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Original Article

Expression of flotillin-2 in human non-small cell lung cancer and its correlation with tumor progression and patient survival

Yong-Lian Wang[1], Wen-Jian Yao[1], Ling Guo[1], Hui-Fang Xi[2], Song-Yue Li[1], Zhong-Min Wang[1]

Departments of [1]Thoracic Surgery, [2]Pediatric Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, China

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Abstract: Introduction: Recent studies have revealed that flotillin-2 (FLOT2) played important roles in cancer progression. The aim of this study was to investigate the clinicopathologic and prognostic significance of FLOT2 expression in human non-small cell lung cancer (NSCLC). Methods: Quantitative real-time PCR (qRT-PCR) was performed to detect FLOT2 mRNA expression in lung cancer cell lines, normal bronchial epithelial cells, 24 pairs of NSCLC tissues and matched adjacent non-tumor tissues. Immunohistochemistry (IHC) was performed to examine FLOT2 protein expression in paraffin-embedded tissues from 90 NSCLC patients. Statistical analyses were performed to evaluate the clinicopathological significance of FLOT2 expression. Results: FLOT2 mRNA expression was evidently up-regulated in lung cancer cell lines and NSCLC tissues compared with normal bronchial epithelial cells and adjacent non-tumor tissues. In the 90 cases of tested NSCLC samples, FLOT2 protein level was positively correlated with tumor stage, and lymph node metastasis. Patients with high FLOT2 expression had shorter overall survival compared with the low FLOT2 expression group. Univariate and multivariate analyses indicated that high FLOT2 expression was an independent poor prognostic factor for NSCLC patients. Conclusions: Our findings provided that high FLOT2 expression was associated with poor outcomes in NSCLC patients, and FLOT2 could be a potential prognostic biomarker for lung cancer progression.

Keywords: FLOT2, lung cancer, non-small cell lung cancer, prognosis

Introduction

Lung cancer is the leading cause of cancer-related death worldwide and non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases [1]. Surgical resection, when possible remains the only curative treatment for early stage of NSCLC. However, nearly 50% of resected patients experience recurrence [2]. The overall 5-year survival for all lung cancer patients is 15%; however, the 5-year survival for patients with pathologic stage I NSCLC is 58-73%, underscoring the importance of the early detection of this disease [3]. Therefore, a better understanding of the molecular mechanisms involve in NSCLC progression will likely contribute to providing useful prognostic biomarker and therapeutic target for NSCLC therapy.

The flotillin family of proteins (also known as the reggie family) is markers of lipid rafts that contain 2 homologous isoforms, flotillin-1 (FLOT1) and flotillin-2 (FLOT2) [4]. FLOT2 is a highly conserved 47 kDa protein which is a marker for caveolae/lipid raft domains that tether growth factor receptors linked to signal transduction pathways [5]. Lots of studies showed that FLOT2 have various functions. For instance, it regulated neuronal differentiation and axonal regeneration, participates in the endocytosis, and was involved in the polarization of primitive and mature hematopoietic cells, as well as the development of neurodegenerative diseases [6-9]. Recently, accumulating evidence has suggested that FLOT2 may play key roles in the development and progression of human malignant tumors. For example, Wang et al found that over-expression of FLOT2 was associated with poor prognosis and reduced survival of patients with breast cancer; multivariate analysis indicated that FLOT2 could be used as an independent prognostic predictor for breast cancer patients [10]. Cao et

Table 1. Correlation of FLOT2 expression with clinicopathologic features in NSCLC patients

Variable	Number	FLOT2 expression		*P* value
		Low	High	
Age (years)				0.996
< 60	23	11	12	
≥ 60	67	32	35	
Gender				0.464
Male	66	30	36	
Female	24	13	11	
Tumor size (cm)				0.605
< 3	61	28	33	
≥ 3	29	15	14	
Histologic type				0.589
Squamous	57	26	31	
Adenoma	33	17	16	
Tumor stage				0.001
I-II	58	35	23	
III	32	8	24	
Lymph nodes metastasis				0.002
No	54	33	21	
Yes	36	10	26	

al revealed that FLOT2 protein expression was significantly correlated with cancer progression and poor prognosis in gastric carcinomas, probably due to its role in the regulation of cell proliferation, migration, and invasion in gastric carcinoma cells [11]. Yan et al proved that FLOT2 was up-regulated in renal cancer and associated with advanced clinical stage and poorer prognosis [12]. Berger et al found FLOT2 deficiency lead to a striking reduction in the number of lung metastasis observed, but had no influence on primary tumor formation in mouse breast cancer model [13]. Moreover, up-regulation of FLOT2 expression was shown to be associated with melanoma progression [14, 15]. These findings suggested that FLOT2 played a dominant positive role in the development and progression of cancers. However, to our knowledge, the expression pattern and prognostic role of FLOT2 expression has not been reported in NSCLC yet.

