RESEARCH ARTICLE

High Expression of Forkhead Box Protein C2 is Related to Poor Prognosis in Human Gliomas

Yao-Wu Wang[1], Chun-Li Yin[2], Hong-Yi Zhang[2], Jin-Min Hao[1], Yue-Ye Yang[2], Heng Liao[2], Bao-Hua Jiao[1]*

Abstract

Background: Increasing evidence has indicated that high Forkhead box protein C2 (FOXC2) level is closely associated with the development, progression, and poor prognosis of a variety of tumors. However, the relationship between FOXC2 and the progression of human gliomas remains to be clarified. The aim of present study was to assess FOXC2 expression and to explore its contribution in human gliomas. Materials and Methods: Realtime quantitative PCR was performed to examine FOXC2 expression in 85 pairs of fresh frozen glioma tissues and corresponding non-neoplastic brain tissues. Associations of FOXC2 expression with clinicopathological factors and prognosis of glioma patients were statistically analyzed. Results: The relative mRNA expression of FOXC2 was significantly higher in glioma tissues than the corresponding non-neoplastic brain tissues (p<0.001). In addition, high FOXC2 expression was significantly associated with advanced pathological grade (P=0.005) and the low Karnofsky performance score (KPS) (p=0.003), correlating with poor survival (p<0.001). Furthermore, multivariate Cox regression analysis showed that high FOXC2 expression was an independent predictor of overall survival (p=0.006). Conclusions: FOXC2 may act as an oncogenic gene and represent a potential regulator of aggressive development and a candidate prognostic marker in human gliomas.

Keywords: Human glioma - forkhead box protein C2 - prognosis

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Introduction

Human gliomas are the most common and aggressive form of primary brain tumors for both children and adults (Bansal et al., 2006; Meyer, 2008). According to the World Health Organization (WHO) classification which is based on histomorphological criteria, human gliomas includes well-differentiated low grade astrocytomas [grade I~II], anaplastic astrocytomas (grade III), anaplastic astrocytomas (grade III) and glioblastoma multiforme (GBM, grade IV) (Louis et al., 2007). Although the survival of patients with gliomas has been improved due to great progress in therapeutic technologies, such as surgery, radiotherapy, photodynamic therapy, and chemotherapy, the clinical outcome of patients with gliomas remains poor (Reni et al., 2005; Feng et al., 2014). For example, GBM, the most malignant and most common glioma, is associated with an average life expectancy as short as only 15 months (Van Meir et al., 2010). The poor prognosis and high lethality of the disease is largely due to the high rate of tumor recurrence and/or metastasis (Meyer, 2008). Therefore, to investigate the molecular genetics of gliomas may help to improve the prognosis of the patients with gliomas.

Forkhead box protein C2 (FOXC2), also known as mesenchyme fork head protein 1 (MFH1), is a gene encoding a transcription factor that controls the generation of mesodermal tissue such as vascular tissue and lymphatic tissue (Wu and Liu, 2011; Kume, 2012). FOXC2 is an important regulator of epithelial to mesenchymal transition (EMT) in cancer cells. Expression of FOXC2 protein was detected in a variety of cancers, including breast adenocarcinomas (Mani et al., 2007), ovarian cancer (Liu et al., 2014), colorectal cancer (Watanabe et al., 2011), cervical cancer (Zheng et al., 2014), gastric cancer (Zhu et al., 2013) and non-small-cell lung cancer (Jiang et al., 2012). Overexpression of FOXC2 has been reported in subtypes of breast cancer which are highly metastatic, suppression of FOXC2 expression using shRNA in a highly metastatic breast cancer model blocks their metastatic ability (Hollier et al., 2013). FOXC2 expression has also been reported in esophageal squamous cell cancer and could be used as a novel independent prognosis factor (Nishida et al., 2011). Therefore, FOXC2 may act as an oncogene and as a potential target for cancer therapy.

To the best of our knowledge, no previous reports exist concerning the expression status of FOXC2, the prognostic value and the role of this protein in gliomas.

