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Original Article

Flot2 correlates to poor outcome and promotes proliferation and metastasis of colorectal cancer

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Abstract: Background: Flot2 was reported in several malignancies, such as lung cancer and breast cancer. Our project aimed to investigate its clinicopathological expression and role in CRC (colorectal cancer). Methods: Immunohistochemistry and Western-Blot were performed to detect expression patterns of Flot2 in 130 paraffin-embedded CRC sample and 24 pairs of matched fresh CRC. Serials of in vitro and in vivo functional assays were performed to detect the role of Flot2 in proliferation and metastasis of CRC cell lines. Results: Our data showed that Flot2 was significantly up-regulated in CRC tissues and had close correlation with bad clinical outcomes. Over-expression of Flot2 promoted proliferation and invasion of CRC cells in vitro, while depletion of Flot2 caused inhibitory effects in vitro. Nude mice in vivo assays showed that Flot2 improved proliferation and metastasis ability of CRC cells in vivo. Conclusion: Flot2 was high-expressed in CRC and correlated to fetal outcomes of clinical patients. Flot2 promoted proliferation and metastasis of CRC cell lines, which might be a biomarker for clinical diagnosis and therapy for CRC.

Keywords: CRC, FLOT2, proliferation, invasion, metastasis

Introduction

Colorectal cancer ranked third among all the cancer-related deaths, only behind breast cancer and lung cancer [1]. Metastasis directly contributes its high mortality, while surgical section remains useless when metastasis developed. Besides, chemotherapy and radiotherapy burden a lot to patients and society [2, 3]. Thus, early diagnosis and therapy for CRC are essential for reduction of CRC death rate.

Flot1 and Flot2 were firstly identified in fish retinal ganglion cells [4]. As members of lipid raft proteins, Flot1/2 were reported in regulation of cell adhesion and actin dynamics and in growth factor signaling [5]. Recent studies about tumor progression revealed Flot2 had close relationship with cancer progression and metastasis [6, 7]. Flot2 is associated with breast cancer progression and correlates with patients' poor prognosis, mice models also show that deficiency of Flot2 reduces lung metastasis of breast cancer [8, 9]. Yang reported that knockdown of FLOT2 reduced renal cell carcinoma (RCC) cell proliferation, migration, and invasion and correlated with bad outcomes of RCC patients, might act as a potential prognostic biomarker of RCC [10]. However, its function in colorectal cancer remains unclear.

In our study, we aimed at detecting Flot2 expression in CRC clinical samples and analyzing its biological effects in CRC cell lines for early diagnosis and therapy of CRC.

Materials and methods

Plasmid and transfection

CDS vector of Flot2 were cloned from cDNA of normal colorectal tissue and inserted into pEGFP-C1 after being double digested by KpnI and XhoI. Connected plasmid was transformed into DH5α and then extracted from bacterial suspension with plasmid extraction kit (Tiangen, Beijing). SiRNAs targeted Flot2 were designed and synthesized by Riobio in Guangzhou. Both plasmid and siRNA were transfected by Lipofamine2000. Flot2 over-expression and de-

pletion lenti-viruses were packed by Jikai in Shanghai. Lenti-virus supernatant was transfected as manufacturer directed.

Cell culture

Colorectal cancer cell lines SW620, RKO, DLD1, HCT116, LOVO, HT29 were purchased from the American Type Culture Collection (ATCC, USA) and maintained in RPMI-1640 with 10% fetal bovine serum (Gibco, America).

qRT-PCR

RNA was extracted from tissues or cell lines by Trizol reagent and reversed into cDNA as protocol directed (Toyobo, Japan). Primers were synthesized by Invitrogen (Flot2 sense 5'-CCC CAG ATT GCT GCC AAA-3', antisense 5'-TCC ACT GAG GAC CAC AAT CTC A-3'). qRT-PCR was performed with the Fast SYBR Green Master Mix System (Takara, Japan) and cycled in 7500 Real-Time PCR system, the circle condition was 95°C for 10 min, followed by 35 cycles of amplification (95°C for 10 s, 60°C for 20 s, 72°C for 34 s). Relative mRNA expression levels were standardized to human GAPDH levels.