In the present study, qRT-PCR assay and ICH assay were performed to detect the expression of FLOT2 in lung cancer. Moreover, the correlations of FLOT2 expression with clinicopathologic features of NSCLC patients were statistically analyzed. Finally, we determined the potential role of FLOT2 in NSCLC prognostic prediction. Our results showed that FLOT2 was significantly up-regulated in lung cancer cell lines and NSCLC tissues and could be served as a potential molecular biomarker for the prediction of poor prognosis.

Methods

Patients and surgical specimens

For qRT-PCR analysis, we collected 24 paired fresh NSCLC tissues and adjacent non-tumor tissues from patients who underwent surgery between Jan 2013 and Dec 2013. In addition, a cohort of 90 formalin-fixed and paraffin-embedded tissues of NSCLC diagnosed between Jan 2005 and Jan 2009 at the Department of Thoracic Surgery, First Affiliated Hospital of Xinxiang Medical University were retrieved. Clinical and clinicopathological classification and staging were determined according to the American Joint Committee on Cancer (AJCC) criteria [16]. Patient consent was gained prior to the use of these clinical materials for research purposes, prior patients' consents, and the protocol was approved from the Institutional Research Ethics Committee. Clinical information on the samples was summarized in **Table 1**. The follow-up time of the primary NSCLC cohort ranged from 5 to 60 months, and the median follow-up time was 44 months.

Cell culture

The human lung cell lines A549, H157, HEK-293T and normal bronchial epithelial cell line 16HBE were purchased from the American Type Culture Collection (ATCC, USA). All cell lines were routinely maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin sodium, and 100 mg/ml streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% CO_2.

Quantitative real-time PCR

Total RNA was isolated from tissues or cells using TRIZOL reagent according to the manufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to

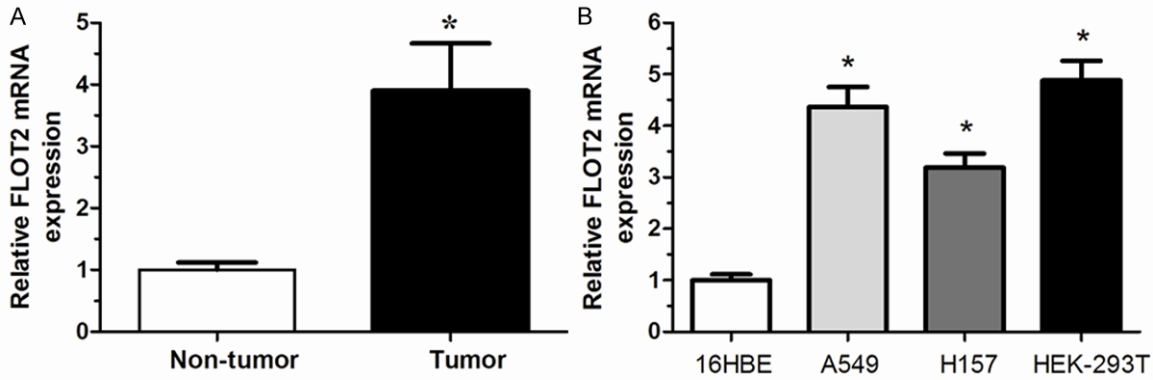

Figure 1. Expression of FLOT2 mRNA in the human NSCLC tissues and lung cancer cell lines. A. Relative mRNA expression of FLOT2 was higher in NSCLC tissues than in matched adjacent non-tumor tissues. B. Relative mRNA expression of FLOT2 was higher in lung cancer cell lines than in normal bronchial epithelial cell line. Results are expressed as mean ± SD for three replicate determination. *$P < 0.05$.