*Department of Neurosurgery, [1]The Second Hospital of Hebei Medical University, Shijiazhuang, [2]Tangshan Gongren Hospital, Tangshan, Hebei, China *For correspondence: bhjiaohmu@126.com*

In the present study, we examined the expressions of FOXC2 mRNA in human gliomas and nonneoplastic brain tissues, and investigated the relationships of FOXC2 with clinicopathological factors as well as the prognosis of the patients with gliomas. Our findings may provide the better understanding on the roles and its clinic implications of FOXC2 in the development and progression of gliomas.

Materials and Methods

Patients, specimens and follow-up

This study was approved by the Research Ethics Committee of the Second Hospital of Hebei Medical University, P. R. China. Written informed consent was obtained from all of the patients according to the committee's regulations. All specimens were handled and made anonymous according to the ethical and legal standards. Eighty-five pairs of glioma and adjacent non-neoplastic brain tissues from 2007-2013 were provided by the Department of Neurosurgery of the Second Hospital of Hebei Medical University, China, Which were re-evaluated according to WHO classifications by two pathologists (Louis et al., 2007). A total of 49 males and 36 females were enrolled in this study, and the median age was 47 years (range, 19-67). Of the 85 enrolled patients, 28 were classified as low-grade [10 pilocytic astrocytomas (WHO I), and 18 diffuse astrocytomas (WHO II)], 57 were classified as high-grade gliomas [31 anaplasia astrocytomas (WHO III), and 26 primary glioblastomas (WHO IV)]. None of the patients had received chemotherapy or radiotherapy prior to surgery. All the tissues were quickly frozen in liquid nitrogen and stored at -80°C until RNA isolation. Clinical characteristics of all patients were summarized in Table 1. Clinical follow-up was performed for all patients every 3 months (median, 32 months; range, 3-64 months). During the follow *p* period, overall survival (OS) was measured from diagnosis to death. Patients who died of diseases not directly related to their gliomas or due to unexpected events were excluded from this study.

Quantitative real-time polymerase chain reaction (PCR)

Total mRNA from frozen samples was extracted using TRIzol reagent (Invitrogen, USA) according to the users' instruction. First-strand cDNA was synthesized from 1 μg mRNA using reverse transcriptase (Fermentas, Glen Burnie, MD) and oligo (dT) primers. Quantitative real-time PCR was performed using the ABI 7300 Sequence Detection System with primer pairs and SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used were as follows: FOXC2forward: 5'-TACCTGAGCGAGCAGAAT-3' and reverse: 5'-CTTGACGAAGCACTCGTT-3'; β-actin forward: 5'-CACCATTGGCAATGAGCGGTTCC-3' and reverse: 5'-GTAGTTTCGTGGATGCCACAGG-3'. The mRNA expression was normalized to the expression of the β-actin housekeeping gene using the $2^{-\Delta Ct}$ (comparative threshold cycle, or CT) method.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS, Chicago, IL).

Data were expressed as means±standard deviation (SD). Paired samples T test was performed to compare the expression levels of FOXC2 between glioma and paired non-neoplastic brain tissues. One-way ANOVA was used to analyze the relationship between FOXC2 expression and clinicopathological characteristics. Survival curves were plotted using the Kaplan-Meier product-limit method, and differences between survival curves were tested using the log-rank test. Cox regression analysis in a forward stepwise method was used to evaluate the effect of multiple independent prognostic factors on survival outcome. Differences were considered to be statistical significant when P value was less than 0.05.

