Int J Clin Exp Pathol 2016;9(2):1752-1757
www.ijcep.com /ISSN:1936-2625/IJCEP0017465

Original Article

High expression of Flotillin-2 is associated with poor prognosis in Chinese patients with colorectal cancer

Guang-Yao Ye*, Yang Luo*, Shao-Lan Qin, Yi-Fei Mu, Ming Zhong

*Department of Gastrointestinal Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, P. R. China. *Equal contributors.*

Received October 8, 2015; Accepted November 20, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Flotillin-2 (Flot-2), which is frequently up-regulated in multiple human malignancies, has been demonstrated to play a critical function in neuronal differentiation and tumorigenesis. However, limited knowledge is known about the expression pattern and prognostic value of Flot-2 in colorectal cancer (CRC). In this study, we firstly observed Flot-2 mRNA is commonly upregulated in 32 paired CRC tissues compared with their normal counterparts. Furthermore, by a large scale of immunohistochemical analysis containing 310 cases of CRC specimens, we demonstrated that Flot-2 protein expression is upregulated in 62.58% (194/310) samples. And we detected that decreasing Flot-2 expression is closely positively correlated with enhanced tumor size, CEA level, TNM stage. Meanwhile, Kaplan-Meier survival analysis showed that CRC patients with a higher Flot-2 expression have a poorer clinical outcome than those with a lower Flot-2 expression. Multivariate Cox regression analysis revealed that Flot-2 and TNM stage are two independent prognostic factors for overall survival rate of CRC patients. Taken together, our studies revealed the prognostic value of Flot-2 in CRC and supported Flot-2 may act as a molecular target for CRC treatment.

Keywords: Flot-2, colorectal cancer, immunohistochemistry, prognosis

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide, which is a multistep process involving apoptosis, differentiation and survival mechanisms [1]. Although current novel monoclonal antibody-based therapies have improved the prognosis of CRC patients, a significant proportion of patients still die from the disease, and the clinical outcome and prognosis of CRC patients remains poor [2, 3]. Consequently, to provide better treatment strategies, there is an urgent need to further understand the precise molecular mechanism of CRC and to identify new prognostic biomarkers and therapeutic targets for colorectal cancer.

Flotillin-2 (Flot-2) is a highly conserved protein and belongs to the SPFH (Stomatin/Prohibitin/Flotillin/HflK/C) protein superfamily [4, 5]. Flot-2 could be associated with growth factor receptors which are linked to signal transduction pathways in human cells. Lots of studies showed that Flot-2 have various functions. For instance, it regulated neuronal differentiation and endocytosis, cell proliferation and invasion [6-8]. Recently, accumulating evidence has suggested that Flot-2 may play key roles in the development and progression of human malignant tumors, such as renal cancer [9], lung cancer [10], breast cancer [11], nasopharyngeal cancer [12] and melanoma [13]. However, to our knowledge, the expression pattern and prognostic role of Flot-2 expression has not been reported in CRC yet.

Materials and methods

Tumor specimens

Thirty-two freshly frozen CRC samples and corresponding non-tumor tissues were obtained from Renji hospital. In addition, we collected 310 paraffin embedded CRC specimens from our hospital between January 2005 and De-

