

Original Article

High expression of flotillin-2 is associated with poor clinical survival in cervical carcinoma

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Abstract: Aims: To investigate the expression and clinical significance of flotillin-2 (FLOT2) in cervical cancer (CC). Methods: We examined FLOT2 mRNA levels in 10 pairs of cervical cancer and adjacent normal tissues. Immunohistochemistry was performed to analyze FLOT2 protein expression in 115 archived cervical cancer samples. The association between FLOT2 levels, clinicopathologic factors and prognosis was analyzed statistically as well. Results: The cancer tissues of CC patients had clearly increased expression of FLOT2 at mRNA level as compared to adjacent nontumorous tissues. Survival analysis of CC patients indicated that FLOT2 expression was significantly associated with poor overall and local recurrence-free survival ($P = 0.025$ and $P = 0.028$, respectively). Moreover, FLOT2 expression was significantly correlated with clinical stage, tumor differentiation, and lymph nodes metastasis. Multivariate analysis revealed that FLOT2 expression was an independent prognostic factor for overall survival in CC patients. Conclusion: FLOT2 may serve as an oncogene in the development of CC, and may serve as a clinicopathologic biomarker for prognosis in CC patients.

Keywords: Cervical cancer, FLOT2, prognosis

Introduction

Cervical cancer (CC) is one of the most frequently occurring malignancies worldwide. It develops majorly from cervical intraepithelial neoplasia (CIN), and the most important risk factor is chronic infection by the human papillomavirus [1]. The molecular pathogenesis of CC includes aberrant expression dominant oncogenes and tumor-suppressor genes and alterations in signaling pathways such as the Wnt/beta-catenin, mTOR, and Notch signaling pathways [2]. Unfortunately, the exact cellular mechanisms of cervical carcinogenesis remain poorly understood. Therefore, understanding the molecular events associated with CC tumorigenesis and prognosis may lead to earlier diagnosis and the development of novel therapeutic strategies.

FLOT2 is a highly conserved protein of the SPFH domain containing proteins family. It was originally isolated from caveolae/lipid raft domains

that tether growth factor receptors linked to signal transduction pathways [3]. The FLOT2 gene is located in a region on human chromosome 17q11.2, and has been suggested to be a transcriptional target of MAPKs and ERK1/2, and could be regulated by ERK1/2 downstream targets Egr1 and SRF [4]. Studies have shown that FLOT2 was expressed ubiquitously in various tissues including brain, heart, lung, and placenta [5], and was associated with various types of human cancers. By binding to PAR-1, FLOT2 promotes tumor cell growth and metastasis, which may influence tumor progression. In addition, FLOT2 was shown to correlate with poor prognosis and clinical survival in several cancers. It was initially reported to be associated with metastatic potential in melanoma [6]. Zhu et al. recently showed that FLOT2 may be involved in stabilizing erbB2 at the plasma membrane, and that FLOT2 expression positively correlates with HER2 level and poor prognosis in gastric cancer [7]. Rickman et al. revealed that FLOT2 has a predictive value for

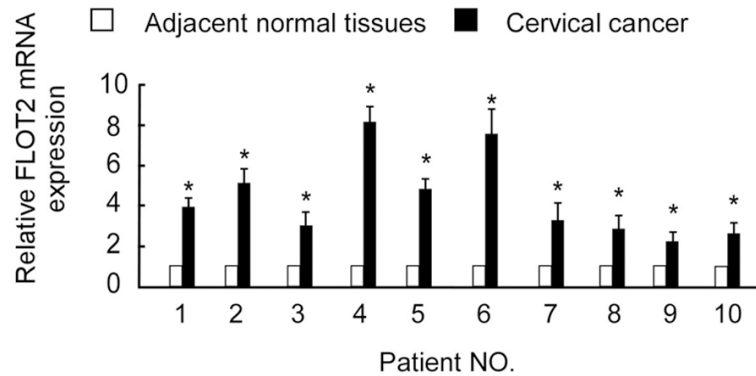


Figure 1. Expression of FLOT2 mRNA in 10 matched pairs of cervical cancer tissues and adjacent normal tissues were examined by Real time-PCR.

the development of metastases in head and neck cancer [8]. Yan et al. reported that FLOT2 is a novel molecule involved in renal carcinoma progression and is closely correlated with poor prognosis in patients with renal carcinoma [9]. However, the role played by FLOT2 in the progression and predicting the prognosis of CC has not yet been determined.