Results

Overexpression of FOXC2 in human glioma tissues

To understand the expression of FOXC2 in the intratumor and peritumor tissues, we first examined the mRNA level of FOXC2 in 85 pairs of glioma and adjacent non-neoplastic brain tissues normalized to beta-actin by using real-time quantitative RT-PCR assay. As shown in Figure. 1A, the expression of FOXC2 was significantly increased in glioma tissues when compared to corresponding non-neoplastic brain tissues (mean±SD: 9.2±3.8 *vs* 4.7±2.8, $p<0.001$). In addition, FOXC2 expression in high-grade (WHO III-IV; 10.8±3.5) and low-grade (WHO I-II; 5.9±1.8) gliomas were both significantly higher than that in corresponding non-neoplastic brain tissues (4.7±2.8; $p<0.001$ and 0.029, respectively, Figure 1B). Moreover, there was also a significant difference in miR-372 expression between high-grade (WHO III-IV) and low -grade (WHO I-II) glioma tissue specimens ($p<0.001$, Figure 1B).

FOXC2 overexpression associates with advanced clinicopathological features of gliomas

To evaluate the association of FOXC2 with tumor biology, comparisons of the clinical pathological variables with intratumoral FOXC2 expression were made.

Table 1. The Clinic Pathological Characteristics of 85 Patients with Glioma

Features	WHO I	WHO II	WHO III	WHO IV
Case No.	10	18	31	26
Mean age (years)	47.3	48.4	49.2	51.4
Gender				
Male	5	13	15	16
Female	5	10	11	10
KPS				
≥70	7	12	7	9
<70	3	6	24	17
Surgery				
Gross total resection	10	18	27	20
Partial resection	0	0	3	5
Biopsy	0	0	1	1
Adjuvant treatment				
Chemotherapy	0	5	28	3
Radiotherapy	0	0	0	5
Chemotherapy and Radiotherapy Combination	0	0	3	18

Glioma tissues expressing FOXC2 at levels less than the median expression level (8.6) were assigned to the low expression group (mean expression value 6.3, n=43), and those samples with expression above the median value were assigned to the high expression group (mean expression value 12.1, n=42). As expected, The high level of FOXC2 expression were more likely to exhibit advanced pathologic grade than those with low pathologic grade (p=0.005, Table 2). In addition, high expression of FOXC2 occurred more frequently in tumors with low Karnofsky Performance Score (KPS) than those with high KPS (p=0.003), whereas other clinical characteristics, including gender, age were not directly related to the high expression of FOXC2 (Table 2).

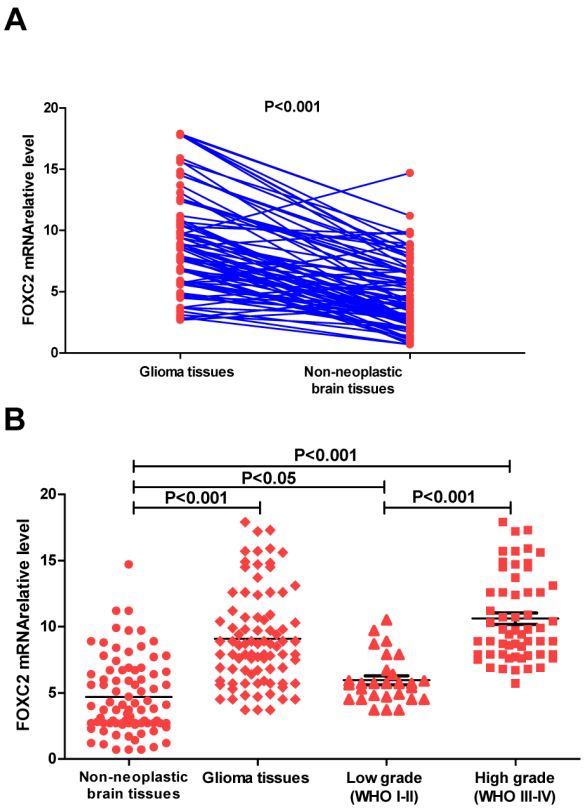

Figure 1. FOXC2 Expression in 85 Pairs of Glioma and Adjacent Non-neoplatic Brain Tissues Detected by Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis. (A) Expression levels of FOXC2 in glioma and paired non-neoplastic brain tissues. **(B).** Expression levels of FOXC2 in non-neoplastic brain tissues and glioma tissues with low (WHO I-II) or high pathological grades (WHO III-IV)