Western blot

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed on ice in RIPA lysis buffer containing complete protease inhibitor and PMSF. Equal protein samples (20 μg) were separated on 10% SDS PAGE gels and transferred to PVDF membranes (Millipore, America). Membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with anti-Flotillin-2 antibody (1:1000, Abcam) overnight at 4°C, and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). ECL Western blotting detection reagent (Fudebio, China) was used to detect protein bands.

Immunohistochemistry

130 cases of CRC patients diagnosed in the First Affiliated Hospital of Wenzhou Medical University from 2005-2009 were enrolled in our study. The tissues were fixed in 10% formalin and paraffin-embedded. The 4 μm-thick sections were deparaffinized and rehydrated, and endogenous peroxidase was inhibited with 0.3% H_2O_2 methanol. Sodium citrate buffer was used for antigen restoration. After being blocked by 5% goat serum for 1 h, sections were incubated with Flot2 antibody at 4°C overnight (1:200, Abcam, Britain) and incubated with corresponding secondary antibody (ZSGB, China). Visualization signals were detected by DAB (ZSJB, China).

In vitro proliferation assay

800 cells were seeded in 96-well plates per well and incubated for 7 days. At each time of per 24 hours, cells were incubated in 100 μl new normal culture medium containing 10 μl CCK8 reagent for 2 hours without lights. Two hours later, the absorbance value of each well at 570 μm was read by a microplate reader.

In vitro invasion assay

In vitro invasion assay was performed by Borden transwell. The matrigel was added into upper side of Borden membrane and then inserted it into Borden chamber. 10^5 cells suspended into empty 200 μl RPMI-1640 were added into upper compartment of Borden chamber and 600 μl RPMI-1640 with 10% FBS were added into lower compartment. After 48 hours, transwells were fixed and stained; cells invaded onto lower side of Borden membrane were counted.

Nude mice model

10^7 cells were injected subcutaneously into 6-week-old nude mice (n=5) to evaluate its in vivo proliferation ability. Tumor sizes were measured from day 5 to day 28. 10^7 cells were injected subcutaneously into 6-week-old nude mice (n=5) to evaluate its in vivo metastasis ability. After 8 weeks, animals were executed and organs were dissected, fixed, paraffin-embedded, sectioned and HE stained. Number of total metastasis nodes was calculated under microscope.

Results

Expression of Flot2 in CRC tissues and cell lines

Immunohistochemistry was performed in 130 cases of paraffin-embedding CRC tissues. Our results showed that signal was mainly detected in cytoplasm; besides, expression in tumor areas was much higher than in corresponding

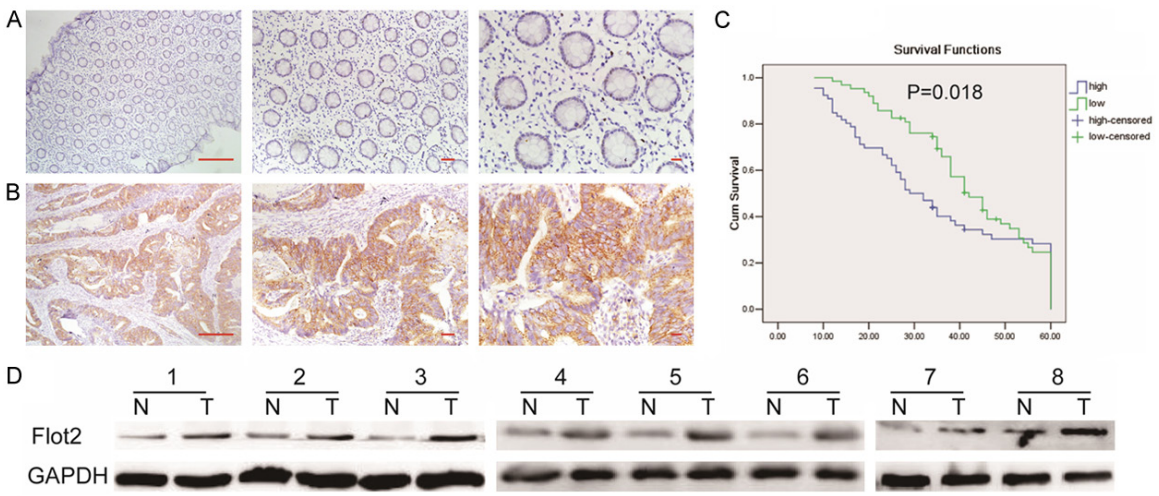

Figure 1. Flot2 was high expressed in colorectal cancer. A, B. Strong positive expression of Flot2 was observed in cytoplasm of colorectal cancer areas, while weak signals were detected in normal colorectal tissues. C. Kaplan-Meier survival analysis of CRC patients with high and low Flot2 expression (P=0.018). D. Flot2 protein expression of 24 CRC fresh tissues was detected by Western blot; Flot2 was high expressed in CRC tissues.

