Int J Clin Exp Pathol 2014;7(11):8071-8076
www.ijcep.com /ISSN:1936-2625/IJCEP0002584

Original Article

Down-expression of F box only protein 8 correlates with tumor grade and poor prognosis in human glioma

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Received September 17, 2014; Accepted November 1, 2014; Epub October 15, 2014; Published November 1, 2014

Abstract: F box only protein 8 (FBX8) is a novel component of F-box proteins which involved in the ubiquitin-dependent proteolytic pathway. Recent studies have revealed that FBX8 was unregulated in tumor cells and was closely associated with tumor progression and metastasis of other cancer, but little research has been done yet to test its usefulness as a prognostic marker in human glioma. In the present study, we investigated the expression of FBX8 in glioma tissues using immunohistochemical analysis and evaluated its prognostic significance in glioma. We found that 44/77 (57.14%) gilomas had positive expression of FBX8, while 65/77 (84.42%) normal brain tissue had positive expression of FBX8. The expression level of FBX8 was remarkably down-regulated in glioma tissues compared with normal brain tissues ($P < 0.001$). The down-expression of FBX8 in tumor cells was strongly correlated with tumor grade of patients with glioma ($P < 0.05$). Patients with lower expression of FBX8 protein had shorter overall survival time than those with higher level expression of FBX8 ($P < 0.05$). Multivariate analysis showed that FBX8 down-expression was an independent prognostic indicator for glioma patient's survival. Our results suggest that a potential application of FBX8 in prognosis prediction and therapeutic application in glioma.

Keywords: F box only protein 8, glioma, prognosis, tumor grade

Introduction

Glioma is the most common form of primary brain tumor and make up 80% of all malignant brain tumors, involving astrocytes, oligodendrocytes, ependyma, and choroids plexus epithelium [1]. It is further classified into four malignancy grades based on consensus criteria established by the World Health Organization [2]. Glioma is typically portended a poor clinical outcome due largely to their highly infiltrative nature, which renders them unsuitable for complete surgical resection. It should be noted that glioblastoma portends the worst prognosis in all gliomas, with a median survival time ranging from 9 to 10 months [3]. Recent studies have shown that many molecular mechanisms, such as the EGFR [4], AKT [5], STAT3 [6], and beta-catenin [7] pathways, are involved in glioma. The molecular pathology of glioma, however, is not sufficient to enable significant progress in individualized and targeted therapy of glioma patients. Therefore, it is quite necessary to identification an effective biomarker for early diagnosis and accurate prognostication.

F box only protein 8 (FBX8), an F-box and Sec7 domain-containing protein, is a novel component of F-box proteins. Recent research revealed that FBX8 has E3 ligase activity mediating the ubiquitination of the GTP-binding protein ARF6; further, it was shown that ubiquitination of ARF6 does not induce the degradation of ARF6 but inhibits ARF6 function [8]. ARF6 is a small GTPase and one of the Ras superfamily proteins [9]. The ARF6-GTPase cycle can affect the invasive potential of tumor cells [10, 11]. In addition, c-Myc protein interacted with FBX8 by the c-Myc box II region to influence on cell invasion by regulating FBX8 on the activation of the ARF6 protein [12]. Expression of FBX8 has been reported to be lost in some tumor cells, such as breast cancer [8], lung cancer [13] and hepatocellular carcinoma (HCC) [14]. FBX8 overexpres-

sion was found to inhibit ARF6-mediated cell invasion activity in breast cancer cells, and breast cancer cells, which lack invasion activity, were found to have reduced levels of FBX8 protein [8]. FBX8 downregulation correlated significantly with poor prognosis of patients with HCC, and over-expression of FBX8 decreased proliferation, migration and invasion in HCC cells [14]. However, the potential prognostic relevance of FBX8 expression in glioma has not been investigated yet.

In this study, we attempted to investigate the expression of FBX8 in glioma tissues using immunohistochemical analysis and identify its relationship to clinicopathological features and evaluate its prognostic value to survival in glioma.

Materials and methods

Patients and tissue samples

In this study, a total of 77 glioma cases of formalin-fixed and paraffin-embedded were obtained from Department of Pathology, The First Hospital, Jilin University, China, from 2006 to 2011. All patients were confirmed by histopathology diagnosis. For the use of these clinical materials for research purposes, prior patient's consent and approval from the Ethics Committee of Jilin University were obtained. In accordance with the WHO classification, all cases were classified, including 15 grade I, 27 grade II, 22 grade III, and 13 grade IV. The mean age of patients at diagnosis was 38.01 ± 13.24 (range, 11-65 years old), with 36 female and 41 male. The mean size of tumor is 35.56 ± 42.58 cm^3. The median follow-up time for overall survival was 23 months for patients still alive at the time of analysis, and ranged from 4 to 52 months.

