FOXL1 overexpression is associated with poor outcome in patients with glioma

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Abstract. Gliomas are the most common primary tumors in adult central nervous system and result in disappointing survival outcomes. FOXL1, as a transcription factor, plays an important role in regulating the expression of genes involved in cell metabolism, proliferation and differentiation. In this study, we investigated the relationship between FOXL1 expression and prognosis of patients with glioma. We selected 611 glioma patients from The Cancer Genome Atlas (TCGA) database and 132 glioma patients from Huai'an First People's Hospital (PFHH). The prognostic values of FOXL1 in glioma were analyzed in both cohorts. In TCGA cohort, the median (10.2389) was used as the cut-off value of FOXL1 mRNA levels in tumor tissue. Kaplan-Meier analysis showed that higher WHO glioma grade (P<0.001) and expression of FOXL1 (P<0.001) were associated with worse overall survival (OS). The univariate Cox regression model revealed that age (P<0.001), WHO grade (P<0.001), histological type (P<0.001) and FOXL1 expression (P<0.001) were associated with prognosis of glioma patients. In PFHH cohort, expression of FOXL1 in tumor cells was detected by immunohistochemistry (IHC) staining based on a tissue microarray (TMA) sample. Kaplan-Meier analysis also showed that WHO glioma grade (P<0.001) and expression of FOXL1 (P=0.012) were associated with OS in glioma patients. The univariate Cox regression showed that WHO grade (P=0.001), histological type (P<0.001) and FOXL1 expression (P=0.013) were associated with prognosis of glioma patients. In both cohorts Kaplan-Meier subgroup analyses showed FOXL expression correlated with OS in high WHO grade subgroup, while low grade subgroup showed no such correlation. This study showed that higher expression of FOXL1 is associated with poor OS of glioma patients in TCGA and PFHH cohorts. Especially, FOXL1 overexpression is associated with worse outcomes in high WHO grade subgroup. Our findings suggest that FOXL1 expression is a candidate predictor of clinical outcome in glioma patients and may act as an effective molecular marker for immunotherapeutic strategies of glioma patients in clinical practice.

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Introduction

Gliomas, which arise from glial cells, make up approximately 30% of all brain and central nervous system tumors and 80% of all malignant brain tumors (1). According to the WHO pathologic grading system, which is the most common of numerous grading systems in use, gliomas are further categorized as four grades (I-IV) (2). Gliomas with lower grade indicate better prognosis while higher grade gliomas indicate worse prognosis and increased fatality (3). At present, the standard treatment for gliomas includes maximal surgical resection and concurrent chemo-radiotherapy. However, the prognosis of glioma is still poor. The survival time of glioblastoma multiform (GBM, grade IV) patients is only approximately one year (4), and for grade II and III gliomas, the survival time is 2 and 2-5 years, respectively (5). Therefore, it is of great importance to find more effective molecular prognostic markers for the treatment of glioma patients in clinical practice.

FOX (Forkhead box) proteins are a super family of transcription factors that play crucial roles in regulating the expression of genes involved in cell metabolism, proliferation, differentiation and apoptosis (6). Many FOX proteins are at the junction of multiple signaling pathways, thus are important to embryonic development (7). Due to the vital roles in growth and development, the malfunctions of FOX proteins play important roles in a variety of pathological processes including cancer. For instance, FOXOs can initiate apoptosis and cause cell cycle arrest (8) and increase.

FOXM1 gene expression is often found in various human cancers (9). In the context of FOXL1 protein, it was first discovered in the mesenchyme of the gastrointestinal tract (10) and it played an important role in gut maintenance (11). Thus far, several studies have reported the associations of FOXL1 with gastrointestinal cancer including stomach, colon and pancreas (12-14), and urinary cancer (10,15). In addition, Nakada *et al* reported that FOXL1 could regulate central-nervous system development by suppressing Sonic Hedgehog

protein expression in zebrafish (16), suggesting that FOXL1 may be also involved in brain cancer.

This study investigated the expression of FOXL1 by immunohistochemistry (IHC) on a tissue microarray (TMA) including 132 glioma specimens in Huai'an First People's Hospital (Huai'an, China) and investigated its association with the survival outcome. Besides, 611 glioma patients from The Cancer Genome Atlas (TCGA) database were also included. To our knowledge, this is the first study to investigate the prognostic value of FOXL1 in glioma patients.

Patients and methods

TCGA database. The FOXL1 expression and clinical data in glioma patients of TCGA database were downloaded from the TCGA Research Network: http://cancergenome.nih.gov/. According to parameters defined in previous studies (17,18), 611 glioma patients with detailed FOXL1 RNA seq information, fully characterized tumors, intact overall survival (OS) were included in our study. Clinicopathological characteristics, including age, sex, historical type and WHO grade were collected.