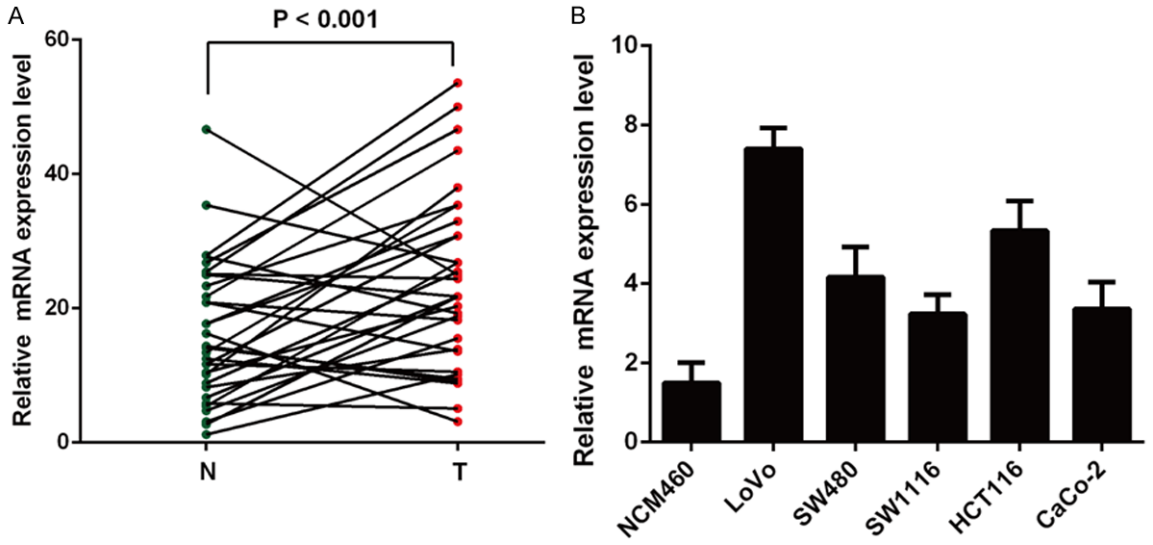

Figure 1. Flot-2 expression is commonly up-regulated in CRC tissues and cell lines at mRNA level. A: The mRNA expression of Flot-2 in 32 pairs of CRC tumor and non-tumor tissues was detected by qPCR. B: The mRNA expression of Flot-2 in 5 CRC cell lines and the normal control cells. *P*-values were calculated by Paired t-test.

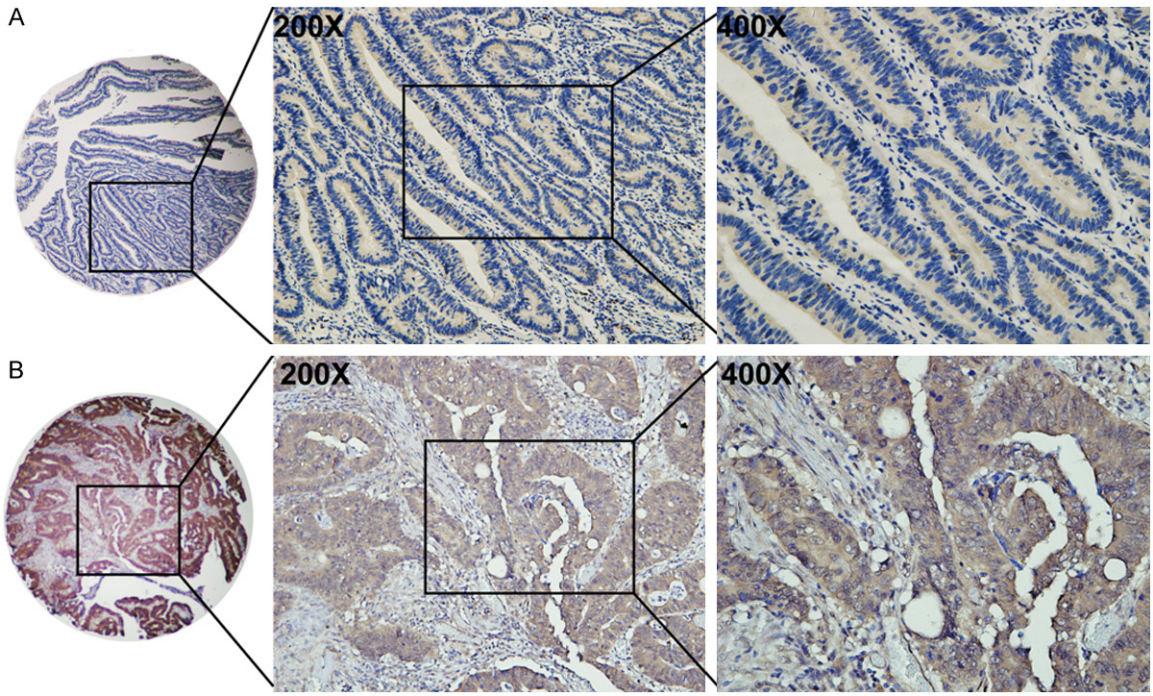

Figure 2. Flot-2 expression in CRC was determined by immunochemistry. A: Negative expression of Flot-2. B: Positive expression of Flot-2. Representative images are shown at 200 × and 400 × magnification, respectively.

cember 2014. Tumor staging for the specimens was carried out according to the American Joint Committee on Cancer staging criteria. The median follow-up time was 50.51 months (range from 6 to 120 months). The follow-up duration was calculated from the date of surgery to the date of death or the last known follow-up. The study was approved by the Institute Research Ethics Committee at the Shanghai Jiao Tong University, School of Medicine, Renji

Table 1. Correlations between Flot-2 expression and clinicopathologic features in 310 colorectal cancer patients