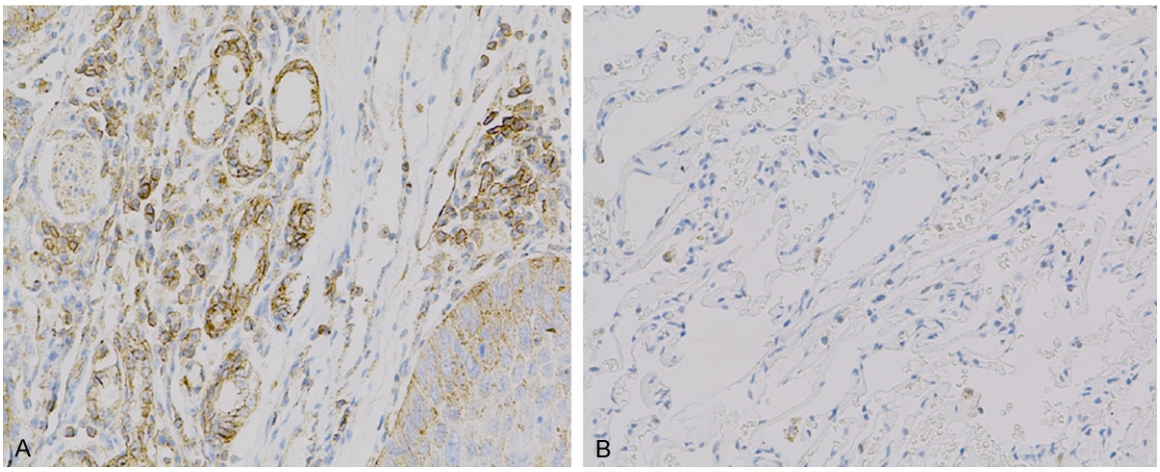

Figure 2. Immunohistochemical analysis of FLOT2 in NSCLC patients. A. High FLOT2 expression in NSCLC tissues. B. Negative FLOT2 expression in adjacent non-tumor tissues.

the manufacturer's instructions. The FLOT2 sense primer was 5'-CCCCAGATTGCTGCCAAA-3', and the antisense primer was 5'-TCCACTGAGGACCACAATCTCA-3'. For the GAPDH gene, the sense primer was 5'-TGCACCACCAACTGCTTAGC-3', and the antisense primer was 5'-GGCATGGACTGTGGTCATGAG-3'. The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicates and repeated three times.

Immunohistochemistry

Altered FLOT2 protein expression was also studied in 90 human NSCLC tissues by immunohistochemistry (IHC). Briefly, the tissue sections were deparaffinized, rehydrated, endogenous-peroxide-blocked and antigen-retrieved sequentially and were then incubated with a rabbit anti-FLOT2 antibody (1:200; Abcam) overnight at 4°C. Then, the tissue sections were washed with PBS and treated with anti-rabbit secondary antibody for

20 min, followed by further incubation with the streptavidin horseradish peroxidase complex. The sections were developed with diaminobenzidine tetrahydrochloride (DAB) and further counter stained with hematoxylin. The degree of immunostaining was evaluated by two inde-