Patients from Huai'an First People's Hospital (PFHH). The TMA used for this study includes 132 unselected, non-consecutive, primary, and sporadic gliomas treated between March 2009 and August 2015 in Department of Neurosurgery, Huai'an First People's Hospital. Formalin-fixed, paraffin-embedded tissue blocks from resected glioma were made. Tissue cylinders with a 2.0 mm diameter were punched from representative tissue areas. The histological types were confirmed by experienced pathologists. The TMAs contained well-documented demographic and clinicopathological information, including patients' age, sex, WHO grade, histology types.

This study was approved by the Ethics and Research Committees of Huai'an First People's Hospital, (Project identification code: IRB-KPJ2017-003-01). Patients who participated in this research had complete clinical data. The signed informed consents were obtained from the patients or the guardians.

Immunohistochemical staining. IHC was performed according to the standard streptavidin-peroxidase (S-P) method (Zymed, San Francisco, CA, USA). The tissue wes fixed with 4% formaldehyde at 22˚C for 12 h and then embedded with paraffin. The thickness of sections was 3 μm. Briefly, in a xylene and alcohol bath solution TMAs were dewaxed and dehydrated. Heat mediated antigen retrieval was performed with Tris/EDTA buffer pH 9.0. The endogenous peroxidase activity was then blocked using 0.3% hydrogen peroxide for 10 min at 22˚C. At room temperature the slides were cooled and blocked by incubating with normal goat serum for 1 h. After that, the slides were subsequently incubated overnight at 4˚C with rabbit anti-human FOXL1 polyclonal antibody (cat. no. ab190226; dil, 1:500; Abcam, Cambridge, UK). The sections were next incubated with biotinylated secondary goat anti-rabbit polyclonal antibody (cat. no. ab6720; dil, 1:800; Abcam, Cambridge, UK) for 30 min at room temperature, followed by incubation with streptavidin horseradish peroxidase complex. Finally, sections were visualized by 3,3'-diaminobenzidine staining. Then the slides were stained with hematoxylin and eosin (H&E).

Scoring of IHC. The immunostaining signals were evaluated with microscope (Olympus, Tokyo, Japan) by two experienced pathologists who were blinded to the patients' clinical and pathological features. FOXL1 expression was scored according to staining intensity and the percentage of positive cells as described before (19). Briefly, the staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) or 3 (strong). The percentage of positive cells was scored as follows: <5% (0), 5-25% (1), 25-50% (2), 50-75% (3) and 75-100% (4) according to the percentages of the positive staining areas in relation to the whole glioma area. Scores for staining intensity and percentage positivity of cells were multiplied to generate the immune-reactivity score (IRS) for each case. Samples with a final staining score of ≤6 were classified as low expression, while those with score of >6 were considered to be high expression.

Statistical analysis. All statistical analyses were carried out by the SPSS v.16.0 (SPSS, Inc., Chicago, IL, USA). Chi-square test and Fisher's exact test were used to analyze the association between clinicopathological parameters and FOXL1 expression. OS was defined as the interval from date of diagnosis until death from any cause. Data were censored for living patients and patients lost between follow-ups. Survival analysis was performed using the Kaplan-Meier method with log rank test and Cox regression model. All confidence intervals (CIs) were stated at the 95% confidence level. All statistical tests were two sided. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics of glioma patients in TCGA cohort and PFHH cohort. In the TCGA cohort, the age of the 611 glioma patients ranged from 14 to 87 years, with a median value of 46. Of the patients, 256 (41.9%) were females and 355 (58.1%) were males. The median follow-up time was 13.4 months and 182 patients died during follow-up. In the PFHH cohort, the age of 132 glioma patients ranged from 7 to 75 years, with a median value of 51 years. Of the total 56 (42.4%) were females and 76 (57.6%) were males. The median follow-up time was 10.0 months and 50 patients died during follow-up. Table I showed the relationship between FOXL1 expression and clinicopathological features of glioma patients in both cohorts. In the cohort of TCGA, FOXL1 expression was associated with age (P<0.001), WHO grade (P<0.001) and histological type (P<0.001). In the PFHH cohort there were no significant associations found between FOXL1 expression and clinicopathological features (Table I).

Expression pattern of FOXL1 in TCGA and PFHH cohorts. In the TCGA cohort, the median (10.2389) was used as the cut-off value of FOXL1 mRNA levels in tumor tissue of eligible patients. Patients were divided into low FOXL1 and high FOXL1 groups for further analysis (for low FOXL1 group, median: 6.2389, IQR: 3.54; for high FOXL1 group,

Table I. Clinical characteristics of glioma patients in TCGA cohort and PFHH cohort.