Clinicopathological feature	Total 310	Expression of Flot-2		*P* value (χ^2 test)
		Low (n = 116, 37.42%)	High (n = 194, 62.58%)	
Age (years)				
< 65	171	70 (40.94)	101 (59.06)	0.159
≥ 65	139	46 (33.09)	93 (66.91)	
Gender				
Male	178	65 (36.52)	113 (63.48)	0.723
Female	132	51 (38.64)	81 (61.36)	
Tumor location				
Rectum	176	67 (38.07)	109 (61.93)	0.814
Colon	134	49 (36.57)	85 (63.43)	
Tumor size				
≤ 5 cm	169	76 (44.97)	93 (55.03)	**0.003**
> 5 cm	141	40 (28.37)	101 (71.63)	
CEA level				
≤ 5 ng/ml	175	86 (49.14)	89 (50.86)	**0.000**
> 5 ng/ml	135	30 (22.22)	105 (77.78)	
TNM stage (AJCC)				
Stage I	45	20 (44.44)	25 (55.56)	**0.000**
Stage II	107	57 (53.27)	50 (46.73)	
Stage III	122	30 (24.59)	92 (75.41)	
Stage IV	36	9 (25.00)	27 (75.00)	

Values in parentheses indicate percentage values. The bold number represents the *P*-values with significant differences.

Hospital, and written informed consent was obtained from each patient.

Immunohistochemistry

Sections of paraffin-embedded CRC specimens were prepared and standard immunohistochemical procedures were carried out as previously described [14]. The Flot-2 antibody was purchased from Abcam (1:100, Cambridge, UK). Protein expression was quantified using a visual grading system based on the extent and intensity of staining. The staining results were scored by two pathologists blinded to the clinical data. Staining index was calculated as the product of the staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the proportion of positive cells (0, no positive tumor cells; 1, < 25%; 2, 26-50%; 3, 51-75%; 4, > 75%). The final score was designated as low or high expression group using the percent of positive cell score × staining intensity score, and low expression was defined as a total score < 6 and high expression with a total score ≥ 6.

Cell culture

The human CRC cell lines (CaCo2, HT29, SW1116, HCT116 and SW480) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The normal control cell line NCM460 was obtained from American Type Culture Collection (ATCC). All cell lines were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified incubator containing 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Takara, Japan). The reverse-transcription PCR (RT-PCR) was performed using a PrimeScript RT-PCR kit (Takara, Japan), and the quantitative real-time PCR (qPCR) was performed in the StepOne Real-Time PCR System (Applied Biosystems, Grand Island, USA) using a SYBR Premix ExTaq kit according to the manufacturer's instructions. Primers for qPCR are as follows. Flot2: Forward, 5'-CCCCAGATTGCTGCCAAA-3', Reverse 5'-TCCACTGAGGACCACAATCTCA-3'. GAPDH: Forward, 5'-CTCCTC CTGTTCGACAGTCAGC-3', Reverse, 5'-CCCAATACGACCAAA TCCGTT-3'. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. Each assay was carried out in triplicate.

Results

Flot-2 expression is commonly up-regulated in CRC tissues and cell lines at mRNA level

To observe the expression pattern of Flot-2 in CRC, we first examined Flot-2 mRNA expression in 32 CRC tissues and paired adjacent normal tissues using qPCR. The results showed that Flot-2 mRNA expression was upregulated in 59.38% (19/32) of patients (**Figure 1A**). Consistent with this, the mRNA expression of Flot-2 was also up-regulated in 5 CRC cell lines compared with the normal colonic epithelial cells

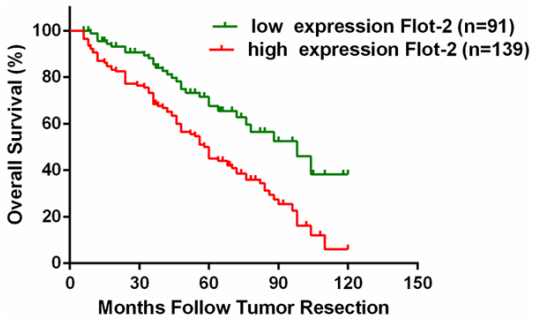

Figure 3. Flot-2 is correlated with overall survival rate in CRC patients. Kaplan-Meier survival curves show high expression level of Flot-2 was significantly correlated with poor survival of CRC. *P*-values were calculated by log-rank test.

NCM460 (**Figure 1B**). Collectively, these data above suggested that Flot-2 was up-regulated in CRC tissues and cell lines.