Table 2. Correlation between FOXC2 Expression in Human Glioma Tissues and Clinicopathological Variables

Clinicopatho-logical Variable	Cases 85	FOXC2 expression High (42)	Low (43)	p value
Age (years)				
≤52	38	20 (52.6%)	18 (47.4%)	0.593
>51	47	22 (46.8%)	25 (53.2%)	
Gender				
Male	49	27 (55.1%)	22 (44.9%)	0.221
Female	36	15 (41.7%)	21 (58.3%)	
KPS				
<70	50	32 (64%)	18 (36%)	0.003
≥70	35	10 (28.6%)	23 (71.4%)	
WHO grade				
I	10	2 (20%)	8 (80%)	0.005
II	18	3 (16.7%)	15 (83.3%)	
III	31	19 (61.2%)	12 (38.7%)	
IV	26	18 (69.2%)	8 (30.7%)	

Overexpression of FOXC2 correlates with poor prognosis in glioma patients

In order to investigate the relationship between FOXC2 expression and clinical outcome in gliomas, the clinical information of the glioma patients was reviewed. As shown in Figure 2A, Overall survival of patients with a high intratumoral FOXC2 level was significantly shorter than survival of those with a low FOXC2 level (p<0.05). Median survival time of patients with high and low FOXC2 levels was 28.8 and 37.2 months, respectively (p<0.05). More importantly, subgroup analyses according to tumor pathological grade revealed that the overall survival of glioma patients with advanced pathological grade (WHO III~IV) was significantly worse for high FOXC2 expression group than for low FOXC2 expression group (p=0.009, Figure 2B), but no significant difference was found for patients with low pathological grades (WHO I~II, p=0.150, Figure 2C). In multivariate analysis, the Cox regression analysis revealed that intratumoral FOXC2 overexpression was an independent prognostic factors for OS (Risk ratio [HR]=5.27; 95% confidence interval [CI],

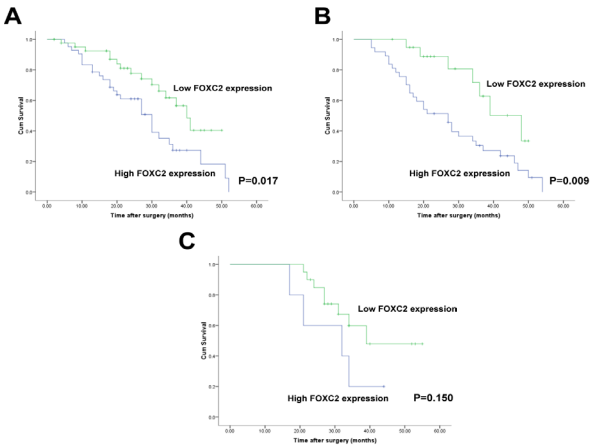

Figure 2. Kaplan-Meier Survival Curves for Glioma Patients with High or Low Expression of FOXC2. (A) The overall survival rate of all 85 glioma patients with high or low FOXC2 expression; **(B)** The overall survival rate of 57 glioma patients with advanced pathological grades (WHO III~IV) in high or low FOXC2 expression group; **(C)** The overall survival rate of 28 glioma patients with low pathological grades (WHO I~II) in high or low FOXC2 expression group

Table 3. Cox Multivariate Analysis Of Factors Associated With Overall Survival Of Glioma Patients

Factors	Risk ratio	95%CI	p value
Age	0.47	0.37-0.76	0.88
Gender	0.91	0.71-1.83	0.41
KPS	2.34	1.31-3.06	0.07
Extent of resection	1.14	0.91-1.67	0.24
Type of adjuvant treatment	1.28	0.79-2.11	0.19
FOXC2 expression	5.27	2.57-9.73	0.006

2.57-9.73; p=0.006). Statistical values of the expression of FOXC2 and other clinical parameters derived from Cox stepwise proportional hazards model were indicated in Table 3.