A, TCGA

Variables	Cases, no. (%)	FOXL1		P-value
		Low	High	
Age (years)				<0.001
<60	471 (77.1)	256 (85.3)	215 (69.1)	
≥60	140 (22.9)	44 (14.7)	96 (30.9)	
Sex				0.909
Female	256 (41.9)	125 (41.7)	131 (42.1)	
Male	355 (58.1)	175 (58.3)	180 (57.9)	
WHO grade				<0.001
Low	222 (36.3)	155 (51.7)	67 (21.5)	
High	389 (63.7)	145 (48.3)	244 (78.5)	
Histological type				<0.001
Oligoastrocytoma	117 (19.1)	74 (24.7)	43 (13.8)	
Oligodendroglioma	178 (29.1)	97 (32.3)	81 (26.0)	
Astrocytoma	171 (28.0)	110 (36.7)	61 (19.6)	
Glioblastoma	145 (23.7)	19 (6.3)	126 (40.2)	

B, PFHH

Variables	Cases, no. (%)	FOXL1		P-value
		Low	High	
Age (years)				0.075
<60	95 (72.0)	59 (67.0)	36 (81.8)	
≥60	37 (28.0)	29 (33.0)	8 (18.2)	
Sex				0.213
Female	56 (42.4)	34 (38.6)	22 (50.0)	
Male	76 (57.6)	54 (61.4)	22 (50.0)	
WHO grade				0.366
Low	46 (34.8)	33 (37.5)	13 (29.5)	
High	86 (65.2)	55 (62.5)	31 (70.5)	
Histological type				0.504
Oligoastrocytoma	8 (6.1)	6 (6.8)	2 (4.5)	
Oligodendroglioma	2 (1.5)	1 (1.1)	1 (2.3)	
Astrocytoma	67 (50.8)	48 (54.5)	19 (43.2)	
Glioblastoma	55 (41.6)	33 (37.4)	22 (50.0)	

TCGA, The Cancer Genome Atlas; PFHH, patients from Huai'an First People's Hospital.

median: 19.6245, IQR: 21.27). In the PFHH cohort, expression of FOXL1 in tumor cells was detected by IHC. For further analysis, patients were divided into two groups with low expression of FOXL1 (IRS ≤6) and high expression of FOXL1 (IRS >6) (Fig. 1).

Prognostic significance of FOXL1 expression in glioma patients. As shown in Fig. 2, in the TCGA cohort Kaplan-Meier analysis showed that WHO glioma grade (P<0.001; Fig. 2A) and expression of FOXL1 (P<0.001; Fig. 2B) were associated with OS. We further made subgroup analyses of FOXL1 expression according to patients' WHO grade. The results indicated that FOXL1 expression correlated with OS in high grade subgroup (P<0.001; Fig. 2D), while low grade subgroup had no such correlation (P=0.250; Fig. 2C). The univariate Cox regression model revealed that age (P<0.001), WHO grade (P<0.001), histological type (P<0.001) and FOXL1 expression (P<0.001) were associated with prognosis

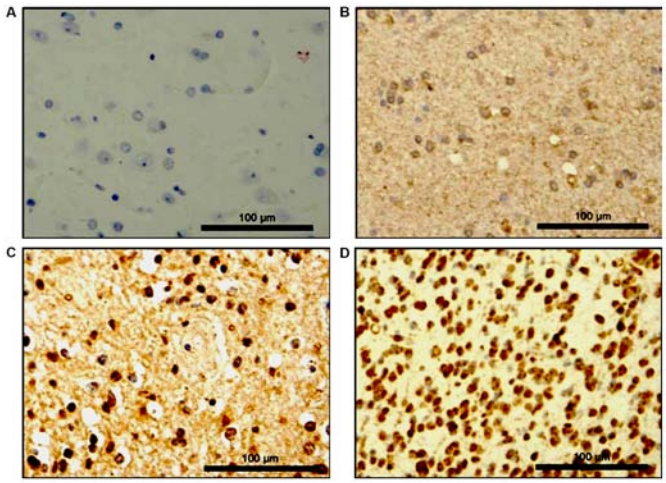

Figure 1. IHC staining characteristics of FOXL1. (A) Absent FOXL1 expression; (B) weak staining of FOXL1; (C) moderate staining of FOXL1; (D) strong staining of FOXL1. (A-D) Magnification, x200. IHC, immunohistochemistry.