In the present study, we aimed to investigate the role of FLOT2 in cervical cancer. We examined the expression of FLOT2 in CC tissues by Real-time PCR and immunohistochemistry. We then investigated the correlation of FLOT2 expression with clinicopathological features, and patients' clinical survival. Our results suggest that FLOT2 has potential prognostic and therapeutic value for cervical cancer.

Materials and methods

Patients and specimens

We collected tumors from 115 consecutive patients who had undergone surgery for CC at the Guangzhou Women and Children's Medical Center from January 2004 to October 2009. For the use of these clinical materials for research purposes, written informed consent from all patients and approval from the Institutional Research Ethics Committee were obtained. Histopathological diagnoses were made according to the pathological classification system of the International Federation of Gynecology and Obstetrics (FIGO). The available patient clinicopathological information included patients' age, FIGO stage, tumor size, histological grade and survival time.

RNA extraction and Real-time reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from frozen CC tissue samples. All tumors and adjacent nontumorous tissues were manually microdissected under direct visual control through a dissecting microscope, and total RNA in the tissues was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was digested with DNase I (Invitrogen) and was used for the first-strand cDNA reaction.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) was carried out using SYBER green kit in a Light Cycler system (Roche Applied Science). Sequences of the primers were as follows: FLOT2, forward 5'-CCCCAGATTGCTGCCAAA-3', reverse 5'-TCCACTGAGGACCACAATCTCA-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-GAAGATGGTATGGGATTTC-3'.

Immunohistochemistry

Immunohistochemical staining was performed on 4 µm sections of paraffin-embedded archival tissue. Each slide was dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in citrate buffer (10 mmol/L, pH = 6.0) for antigen retrieval. Then the sections were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, were blocked with bovine serum albumin for 30 min and incubated overnight at 4°C with primary mouse monoclonal anti-FLOT2 (Abcam, USA; 1:200). After washing with PBS buffer, the slides were incubated with secondary antibody (Abcam), followed by incubation with diaminobenzidine (DAB), and counterstaining with 10% Mayer hematoxylin. Negative control was done by using isotypic mouse immunoglobulin. All control experiments gave negative results.

Evaluation of immunohistochemical staining

The evaluation of the immunohistochemical staining was performed by two independent authors with no knowledge of the clinic pathological information. The degree of immunohis-