Immunohistochemistry (IHC)

Immunohistochemistry was done to study altered protein expression in 77 human glioma tissues. The procedures were done similarly to previously described methods [15]. Briefly, 4-μm sections mounted on aminopropylethoxysilane (APES) slides and pretreated for immunohistochemistry were dewaxed in xylene and brought through graded ethanols to deionized distilled water. An antigen retrieval step was performed in EDTA antigenic retrieval buffer (pH 8.0) and microwaved. The classical avidin-biotin peroxidase complex (ABC) procedure was used for immunohistochemistry. In the ABC system, endogenous peroxidase was quenched by incubation of the sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 30 min at room temperature. Non-specific binding was blocked by incubation with non-immune serum (1% bovine serum albumin for 15 min at room temperature). The sections were incubated overnight with anti-FBX8 monoclonal antibody (ab57056, Abcam, MA, USA) at a dilution of 1:250 at 4°C. After washing, the sections were further incubated with HRP at 4°C for 30 min. For color reactions, diaminobenzidine (DAB) was used. For negative controls, the antibody was replaced by normal goat serum.

Evaluation of immunohistochemical staining

Two observers independently reviewed and assessed the cellular localization and intensity of immunostaining in each section. Staining for FBX8 protein in tumor cells was scored semiquantitatively by a quality control system. The proportion of FBX8-expression cells varied from 0-100%, and the intensity of staining varied from weak to strong. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), according to the percentages of the positive staining areas in tumor area. The sum of the intensity and extent scores was used as the final staining score (0-7) for FBX8. This relatively simple, reproducible scoring method gives highly concordant results between independent evaluators and has been used in previous studies [16, 17]. For the purpose of statistical analysis, tumors with a final staining score of ≥3 were considered to be high expression.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 statistical software package. The Mann-Whitney U test was used to analyze the relationship between FBX8 expression and clinicopathologic characteristics. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. $P < 0.05$ in all cases was considered statistically significant.

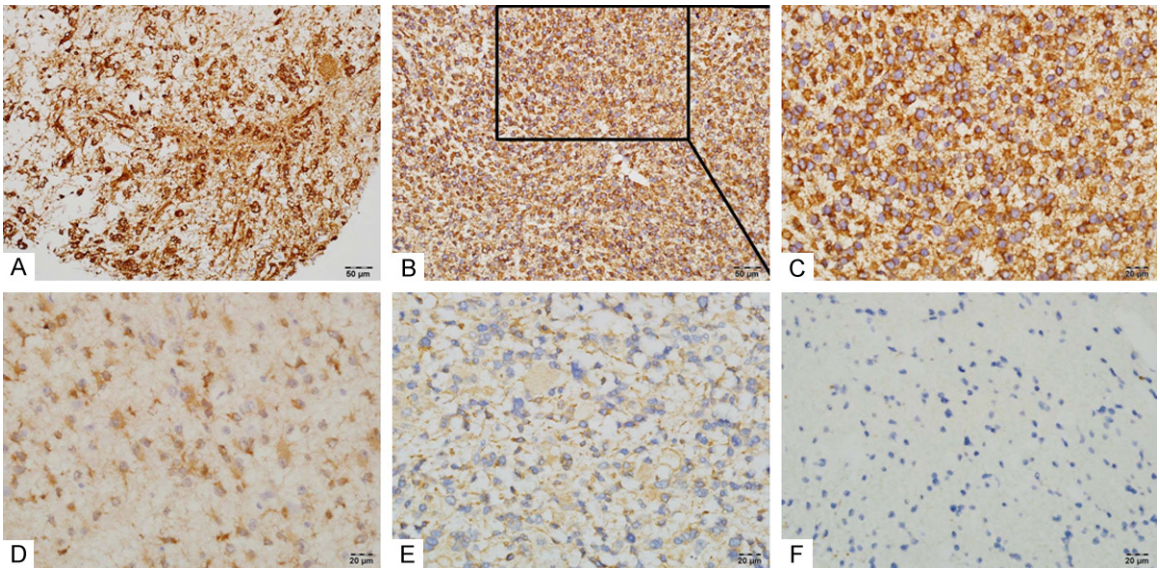

Figure 1. The expression of FBX8 protein in human glioma tissues. A-C. Strong positive expression of FBX8 protein (original magnification 200×, 200× and 400×, respectively). D. Moderated positive expression of FBX8 protein (original magnification 400×). E. Weak positive expression of FBX8 protein (original magnification 400×). F. Negative expression of FBX8 protein (original magnification 400×).