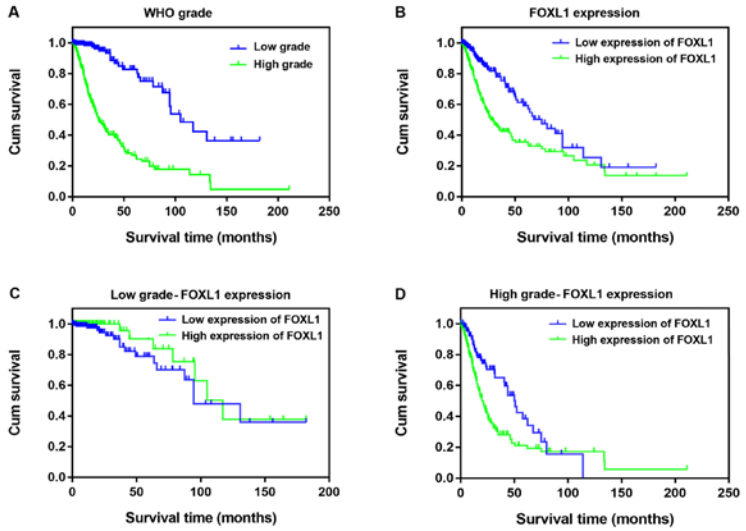

Figure 2. Survival analysis using Kaplan Meier method in TCGA cohort. (A) WHO grade (P<0.001); (B) FOXL1 expression (P<0.001); (C and D) subgroup analyses for FOXL1 expression in low grade (P=0.250) and high grade (P<0.001) glioma. TCGA, The Cancer Genome Atlas.

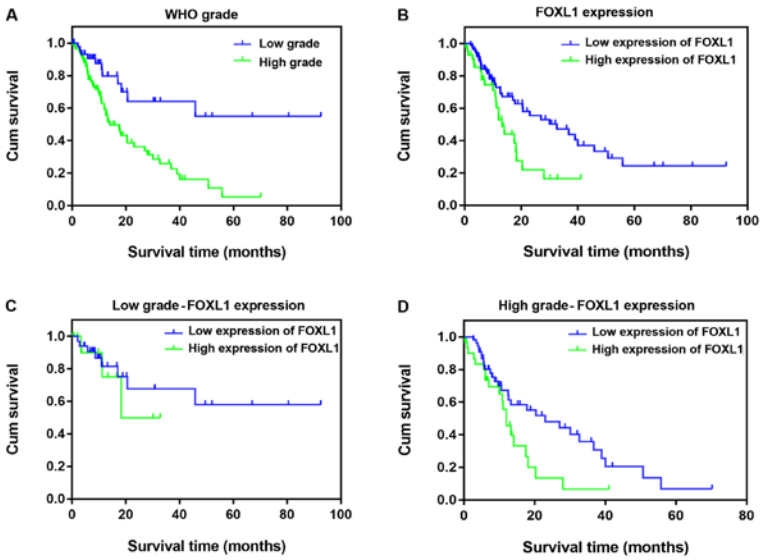

Figure 3. Survival analysis using Kaplan Meier method in PFHH cohort. (A) WHO grade (P<0.001); (B) FOXL1 expression (P=0.012); (C and D) subgroup analyses for FOXL1 expression in low grade (P=0.585) and high grade (P=0.034) glioma. PFHH, patients from Huai'an First People's Hospital.

Table II. Univariate and multivariate Cox regression analysis of overall survival.

A, TCGA

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Age			<0.001			<0.001
<60	1					
≥60	4.983	3.644-6.814		2.504	1.787-3.507	
Sex			0.629			
Female	1					
Male	1.076	0.800-1.446				
WHO grade			<0.001			<0.001
Low	1					
High	6.007	3.891-9.274		2.958	1.831-4.778	
Histological type			<0.001			<0.001
Oligoastrocytoma	1					
Other types	2.752	2.293-3.303		1.823	1.505-2.208	
FOXL1			<0.001			0.281
Low	1					
High	2.274	1.661-3.113		1.203	0.860-1.683	

B, PFHH

Variables	HR	95% CI	P-value	HR	95% CI	P-value
Age			0.571			
<60	1					
≥60	1.172	0.677-2.028				
Sex			0.793			
Female	1					
Male	1.072	0.637-1.805				
WHO grade			0.001			0.040
Low	1					
High	3.068	1.585-5.940		2.177	1.036-4.574	
Histological type			<0.001			0.027
Oligoastrocytoma	1					
Other types	2.396	1.487-3.860		1.688	1.063-2.680	
FOXL1			0.013			0.054
Low	1					
High	1.981	1.152-3.406		1.710	0.990-2.952	

TCGA, The Cancer Genome Atlas; PFHH, patients from Huai'an First People's Hospital.

of glioma patients in terms of OS in the TCGA cohort. Multivariate Cox regression after adjustment indicated that age (P<0.001), and WHO grade (P<0.001), histological type (P<0.001) were independent prognostic factors for OS in glioma patients and FOXL1 expression lost its significance (P=0.281; Table II).