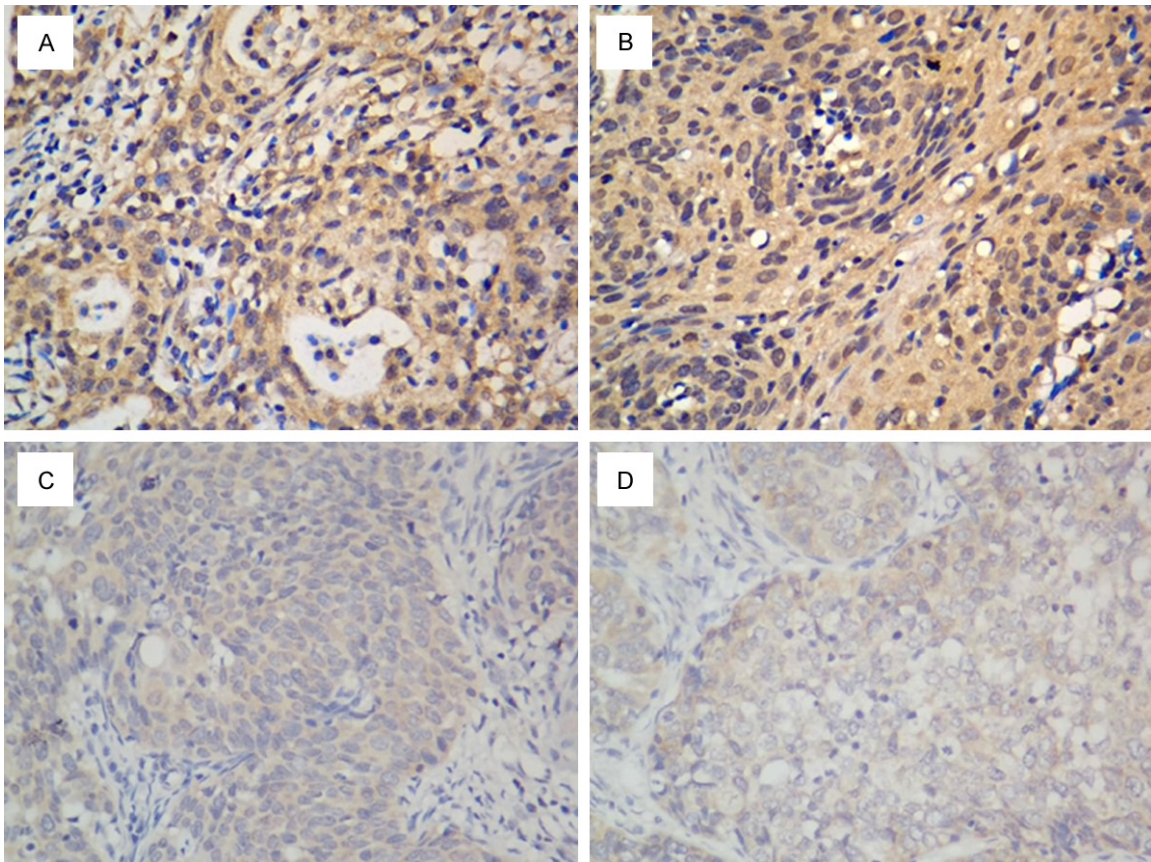


Figure 2. Representative protein expression of FLOT2 in cervical cancer by immunohistochemical staining. A, B. High expression; C, D. Low expression. Original magnification, 400 \times .

tochemical staining was recorded using a semi-quantitative and subjective grading system that considered both the intensity of staining and the proportion of tumor cells that had an unequivocal positive reaction. The intensity of the staining was scored using the following scale: 0: no staining; 1: weak staining; 2: positive staining; and 3: strong staining. The area of staining was evaluated and recorded as a percentage: 0: no staining; 1: positive staining in < 10% of tumor cells; 2: positive staining in 10% to 50% of tumor cells; 3: positive staining in > 50% of tumor cells. The staining index was calculated as by multiplying the positive area and the staining intensity.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 statistical software package. Statistical tests for data analysis included chi-square test, Kaplan-Meier method, and log-rank test. Categorical data were analyzed using the chi-square test, and survival curves were

plotted by using Kaplan-Meier method. Survival data were estimated using multivariate Cox regression analyses. Values of $P < 0.05$ were considered statistically significant.

Results

FLOT2 is upregulated in cervical cancer tissues

To estimate the expression levels of FLOT2 RNA in cervical cancer tissues, Real-time PCR analysis was performed in 10 pairs of cervical cancer and adjacent normal tissues. The expression of FLOT2 mRNA was highly expression in cervical cancer tissues in compared with that in adjacent normal tissues (**Figure 1**).

Correlations between FLOT2 expression and the clinical features of cervical cancer

To investigate the potential functions of FLOT2 in cervical cancer progression, we measured the expression pattern of FLOT2 in 115 archived

Table 1. FLOT2 expression and clinicopathological characteristics in cervical cancer

Parameters	Group	Total	FLOT2		P value
			low	high	
Age (years)	≤ 50	74	30	44	0.874
	> 50	41	16	25	
FIGO Stage	IB	87	40	47	0.021
	> IB	28	6	22	
Tumor size (cm)	≤ 4	79	34	45	0.325
	> 4	36	12	24	
Differentiation (grade)	1/2	91	41	50	0.031
	3	24	5	19	
Histology	SCC	84	31	53	0.307
	AC	31	15	16	
LN Metastasis	No	94	42	54	0.036
	Yes	21	4	17	