Table 1. Correlations between the clinicopathologic features and expression of FBX8

Characteristics	N	FBX8		*P*	χ^2
		Low (%)	High (%)		
Gender					
Male	41	22 (53.66)	19 (46.34)		
Female	36	25 (69.44)	11 (30.56)	0.156	2.009
Age (y)					
≤38	33	17 (51.52)	16 (48.48)		
>38	44	30 (68.18)	14 (31.82)	0.138	2.203
Tumor site					
Frontal lobe	26	16 (61.54)	10 (38.46)		
Temporal lobe	27	15 (55.56)	12 (44.44)		
Fronto-temporal lobe	16	10 (62.50)	6 (37.50)		
Parietal lobe	8	6 (75.00)	2 (25.00)	0.798	1.014
Tumor extension					
One lobe	60	37 (61.67)	23 (38.33)		
More than one lobe	17	10 (58.82)	7 (41.18)	0.832	0.947
Tumor size (3 cm)					
≤35.56	48	27 (56.25)	21 (33.75)		
>35.56	27	20 (74.07)	7 (25.93)	0.268	1.229
Tumor grade					
I	15	4 (26.67)	11 (73.34)		
II	27	17 (62.96)	10 (37.04)		
III	22	16 (72.73)	6 (27.27)		
IV	13	10 (76.92)	3 (23.08)	0.017	10.137
Low-grade (I-II)	42	21 (50.00)	21 (50.00)		
High-grade (III-IV)	35	26 (74.29)	9 (25.71)	0.030	4.735

Results

FBX8 was down-regulated in glioma tissues

We measured FBX8 expression in a cohort of 77 archived paraffin-embedded glioma tissues and adjacent normal brain tissues using IHC. Signals of FBX8 were predominantly detected in the cytoplasm of cells (**Figure 1**). We also observed that 33 (42.86%) exhibited no positivity for FBX8, 20 (25.97%) demonstrated weak positivity, 12 (15.58%) had moderated positivity, and in 12 (15.58%), strong positivity was observed (**Figure 1**). We observed a relative low level of FBX8 protein expression in 57.14% (44/77) of all glioma samples, compared with 84.42% (65/77) of normal brain samples ($P < 0.001$). According to evaluation method as described above, FBX8 expression of tumor cells was further reclassified into high or low expression. FBX8 was evaluated as high expression in 38.96% (30/77) of tumor samples.

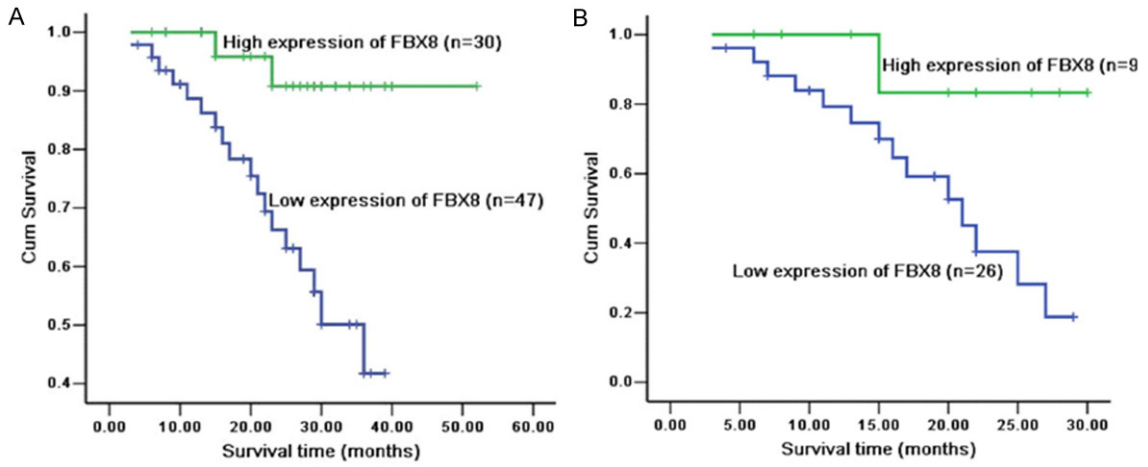

Figure 2. Kaplan-Meier survival analysis for overall survival duration in the 77 gliom patients according to FBX8 expression. A. Kaplan-Meier analysis showing the overall survival of glioma patients with high and low expression of FBX8. B. Kaplan-Meier analysis showing the overall survival of high-grade glioma patients with high and low expression of FBX8. The log-rank test was used to calculate *P* value.