normal areas (**Figure 1A**, **1B**). Survival analysis result showed Flot2 was associated with poor outcomes of CRC patients (P=0.018) (**Figure 1C**). Western-Blot in 24 pairs matched fresh CRC tissues showed protein expression of Flot2 in tumor tissues was higher than in its adjacent normal tissue (**Figure 1D**).

Endogenous expression of Flot2 of CRC cell lines and over-expression and depletion efficiency of transfection

qRT-PCR and Western-Blot were performed in 6 CRC cell lines; results showed that HT29 had relative high expression of Flot2 while HCT116 had low expression (**Figure 2A**, **2B**). Thus, HCT116 was chosen for over-expression by transfecting Flot2 over-expressing lenti-virus, HT29 was chosen for depletion by corresponding lenti-virus. qRT-PCR and Western blot were performed to analyze their transfection efficiency (**Figure 2C**, **2D**).

Role of Flot2 on proliferation ability of CRC cell lines

MTT assay showed that HT29 had decreased proliferation ability when Flot2 expression was knocked-down, while HCT116 had increased proliferation ability with extra Flot2 expression (**Figure 2E**, **2G**). Ten mice were injected with 10^7 HT29 or HCT116 cell subcutaneously for detection of CRC cell proliferation ability in vivo. After four weeks, tumor size in HT29/siFlot2 group was less than in HT29/NC group and HCT116/mock group was less than HCT116/Flot2 group (**Figures 3A**, **4A**). Ki-67, indicating proliferation ability, also showed the same results (**Figures 3B**, **4B**).

Role of Flot2 on invasion ability of CRC cell lines

Borden transwell assay was performed to detect invasion ability of CRC cell lines. Compared with HT29/NC group, HT29/siFlot2 groups had less cells attracted to outside of the membrane. After over-expression of Flot2, more invaded HCT116/Flot2 and HCT116/mock cells were counted (**Figure 2F**, **2H**). For detection of invasion ability of CRC cell lines, 10 mice were injected with 10^7 HT29 or HCT116 cells into tail vein. After 2 months, animals were executed, organs were dissected. Numbers of metastasis nude were counted under microscope after HE staining. Data indicated that mice lung metastasis nodes were more in HT29/NC and HCT116/Flot2 groups than in HT29/si and HCT116/mock groups (**Figures 3D**, **3E**, **4D**, **4E**). Besides, we observed that the borders of subcutaneous tumor in HT29/NC and HCT116/Flot2 groups were more destroyed than in HT29/si and HCT116/mock groups, which meant HT29/NC and HCT116/Flot2 groups were more aggressive. These results indicated that Flot2 promoted invasion ability of CRC cell lines both in vitro and in vivo.