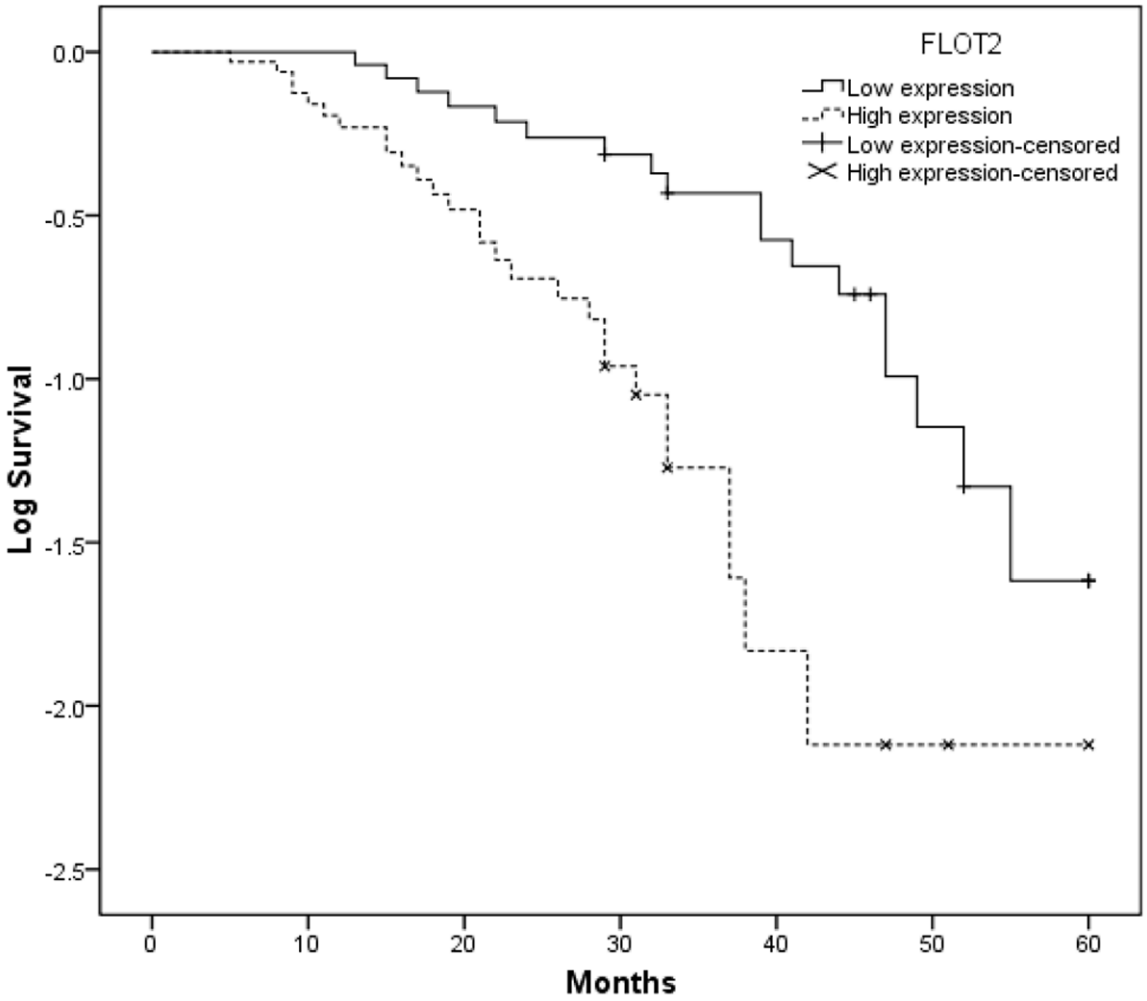

Figure 3. The Kaplan-Meier survival curve for patients with lung cancer and FLOT2 expression.

pendent observers who were blind to the clinical data of the patients. The percent of positive cells was scored as ≤ 10% = 0, >10% to ≤ 25% = 1, > 25% to ≤ 50% = 2, > 50% to ≤ 75% = 3 or > 75% = 4. The intensity of nuclear staining was scored as negative = 0, weak = 1, moderate = 2, or strong = 3. The two scores were then multiplied to calculate the final score. FLOT2 expression was considered low if the final score was equal to or less than four, otherwise, FLOT2 expression was considered high.

Statistical analysis

All data were presented as mean ± SD, analyzed using SPSS 18.0. The significance of differences between groups was estimated by Student's t-test. The relationship between FLOT2 expression and clinicopathologic characteristics was analyzed by Pearson's chi-squared test. Overall survival was estimated by using Kaplan-Meier method and was evaluated for the statistical significance using a log-rank test. The significance of different variables with respect to overall survival was analyzed using the univariate and multivariate Cox proportional hazards model. $P < 0.05$ was considered statistically significant.

Results

Up-regulation of FLOT2 mRNA in lung cancer tissues and cell lines

The transcriptional levels of FLOT2 were determined with qRT-PCR assays in 24 pairs of NSCLC and adjacent non-tumor tissues. The average expression levels of FLOT2 mRNA were significantly higher in NSCLC tissues than that in matched adjacent non-tumor tissues (*P* <

Table 2. Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Age (years)	1.227	0.739-1.528	0.425			
≥ 60 vs < 60						
Gender	1.172	0.526-1.712	0.276			
Male vs Female						
Tumor size	1.794	0.818-2.673	0.186			
≥ 3 cm vs < 3 cm						
Histologic type	1.424	0.739-2.216	0.304			
Squamous vs Adenoma						
Tumor stage	2.117	1.614-4.332	0.011	1.977	1.476-3.995	0.007
III vs I-II						
Lymph nodes metastasis	3.107	1.811-5.327	0.008	2.874	1.583-4.917	0.003
Yes vs No						
FLOT2	2.617	1.403-4.927	0.003	2.317	1.248-4.327	0.001
High vs Low						

0.05, **Figure 1A**). FLOT2 expression was further examined in three lung cancer cell lines (A549, H157, HEK-293T) and a normal bronchial epithelial cell line 16HBE. qRT-PCR results showed that the expression levels of FLOT2 mRNA was higher in three lung cancer cell lines (A549, H157, HEK-293T) than that in normal bronchial epithelial cell line 16HBE ($P < 0.05$, **Figure 1B**).