In the PFHH cohort, using Kaplan-Meier analysis it was found that WHO glioma grade (P<0.001; Fig. 3A) and expression of FOXL1 (P=0.012; Fig. 3B) were associated with OS in glioma patients. Subgroup analyses showed that FOXL1 expression correlated with OS in high grade subgroup (P=0.034; Fig. 2D). The univariate Cox regression showed that WHO grade (P=0.001), histological type (P<0.001) and FOXL1 expression (P=0.013) were associated with prognosis of glioma patients in the PFHH cohort. After adjustment multivariate Cox regression revealed that WHO grade (P=0.040) and histological type (P=0.027) were independent prognostic factors for OS in glioma patients.

Discussion

Previous studies have suggested that FOXL1, a critical transcription factor, plays an important role in regulation of cell proliferation and development of the epithelium in gastrointestinal tracts in mice (20,21). The roles of FOXL1 in gastrointestinal cancers have been widely investigated (12-14). Furthermore, FOXL1 has been reported to be associated with regulation of central nervous system development in zebrafish (16), suggesting that FOXL1 may also have an effect on brain cancers. However, limited evidence is available on the role of FOXL1 in brain cancers. To the best of our knowledge, this is one of the first studies to demonstrate the associations of FOXL1 with clinicopathological features in glioma patients. Our data showed for the first time that a higher FOXL1 expression is associated with worse clinical outcome in glioma patients from TCGA and PFHH cohorts. These findings suggested that FOXL1 may be involved in tumorigenesis and progression of glioma and may serve as a candidate predictor of clinical outcome in glioma patients undergoing surgery.

Downregulation of FOXL1 has been studied in several malignant tumors. In a mechanistic research using ApcMin mice, Perreault *et al* demonstrated that FOXL1 was the first mesenchymal modifier of Min and plays a key role in gastrointestinal tumorigenesis (12). In another study, Zhang *et al* reported that FOXL1, as a novel tumor suppressor candidate, could inhibit tumor aggressiveness and predict better clinical outcome in human pancreatic cancer (13). They also revealed that FOXL1 promoted apoptosis was partly through the induction of TNF-related apoptosis-inducing ligand (TRAIL) in pancreatic cancer cells (13). Qin *et al* reported that FOXL1 could also suppress tumorigenicity in gallbladder cancer. The underlying mechanism may refer to the disruption of mitochondrial transmembrane potential and triggering mitochondria-mediated apoptosis (10). However, the mechanisms for downexpression of FOXL1 in these cancers have not been fully elucidated yet. In addition, some other mechanisms were also proposed to be associated with deregulation of Fox factors, including chromosome translocations (22,23), chromosomal deletion (24), promoter methylation (25), alteration in upstream regulators (26,27) and post-translational modifications. Further functional studies referring to FOXL1 downregulation in cancers are still needed.

It is interesting to note that the results presented here show that higher FOXL1 expression in gliomas is associated with a worse clinical outcome, which is different from other malignant tumors. However, limited information is available on the underlying mechanism concerning the observations. One study using zebra fish reported that FOXL1 was strongly expressed in neural tissues (16). This result may indicate that FOXL1 expression pattern in neural tissues is different from other tissues, which may give a hint to the future investigations. Further studies on genetic and epigenetic mechanisms ought to be carried out to clarify the issue.

There are some limitations that should be considered in our study. Although we have analyzed a large cohort, the present study is a retrospective analysis and there is a potential or selection bias. Additionally, the relatively small sample of histological subgroups in PFHH cohort may result in lack of statistical power for the Cox regression analysis.

In conclusion, this study showed that higher expression of FOXL1 is associated with worse outcome of glioma patients in TCGA and PFHH cohorts. Especially, FOXL1 expression is associated with OS in high grade subgroup. Our findings suggest that FOXL1 expression is a candidate predictor of clinical outcome in glioma patients and may act as an effective molecular marker for immunotherapeutic strategies of glioma patients in clinical practice.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AC wrote the manuscript and helped with immunohistochemical staining. LZ and JL collected and analyzed general information of patients and contributed to statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics and Research Committees of Huai'an First People's Hospital (Huai'an, China), (Project identification code: IRB-KPJ2017-003-01). Patients who participated in this research had complete clinical data. The signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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