SCC: squamous cell cancer; AC: Adenocarcinoma.

cervical cancer specimens. The representative immunostaining of FLOT2 in cervical cancer was shown in **Figure 2**. The associations among the protein expression levels of FLOT2 and the clinical features of cervical cancer patients are shown in **Table 1**. High FLOT2 expression was positively associated with clinical stage ($P = 0.021$), tumor differentiation ($P = 0.031$), and lymph nodes metastasis ($P = 0.036$). There was no significant association between FLOT2 expression and age, tumor size, and tumor differentiation.

FLOT2 expression predicts prognosis in patients with cervical cancer

The overall and recurrence-free survival curves for the remaining 115 CC cases are shown in **Figure 3**. Kaplan-Meier analysis showed that patients with higher FLOT2 expression had shorter overall ($P = 0.025$, **Figure 3**) and recurrence-free ($P = 0.028$, **Figure 3**) survival time than that with lower FLOT2 expression. Multivariate analysis showed that FLOT2 expression was an independent prognostic factor for the overall survival of cervical cancer patients (**Table 2**). These results suggested that FLOT2 expression could be used as a powerful independent prognostic factor in cervical cancer patients.

Discussion

In the present study, we investigated the clinical significance of FLOT2 in cervical cancer. We

found that the expression level of FLOT2 was overexpressed in cervical cancer in compared to adjacent nontumorous tissues. Moreover, FLOT2 expression was closely correlated with tumor stage, tumor differentiation, and lymph nodes metastasis. Survival analysis revealed that high levels of FLOT2 had poorer clinical outcome in cervical cancer patients. Our study may lead to improvement in the diagnosis and treatment of cervical cancer.

It has originally been demonstrated that flotillins are transmembrane proteins that are enriched in caveolae [10], while there are debates because later studies showed that flotillins reside within non-caveolar rafts [11], and that they are attached to the cytosolic leaflet of the plasma membrane rather than traverse the membrane [12]. The expression of flotillins was reported to be particularly high in several tissues including brain, lung, heart, and low in liver and pancreas, and the subcellular localization of flotillins is extremely dynamic [13]. As many signal pathways take place in membrane rafts, flotillins have been suggested to participate in various signaling pathways, in which they exhibit a scaffolding function in the rafts. Recent studies have indicated that the upregulation of FLOT2 was associated with tumor progression and metastasis. Pust et al. showed that depletion of FLOT1 and FLOT2 leads to internalization and degradation of ErbB2, which triggered downstream signaling in breast cancer [14]. Hazarika et al. found that overexpression of FLOT2 is associated with melanoma progression and with transformation of SB2 melanoma cells to a highly metastatic cell line [6]. Doherty et al. demonstrated that high FLOT2 expression is associated with lymph node metastasis and Breslow depth in melanoma [15]. However, the role of FLOT2 in cervical cancer remains unknown.

From the clinicalpathological data, we showed that high levels of FLOT2 expression are significantly correlated with tumor stage, differentiation, and lymph nodes metastasis. Previous studies have shown that flotillins are located predominantly at the plasma membrane and in intracellular structures, and that flotillins expression is often increased in differentiated cells [10, 16]. In vitro study has revealed that transient knockdown of FLOT2 impairs tumor cell spreading, whereas overexpression of FLOT2 enhances cell spreading and induces