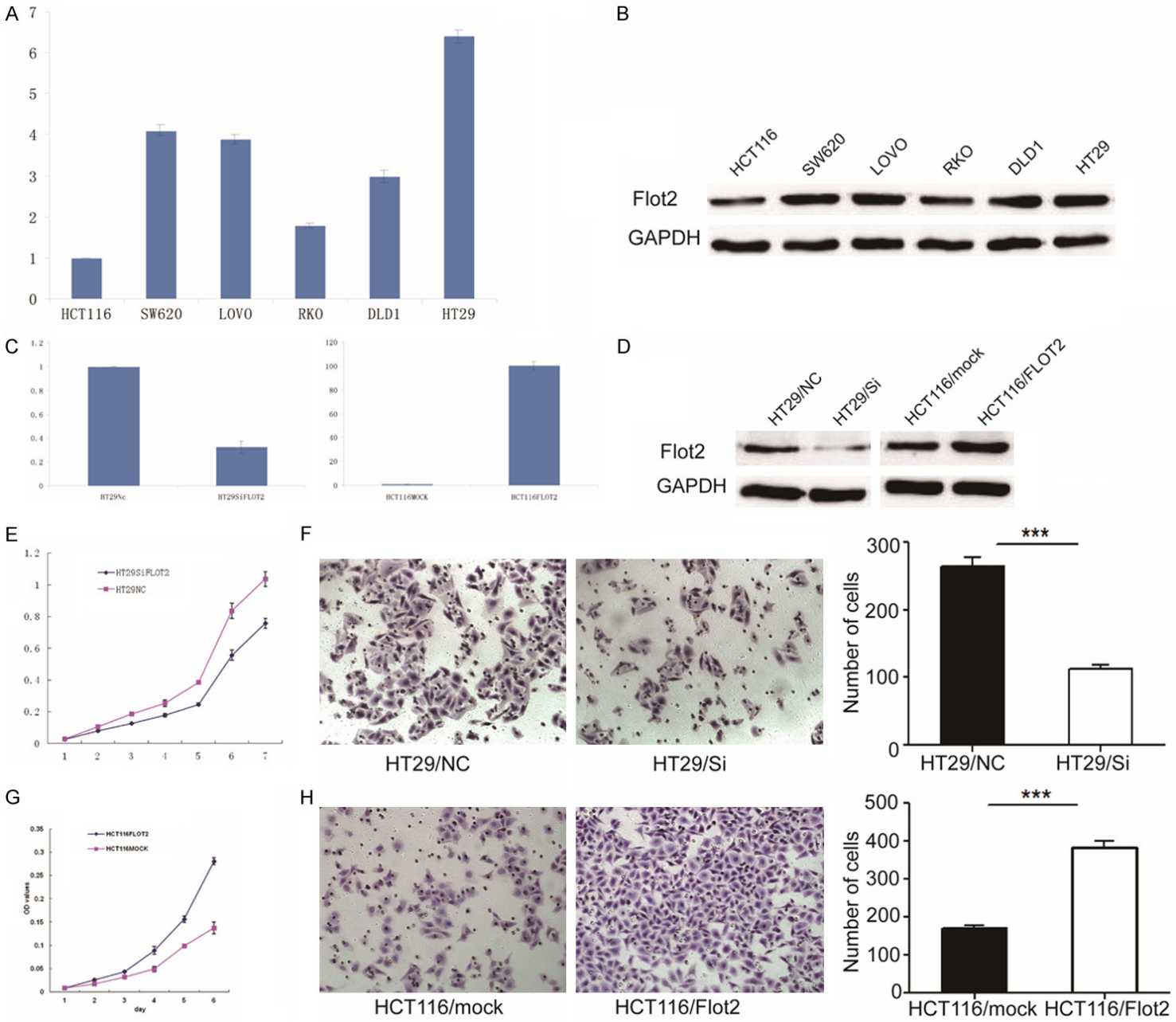
A
HCT116
SW620
LOVO
RKO
DLD1
HT29
B
Flot2
GAPDH
C
D
HT29/NC
HT29/Si
HCT116/mock
HCT116/FLOT2
E
F
HT29/NC
HT29/Si
Number of cells
***
G
H
HCT116/mock
HCT116/Flot2
Number of cells
***

Figure 2. Flot2 promoted proliferation and invasion abilities of CRC cell lines in vitro. A, B. Endogenous expression of Flot2 was detected in 6 CRC cell lines by qRT-PCR and Western Blot. C, D. Transfection efficiency of HT29 and HCT116 was analyzed by qRT-PCR and Western Blot. E, F. Depletion of Flot2 decreased proliferation and invasion abilities of HT29 in vitro. G, H. Over-expression of Flot2 increased proliferation and invasion abilities of HCT116 in vitro.