Correlation between elevated Flot-2 expression and corresponding clinicopathological parameters in patients with CRC

To further investigate Flot-2 expression at protein level, we performed immunohistochemical analysis of a tissue microarray containing 310 cases of CRC specimens. The result showed that Flot-2 protein is highly expressed in 62.58% (194/310) CRC tissues (**Figure 2B**), while the rest 116 (37.42%) samples remained a low expression level (**Figure 2A**). As shown in **Table 1**, Flot-2 protein expression was significantly positively correlated with tumor size, serum CEA level and TNM stage, whereas no significant difference was found in age, gender and tumor location. This result indicates that up-regulated Flot-2 might contribute to the development and progression of CRC.

Prognostic value of Flot-2 in patients with CRC

To determine the prognostic value of Flot-2 for CRC, the relationship between Flot-2 expression and the clinical follow-up data was analyzed using Kaplan-Meier survival curves and the log-rank test. The result revealed that high expression of Flot-2 was inversely associated with overall survival (OS) (n = 230, P = 0.000, **Figure 3**), which indicate that the OS is poor in CRC patients with high Flot-2 expression than in those with low Flot-2 expression.

To directly identify the risk factors associated with OS in CRC patients, univariate and multivariate analysis were performed to confirm that Flot-2 represents an independent risk factor for poor prognosis. Univariate Cox regression analysis showed that the Flot-2 expression level, CEA level, tumor size and TNM stage were significantly associated with OS (**Table 2**). Furthermore, multivariate Cox regression analysis confirmed that the Flot-2 expression level and TNM stage were independent predictors of OS in patients with CRC (**Table 2**). These data indicated that high expression of Flot-2 may be a predictor for diagnosis and prognosis in colorectal cancer patients.

Discussion

Flot-2, as a target gene of p63 and p73, member of the p53 transcription factor family [15, 16], has been proposed as a prognostic marker linked to poor prognosis in several human tumors, such as breast cancer [17], gastric cancer [18], cervical cancer [19]. However, the significance of Flot-2 in colorectal cancer was still seldom reported. In this study, we observed that Flot-2 is frequently up-regulated at mRNA and protein level in both CRC clinical samples and cell lines compared with normal control tissues.

Since it was found that Flot-2 expression was associated with CRC invasion and metastasis, which may determine tumor prognosis, we further evaluated its prognostic value by the Kaplan-Meier analysis. Results showed that CRC patients with high Flot-2 expression tend to have worse overall survival compared with those with low Flot-2 expression. These results indicated that Flot-2 expression in CRC might serve as a new prognostic marker for diagnosis. Jie et al. found Flot-2 exerts a cancerous role in nasopharyngeal carcinoma through NF-κB and PI3K/Akt3 signaling and is involved in tumor progression and metastasis [8]. This finding was consistent with our studies which demonstrated that Flot-2 expression was associated with TNM stage in CRC. Moreover, univariate and multivariate analysis demonstrated that Flot-2 expression was an independent risk factor in the prognosis of CRC patients. However, the molecular mechanisms underlying the oncogenic functions of Flot-2, which contribute to the tumor progression and poor prognosis of CRC, remain further excavation.

In conclusion, our studies demonstrate that Flot-2 is up-regulated in CRC at both mRNA and

Table 2. Univariate and multivariate analyses of prognostic parameters for survival in 230 colorectal cancer patients

	Univariate analysis			Multivariate analysis		
Prognostic parameter	HR	95% CI	*P* value	HR	95% CI	*P* value
Expression of Flot-2 (low vs. high)	2.154	1.420-3.266	**0.000**	1.607	1.035-2.495	**0.034**
Age (< 65 vs. ≥ 65)	1.185	0.822-1.708	0.364	-	-	-
Gender (male vs. female)	1.046	0.727-1.507	0.807	-	-	-
Tumor Size (≤ 5 cm vs. > 5 cm)	1.488	1.028-2.153	**0.035**	1.034	0.689-1.552	0.873
CEA level (≤ 5 ng/ml vs. > 5 ng/ml)	1.517	1.051-2.188	**0.026**	1.416	0.971-2.066	0.071
Tumor location (rectum vs. colon)	1.348	0.932-1.951	0.113	-	-	-
TNM stage (I vs. II vs. III vs. IV)	1.777	1.428-2.212	**0.000**	1.600	1.256-2.039	**0.000**

HR: Hazard ratio; CI: Confidence interval. The bold number represents the *P*-values with significant differences.

protein level and is valuable predictor for poor outcome of CRC patients. In the current era of personalized medicine, a novel prognostic factor may provide clinicians with great opportunities for early interventions and further improve the prognosis of patients with CRC. Thus, our study might help to determine optimal treatment strategies of CRC.