Discussion

In the present study, by using realtime PCR, we confirmed for the first time that the expression of FOXC2 was markedly unregulated in human glioma tissues compared with corresponding non-neoplastic brain tissues. Moreover, our results indicated that the increased expression of FOXC2 in glioma tissues was significantly correlated with advanced tumor progression and aggressive clinicopathological features. Glioma patients with high FOXC2 expression tend to have poorer overall survival, and subgroup analyses showed the significant prognostic value of FOXC2 upregulation for glioma patients especially for those with advanced pathological grade. In addition, multivariate analysis clearly revealed that high intratumoral FOXC2 expression was an independent prognostic factor for OS. Taken together, our study here provides suggests that FOXC2 maybe function as an oncogene in the development of glioma, and maybe represent a candidate prognostic marker for glioma patients, especially for advanced tumors with high pathological grades.

Forkhead box C2 (FOXC2) is a member of the winged helix/forkhead box (Fox) family of transcription factors, which has been demonstrated to play important roles in tumor vasculature, growth, invasion and metastasis (Kume, 2008; Wu and Liu, 2011; Kume, 2012). A high incidence of FOXC2 expression in many cancers and a significant association with tumor proliferation and metastasis has been reported. For example, Nishida et al. revealed that FOXC2 is associated with invasion and metastasis of esophageal squamous cancer cells (SCCs), FOXC2 targeted RNA interference can reduce esophageal SCCs ability to proliferate and migrate (Nishida et al., 2011). Li et al. found that FOXC2 was overexpressed in GBM cell lines and GBM tissues and FOXC2 promoted GBM cell proliferation and invasion properties through EGFR (Li et al., 2013). Zheng et al. revealed that positive rate of FOXC2 in cervical cancer tissues (91.3%) was significantly higher than those in normal cervical tissue (25%), and the FOXC2 positive expression rate was 88.5% in patients with cervical SCC stage I and 100% in stage II, FOXC2 knocked down in human Hela and SiHa cervical cells by siRNA showed reduced cell proliferation and relative migration, suggesting that FOXC2 gene expression increases with malignancy, especially with blood vessel hyperplasia and invasion degree, supporting FOXC2 as a potential target for cancer therapy. (Zheng et al., 2014). It was also reported that FOXC2 was associated with poor prognosis in various human malignancies. Zhu et al. (2013) found high FAT10 expression was related to poor prognosis in patients with gastric carcinoma. Jiang et al. revealed that overexpression of Foxc2 in stage I NSCLC was associated with a worse overall survival and was an independent predictor of recurrence-free and overall survival (Jiang et al., 2012). Consistent with these previous studies, our data also found the upregulation of FOXC2 in glioma tissues compared with paired adjacent nonneoplastic brain tissues. In addition, the aberrant expression of FOXC2 was associated with advanced pathological grades and low KPS of glioma patients, indicating that this FOXC2 may be involved in the development of human gliomas.

Our findings that FOXC2 overexpression was associated with aggressive tumor progression mentioned above prompt us to investigate its possible prognostic value in glioma patients. According to the univariate and multivariate analyses, we identified FOXC2 overexpression as an independent predictor for overall survival of glioma patients, which was in agreement with recent findings in NSCLC and gastric cancer, suggesting that the detection of increased FOXC2 expression might help identify glioma patients with a poor prognosis, and could therefore be a novel prognostic marker for glioma patients. Interestingly, our subgroup analyses further suggested that FOXC2 may act as a significant prognostic factor for glioma patients with high pathological grades (WHO III~IV), but not for those with low pathological grades (I~II). However, the underlying mechanisms warrants further investigation.

In summary, the present study offer the convincing evidence for the first time that FOXC2 may act as an oncogenic gene in gliomas and represent a potential regulator of aggressive development and a candidate prognostic marker for this malignancy, especially for advanced tumors with high pathological grades. Further investigation of the mechanism by which the oncogenic roles of FOXC2 in gliomas is needed.

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