FLOT2 is associated with survival in cervical cancer

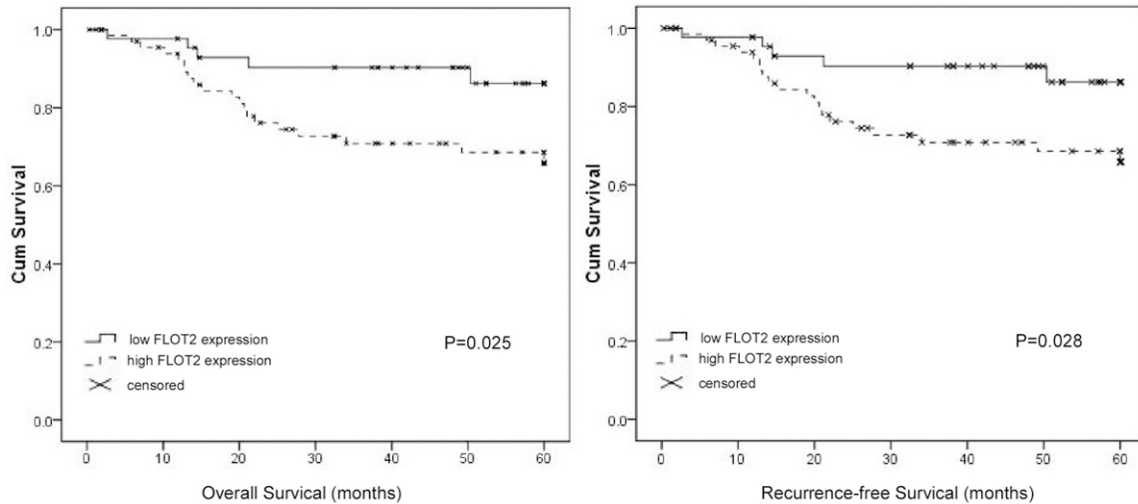


Figure 3. Survival curves of 115 cervical cancer patients. Patients with higher FLOT2 expression in tumor were closely correlated with poorer overall survival (left) and recurrence-free survival (right) than that with tumor with lower FLOT2 expression ($P < 0.05$, respectively).

Table 2. Prognostic factors in Cox proportional hazards model for cervical cancer patients

Prognostic variables	OS		RFS	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Age (> 50 vs. ≤ 50)	1.055 (0.278-4.149)	0.714	1.148 (0.201-4.216)	0.825
FIGO Stage (> IB vs. IB)	1.874 (0.172-5.679)	0.241	1.897 (0.427-5.543)	0.274
Tumor size (> 4 cm vs. ≤ 4 cm)	2.531 (0.384-6.217)	0.591	2.426 (0.229-7.644)	0.672
Differentiation (Grade 3 vs. 1/2)	1.373 (0.194-4.269)	0.410	1.590 (0.209-4.218)	0.382
LN Metastasis (yes vs. no)	4.214 (2.615-10.214)	0.028	4.029 (2.177-12.311)	0.048
FLOT2 (high vs. low)	3.919 (1.927-8.011)	0.037	3.178 (2.417-6.964)	0.089

filopodia-like protrusions in an expression level dependent manner [17]. In addition, flotillin microdomains have been shown to be involved in neutrophil migration [18]. In some cancers, flotillin-1 was shown to be increased and positively correlated with disease stage [19]. Thus, we propose that FLOT2 may also regulate the growth, differentiation and metastasis of cervical cancer cells, which is supported by Wang et al., who have demonstrated that overexpression of FLOT2 is associated with the clinical stage, histological differentiation and receptor tyrosine kinase ErbB2 expression level in breast cancer [20].

To further understand the prognostic role of FLOT2 in cervical cancer, survival analysis was conducted. Our data showed that higher expression of FLOT2 expression is closely correlated with poorer overall and recurrence-free survival. These data are in line with previous

studies in that high expression of flotillins is correlated with poor cancer patient survival. Lin et al. reported that FLOT1 promotes cell proliferation and tumorigenicity and is correlated with the poor clinical survival in breast cancer patients [21]. Song et al. demonstrated that FLOT1 promotes cancer cell proliferation and is correlated with disease stage and survival time in esophageal squamous cell carcinoma [22]. These studies together indicated that FLOT2 could be used as a prognostic marker in cervical cancer.

In conclusion, our study demonstrates that FLOT2 is up-regulated and can promote the malignant phenotypes of in cervical cancer. FLOT2 expression is inversely associated with the tumor stage, differentiation, lymph node metastasis, and clinical survival of cervical cancer patients. Thus, new strategies in cervical cancer treatment could be developed by targeting FLOT2 expression.

Disclosure of conflict of interest

None.

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