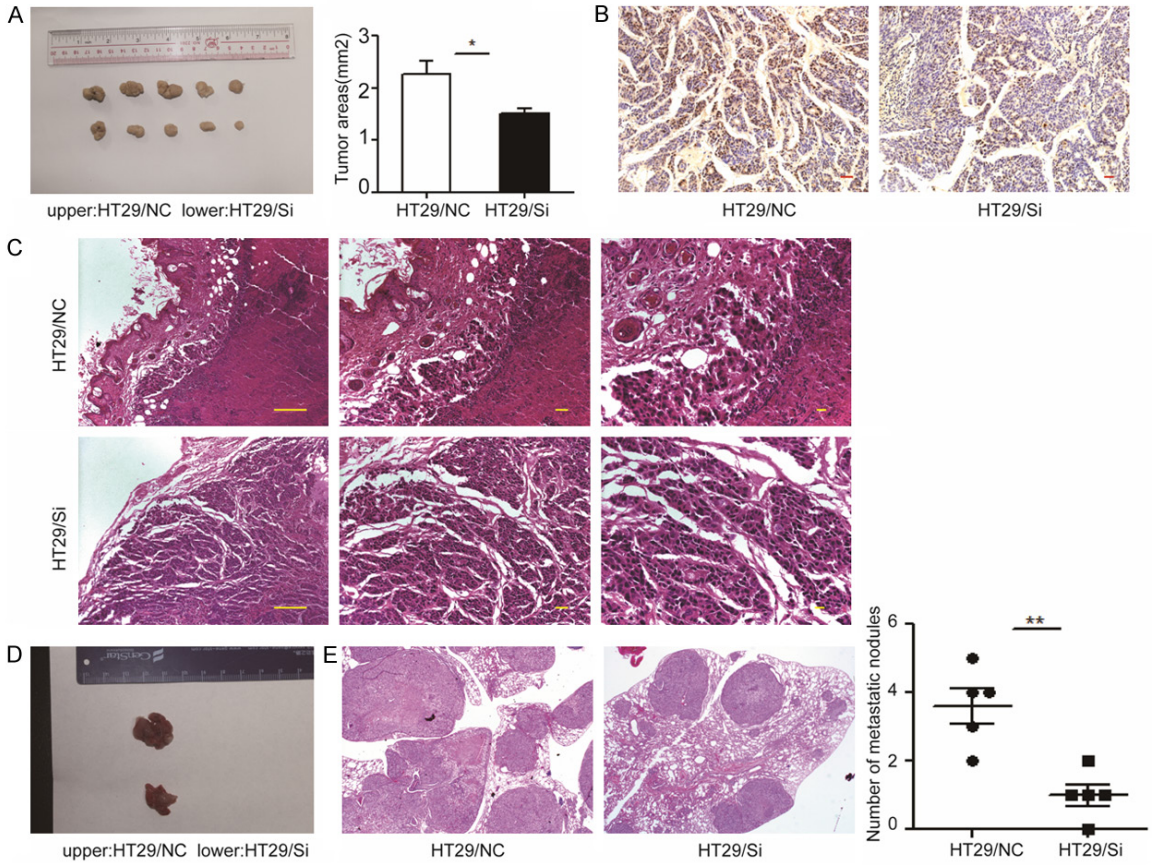

Figure 3. Depletion of Flot2 decreased proliferation and invasion abilities of HT29 in vivo. A. Depletion of Flot2 decreased proliferation ability of HT29 in vivo. B. Ki-67 of subcutaneous tumor was detected by immunohistochemistry assay. C. Depletion of Flot2 group was less aggressive at the border of subcutaneous tumor. D, E. Depletion of Flot2 decreased lung metastasis of nude mice.

Discussion

Colorectal cancer is among the most common magnificent tumors worldwide, besides pathology classification, early diagnosis and therapy methods directly affect its outcomes. Thus, we focused our research on novel biomarkers targeted CRC to provide potential guide for clinical diagnosis and treatment.

It is well-known that Flot2 is a major lipid draft protein, which participates in neuronal differentiation, axonal regeneration, the polarization of primitive and mature hematopoietic cells [11-14]. Recent studies about Flot2 in tumor development showed that it involved in several cancers, such as breast cancer, melanoma and gastric cancer [9, 15-17].

In this study, we investigated Flot2 expression in clinical CRC patients. Immunohistochemistry showed Flot2 mainly expressed in cytoplasm of tumor glands, while normal areas only had weak signals. Survival analysis result showed Flot2 was associated with poor outcomes of CRC patients (P=0.018). Western Blot assays of fresh pairs of CRC samples also improved the same pattern. Together with its expression in other gastrointestinal carcinoma, Flot2 was high expressed in gastric cancer and signifi-