Disclosure of conflict of interest

None.

Address correspondence to: Ming Zhong, Department of Gastrointestinal Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 1630 Dongfang Road, Shanghai 200127, P. R. China. Tel: +86-21-68383985; E-mail: drzhongming@hotmail.com

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
[2] Brenner H, Kloor M and Pox CP. Colorectal cancer. Lancet 2014; 383: 1490-1502.
[3] Clark ME and Smith RR. Liver-directed therapies in metastatic colorectal cancer. J Gastrointest Oncol 2014; 5: 374-387.
[4] Staubach S and Hanisch FG. Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. Expert Rev Proteomics 2011; 8: 263-277.
[5] Salzer U and Prohaska R. Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. Blood 2001; 97: 1141-1143.
[6] Munderloh C, Solis GP, Bodrikov V, Jaeger FA, Wiechers M, Malaga-Trillo E and Stuermer CA. Reggies/flotillins regulate retinal axon regeneration in the zebrafish optic nerve and differentiation of hippocampal and N2a neurons. J Neurosci 2009; 29: 6607-6615.
[7] Meister M and Tikkanen R. Endocytic trafficking of membrane-bound cargo: a flotillin point of view. Membranes (Basel) 2014; 4: 356-371.
[8] Liu J, Huang W, Ren C, Wen Q, Liu W, Yang X, Wang L, Zhu B, Zeng L, Feng X, Zhang C, Chen H, Jia W, Zhang L, Xia X and Chen Y. Flotillin-2 promotes metastasis of nasopharyngeal carcinoma by activating NF-kappaB and PI3K/Akt3 signaling pathways. Sci Rep 2015; 5: 11614.
[9] Zhang Y, Li J, Song Y, Chen F, Pei Y and Yao F. Flotillin-1 expression in human clear-cell renal cell carcinoma is associated with cancer progression and poor patient survival. Mol Med Rep 2014; 10: 860-866.
[10] Wen Q, Wang W, Chu S, Luo J, Chen L, Xie G, Xu L, Li M and Fan S. Flot-2 Expression Correlates with EGFR Levels and Poor Prognosis in Surgically Resected Non-Small Cell Lung Cancer. PLoS One 2015; 10: e0132190.
[11] Xie G, Li J, Chen J, Tang X, Wu S and Liao C. Knockdown of flotillin-2 impairs the proliferation of breast cancer cells through modulation of Akt/FOXO signaling. Oncol Rep 2015; 33: 2285-2290.
[12] Zhao L, Lin L, Pan C, Shi M, Liao Y, Bin J and Liao W. Flotillin-2 promotes nasopharyngeal carcinoma metastasis and is necessary for the epithelial-mesenchymal transition induced by transforming growth factor-beta. Oncotarget 2015; 6: 9781-9793.
[13] Liu R, Xie H, Luo C, Chen Z, Zhou X, Xia K, Chen X, Zhou M, Cao P, Cao K and Zhou J. Identification of FLOT2 as a novel target for microRNA-34a in melanoma. J Cancer Res Clin Oncol 2015; 141: 993-1006.
[14] Yu MH, Luo Y, Qin SL and Zhong M. Increased expression of Rab5A predicts metastasis and poor prognosis in colorectal cancer patients. Int J Clin Exp Pathol 2015; 8: 6974-6980.
[15] Banning A, Regenbrecht CR and Tikkanen R. Increased activity of mitogen activated protein

kinase pathway in flotillin-2 knockout mouse model. Cell Signal 2014; 26: 198-207.
[16] Sasaki Y, Oshima Y, Koyama R, Maruyama R, Akashi H, Mita H, Toyota M, Shinomura Y, Imai K and Tokino T. Identification of flotillin-2, a major protein on lipid rafts, as a novel target of p53 family members. Mol Cancer Res 2008; 6: 395-406.
[17] Wang X, Yang Q, Guo L, Li XH, Zhao XH, Song LB and Lin HX. Flotillin-2 is associated with breast cancer progression and poor survival outcomes. J Transl Med 2013; 11: 190.
[18] Cao K, Xie D, Cao P, Zou Q, Lu C, Xiao S, Zhou J and Peng X. SiRNA-mediated flotillin-2 (Flot2) downregulation inhibits cell proliferation, migration, and invasion in gastric carcinoma cells. Oncol Res 2014; 21: 271-279.
[19] Liu Y, Lin L, Huang Z, Ji B, Mei S, Lin Y and Shen Z. High expression of flotillin-2 is associated with poor clinical survival in cervical carcinoma. Int J Clin Exp Pathol 2015; 8: 622-628.