Table 2. Summary of Overall survival analyses by univariate and multivariate COX regression analysis

Variables	Univariate analysis			Multivariate analysis		
	P value	HR	95% CI	*P* value	HR	95% CI
Gender	0.964	0.98	0.405-2.370			
Age	0.016	3.854	1.282-11.585	0.288	1.863	0.591-5.867
Tumor site	0.265	0.757	0.464-1.236			
Tumor size	0.071	2.3	0.933-5.672			
Tumor extension	0.082	0.167	0.022-1.251			
Tumor grade	<0.001	11.79	3.368-37.897	0.001	9.095	2.579-32.069
FBX8 expression	0.009	0.142	0.033-0.612	0.017	0.166	0.038-0.730

Down-expression of FBX8 is associated with grade of glioma

Table 1 showed the relationship between the down-expression of FBX8 protein and clinical characteristics. There was no significant correlation between the down-expression level of FBX8 protein and age, gender, tumor site tumor extension and tumor size of patients with glioma ($P > 0.05$). However, the down-expression of FBX8 was closely associated with tumor grade of patients with glioma ($P < 0.05$).

Down-expression of FBX8 predicts patient survival in gliomas

To further evaluate the prognostic value of FBX8 protein for glioma patients, we also analyzed the association between FBX8 protein expression and survival duration using Kaplan-Meier analysis with the log-rank test. The results revealed that low-level expression of FBX8 protein was correlated with short survival time of patients with glioma (Log Rank = 9.338, *P = 0.002*, **Figure 2A**). Low-level of FBX8 was associated with short survival time (28.84 ± 1.85 *vs.* 48.99 ± 2.03). Furthermore, we also evaluated the prognostic value of FBX8 for patients with high-glade glioma. Compared to patients with high-level of FBX8 expression, those with low-level of FBX8 expression had shorter overall survival time (19.49 ± 1.70 *vs.* 27.5 ± 2.08, $P = 0.038$, **Figure 2B**).

Univariate and multivariate analyses of prognostic variables in glioma patients

To determine whether expression of FBX8 is an independent prognostic factor for glioma, univariate and multivariate analyses were performed to determine the prognostic value of clinicopathological variables including sex, age, tumor site, tumor size, tumor extension, and tumor grade in patients with glioma. The results

showed that low-level expression of FBX8 protein is an independent prognostic factor for poor survival of patients with CRC ($P < 0.05$, **Table 2**).

Discussion

The ubiquitin-dependent proteolytic pathway is an important mechanism of protein abundance regulation in eukaryotes. F-box proteins are critical components of the SCF ubiquitin-protein ligase complex and are involved in the ubiquitin-dependent proteolytic pathway. The F-box proteins constitute one of the four subunits of the ubiquitin protein ligase complex called SKP1-cullin-F-box (SCFs), which is involved in phosphorylation-dependent ubiquitination [18]. More than 70 putative F-box proteins have been identified in human genome so far. However, the function and their substrates of most F-box proteins remain unclear. FBX8 is a novel member of the F-box protein family, containing an F-box domain and a putative Sec7 domain. FBX8 was originally identified as a Skp1-binding protein [19]. In addition, c-Myc protein interacted with FBX8 by the c-Myc box II region [12]. The studies revealed that FBX8 was closely associated with tumor progression and metastasis, including breast cancer and HCC [8, 12, 14]. However, less is known about the relationship between the FBX8 expression and the prognosis of patients with glioma. This study focused on the potential relationship between the expression level of FBX8 and various clinicopathological characteristics of glioma patients, as well as overall survival.

In our study based on clinical samples, we observed that a significantly low-level expression of FBX8 protein was found in glioma cells compared with control groups tested. Our results suggest that the loss of FBX8 might play a role in glioma tumorigenesis, though the precise molecular mechanism of FBX8 in glioma remains to be further elucidated. Similar results were also observed in other human cancer, such as breast cancer [8], lung cancer [13] and HCC [14]. Thus, the confirming results help us deducing that FBX8 is a tumor suppressor gene.

Our analysis further showed that FBX8 down-expression correlated with tumor grade of patients with glioma, which suggested that FBX8 might play an important role in the progression of glioma. In agreement with our researches, Wang [14] et al established HCC cell model with FBX8 over-expression, and found that over-expression of FBX8 decreased proliferation, migration and invasion in HCC cells. In addition, Kaplan-Meier survival analysis revealed that low level FBX8 expression was significantly correlated with a poor prognosis of patients with glioma after surgical resection in our study. A multivariate Cox proportional hazard regression analysis revealed that FBX8 down-expression was a worse prognostic impact on the overall survival of glioma patients independent of tumor grade. This finding, similar to that of Wang et al, may suggest that as an independent risk factor, FBX8 could serve as a prognostic marker for the survival of glioma patients as well.

In conclusion, this is the first study showing the expression of FBX8 in glioma, highlighting the clinical significance of FBX8 in glioma. Lower FBX8 expression is strongly correlated with tumor grade and overall survival times of patients with glioma, providing evidence that down-regulation of FBX8 might play an important role in the progression, and that FBX8 could be considered as a novel therapeutic target for patients with glioma.

Disclosure of conflict of interest

None.

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