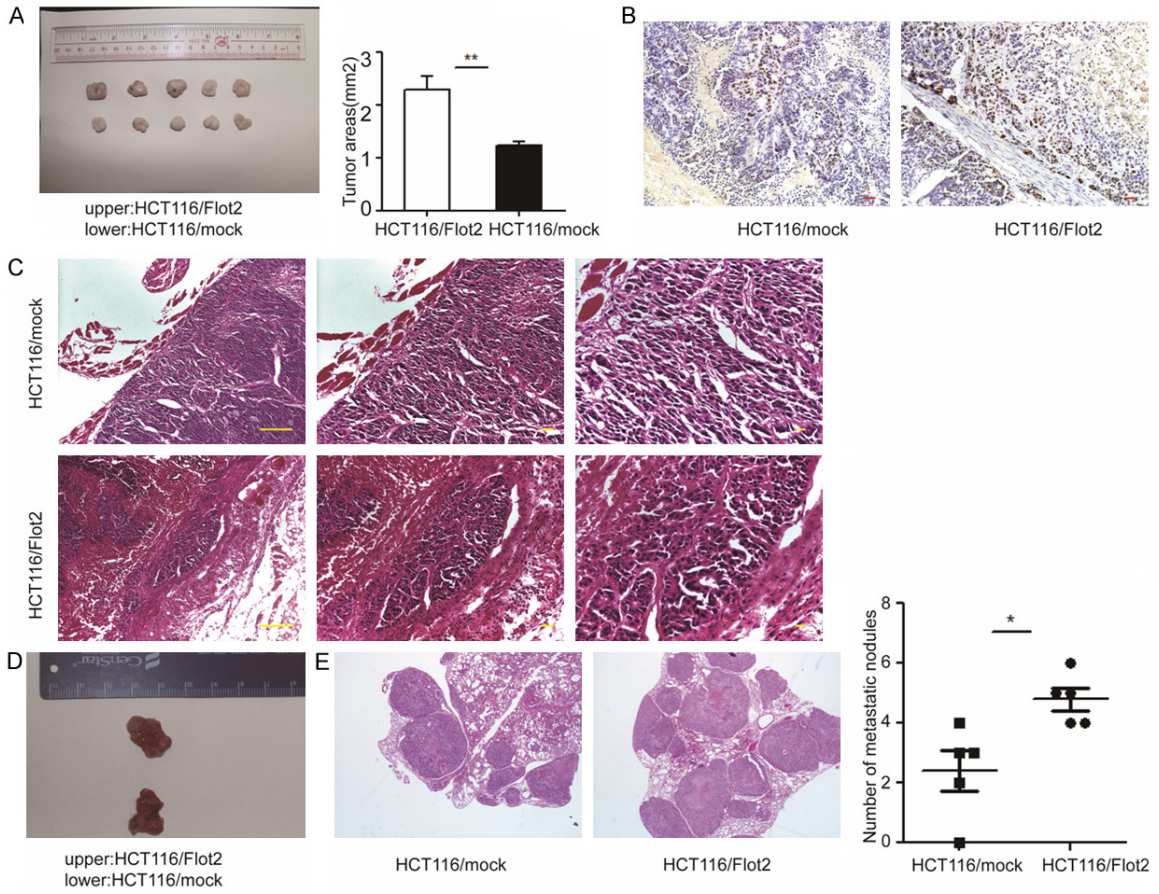

Figure 4. Over-expression of Flot2 increased proliferation and invasion ability of HCT116 in vivo. A. Over-expression of Flot2 increased proliferation ability of HCT116. B. Ki-67 index of subcutaneous tumor was detected by immunohistochemistry assay. C. Over-expression of Flot2 enhanced aggression at the border of subcutaneous tumor. D, E. Over-expression of Flot2 promoted lung metastasis of nude mice.

cantly correlated with cancer progression and prognosis [15].

Next we performed in vitro functional assays to detect biological behaviors of Flot2 in CRC cell lines in vitro. Results indicated that knockdown of Flot2 significantly decreased proliferation and invasion abilities of CRC cell lines in vitro. To further explore Flot2's effect on CRC cells in vivo, we adopted nude mice experiments, results showed that Flot2 promoted proliferation and metastasis of CRC cells in vivo, besides, the borders of subcutaneous tumors of nude mice were much more destroyed in HT29/NC and HCT116/Flot2 groups, which meant more aggressive after gaining of Flot2.

In conclusion, our data showed Flot2 is high expressed in CRC tissues and has correlation with bad outcomes of patients. Flot2 regulates proliferation and invasion of CRC while its delicate mechanism needs deeper research. Our work may provide a new biomarker for clinical diagnosis and treatment for CRC patients.

Disclosure of conflict of interest

None.

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