Immunohistochemical analysis of FLOT2 expression in NSCLC tissues

We investigated the status of FLOT2 expression in 90 paraffin-embedded archived NSCLC tissues by IHC staining. Among the 90 NSCLC samples, 47 showed high FLOT2 expression, whereas the remaining 43 cases displayed low FLOT1 expression (**Table 1**). As shown in **Figure 2**, no signals or only weak signals were detected in the adjacent non-tumor tissues. In contrast, FLOT2 was highly expressed in NSCLC tissues. The subcellular location of FLOT2 was mainly at the plasma membrane.

Relationship between FLOT2 expression and NSCLC patients' clinical features

We further analyzed the correlation between FLOT2 expression and the clinicopathological features of NSCLC patients by the Chi-square test. As shown in **Table 1**, there were no significant correlations between the expression level of FLOT2 protein and patient age, gender, tumor size or histologic type in NSCLC patients ($P > 0.05$). However, the FLOT2 expression level was markedly associated with tumor stage, and lymph nodes metastasis ($P < 0.05$). Taken together, these observations showed that high levels of FLOT2 expression were associated with the clinical development of NSCLC.

Association between FLOT2 expression and NSCLC patients' survival

The overall survival analysis using the Kaplan-Meier method revealed that the prognosis of NSCLC patients with high FLOT2 expression was significantly poorer than those with low FLOT2 expression (**Figure 3**; $P < 0.05$). Univariate analysis of overall survival revealed that the relative level of FLOT2 expression, tumor stage, lymph node metastasis were prognostic indicators (**Table 2**; $P < 0.05$). The other clinicopathological features, such as age, gender, tumor size, and histologic type were not statistically significant prognosis factors ($P > 0.05$; **Table 2**). Variables with a value of $P < 0.05$ were selected for multivariate analysis. Multivariate analysis showed that FLOT2 expression was an independent prognostic indicator for overall survival in NSCLC patients with in addition to high tumor stage, and presence of lymph node metastasis (**Table 2**; $P < 0.05$).

Discussion

Lung cancer ranks one of the most frequent causes of cancer-related mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer

cases [1]. The prognosis of NSCLC cases is usually poor because of late diagnosis and therapeutic limitations [17]. Therefore, exploration of new molecular markers involved in progression of NSCLC can facilitate effective targeted treatment and prognostic assessment [18]. Tumor invasion and metastasis are important issues for understanding tumor biology and further improving the prognosis of patients with carcinomas, including NSCLC. This is a very complex process with multiple promoters or suppressor genes involved [19]. Understanding the genes responsible for either enhancing or suppressing this process would enable novel diagnostic, therapeutic, and prognostic applications to evolve and thus improve the clinical outcome of NSCLC patients.

Recently, accumulating evidence has showed that FLOT2 may play important roles in the development and progression of human cancers. However, the role of FLOT2 expression in the development and progression of NSCLC is not fully understood. In the present study, we found that FLOT2 mRNA level in lung cancer cell lines (A549, H157, and HEK-293T) and NSCLC tissues was significantly higher compared to that in normal bronchial epithelial cell line 16HBE and adjacent non-tumor tissues. It suggested that FLOT2 might play important roles in the tumorigenesis of NSCLC. Furthermore, IHC was performed in 90 archived paraffin-embedded NSCLC samples. Our study showed that FLOT2 protein expression was positively correlated to the tumor stage, and lymph node metastasis, although it showed no association with age, gender, tumor size and histologic type in NSCLC patients, suggesting the possible participation of FLOT2 on NSCLC invasion and metastasis.

Since it was found that FLOT2 expression was associated with NSCLC invasion and metastasis, which may determine tumor prognosis, we further evaluate its prognostic value by the Kaplan-Meier analysis. Results showed that NSCLC patients with high FLOT2 expression tend to have worse overall survival compared with those with low FLOT2 expression. These results indicated that FLOT2 expression in NSCLC might serve as a new prognostic marker for diagnosis. Moreover, Univariate and multivariate analysis demonstrated that FLOT2 expression was an independent risk factor in the prognosis of NSCLC patients.

In summary, we investigated the expression pattern of FLOT2 in clinical NSCLC specimens and its association with prognosis of patients. The expression level of FLOT2 was significantly increased in lung cancer cell lines and tissues and positively correlated to tumor progression. Increased FLOT2 expression was also found to be independently associated with poor overall survival of NSCLC patients. Our results emphasized the importance of FLOT2 in lung cancer tumorigenesis and provided new insights into understanding the molecular mechanism of development.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhong-Min Wang, Department of Thoracic Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, China. E-mail: zhongminwang65@163.com

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