mice, suggesting that activation of FOXO3a may be a potential therapeutic intervention strategy for breast cancer (28). In the current study, we found that silencing FLOT1 decreased phosphorylation, whereas it
significantly increased transactivation activity of FOXO3a, suggesting that FLOT1 may also be a potential therapeutic target for breast cancer.

Several lines of evidence have suggested that the Akt kinase that acts upstream of FOXO3a plays important roles in lipid raft–mediated signal transduction. For example, disruption of lipid rafts inhibits Akt phosphorylation and reduces prostate cancer cell survival, but reconstitution of the lipid rafts with cholesterol can restore PI3K/Akt dependent apoptotic signal (29). Furthermore, activation of Liver X Receptor induces apoptosis of prostate cancer cells through downregulation of Akt signaling via binding lipid rafts protein FLOT2 (30), whereas overexpressing heat shock protein Hsp16.2 in NIH3T3 cells increases lipid raft formation and Akt phosphorylation, as well as protects cells against stress stimuli (31). Zhuang and colleagues found that inhibition of cholesterol synthesis could lower raft cholesterol content, deactivate Akt/PKB pathway signaling, and induce apoptosis in LNCaP PCa cells. However, elevation of cholesterol increases phosphorylation of Akt, reduces apoptosis, and promotes xenograft tumor growth (32). Interestingly, lipid raft disruption does not affect the interaction of PI3K with the cell membrane and the capability of PI3K to convert phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, but it does abrogate the binding of Akt and PDK1 to the cell membrane, suggesting that the integrity of lipid rafts is required for the activity of Akt kinase and cell survival (33). Here, we found that knockdown of FLOT1 decreased the level of phosphorylated Akt, indicating that downregulation of FLOT1 may disrupt the integrity of lipid rafts in breast cancer cells. Apparently, such a hypothesis and whether knockdown of FLOT1 can reduce the binding of Akt to the cell membrane needs to be further investigated.

In summary, we have shown that FLOT1, a marker of lipid rafts, is evidently overexpressed in breast cancers. Moreover, our finding that knockdown of FLOT1 inhibited the proliferation and tumorigenesis of breast cancer cells through Akt/FOXO3a pathway illustrates a new mode of action in the molecular mechanism underlying the tumorigenesis of breast cancer. Understanding the precise role of FLOT1 in breast cancer progression will not only increase our knowledge of the biology of breast cancer but may also enable development of a novel therapeutic strategy via suppression of FLOT1.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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