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

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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

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 (Ren 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.

Asian Pac J Cancer Prev, 15 (24), 10621-10625
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-up 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: FOXC2 forward: 5'-TACCTGAGCGACGAAT-3' and reverse: 5'-CTTGACGAAGCACTCGTT-3'; β-actin forward: 5'-CACCTTTGGAATTAGGCGGTTC-3' and reverse: 5'-GTAGTTCGTCGGATGCGCAGG-3'. The mRNA expression was normalized to the expression of the β-actin housekeeping gene using the 2^ΔΔCT (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 mean±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 intratumor FOXC2 expression were made.

| 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     |
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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 value were assigned to the high expression group (mean expression value 6.3, n=43), 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 expression level (8.6) were assigned to the high expression group (mean expression value 12.1, n=42). As expected, those with high 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).

**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 high 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 1.** FOXC2 Expression in 85 Pairs of Glioma and Adjacent Non-neoplastic 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**

| Clinicopathological Variable | Cases | FOXC2 expression | 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%) |

**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   |
Overall survival (Jiang et al., 2012). Consistent with these and was an independent predictor of recurrence-free and NSCLC was associated with a worse overall survival et al. revealed that overexpression of Foxc2 in stage I poor prognosis in patients with gastric carcinoma. Jiang et al. (2013) found high FAT10 expression was related to poor prognosis in various human malignancies. Zhu al., 2014). It was also reported that FOXC2 was associated with advanced pathological grades and low KPS 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.

References
Bansal K, Liang ML, Rutka JT (2006). Molecular biology of human gliomas. Technol Cancer Res Treat, 5, 185–94.
Feng B, Hu P, Lu SJ, et al (2014). Increased argonaute 2 expression in gliomas and its association with tumor progression and poor prognosis. Asian Pac J Cancer Prev, 15, 4079–83.
Hollier BG, Tinnirello AA, Werden SJ, et al (2013). FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. Cancer Res, 73, 1981–92.
Jiang W, Pang XG, Wang Q, et al (2012). Prognostic role of Twist, Slug, and Foxc2 expression in stage I non-small-cell lung cancer after curative resection. Clin Lung Cancer, 13, 280–7.
Kume T (2008). Foxc2 transcription factor: a newly described regulator of angiogenesis. Trends Cardiovasc Med, 18, 224–8.
Kume T (2012). The role of Foxc2 transcription factor in tumor angiogenesis. J Oncol, 2012, 204593.
Li W, Fu X, Liu R, et al (2013). FOXC2 often overexpressed in glioblastoma enhances proliferation and invasion in glioblastoma cells. Oncol Res, 21, 111–20.
Liu B, Han SM, Tang XY, et al (2014). Overexpressed FOXC2 in ovarian cancer enhances the epithelial-to-mesenchymal transition and invasion of ovarian cancer cells. Oncol Rep, 31, 2545–54.
Louis DN, Ohgaki H, Wiestler OD, et al (2007). The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol, 114, 97–109.
Mani SA, Yang J, Brooks M, et al (2007). Mesenchyme forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci USA*, **104**, 10069-74.

Meyer MA (2008). Malignant gliomas in adults. *N Engl J Med*, 359, 1850.

Nishida N, Mimori K, Yokobori T, et al (2011). FOXC2 is a novel prognostic factor in human esophageal squamous cell carcinoma. *Ann Surg Oncol*, **18**, 535-42.

Reni M, Mazza E, Tosoni A, et al (2005). Novel therapeutics in adult malignant brain gliomas. *Expert Opin Investig Drugs*, **14**, 643-58.

Van Meir EG, Hadjipanayis CG, Norden AD, et al (2010). Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin*, **60**, 166-93.

Watanabe T, Kobunai T, Yamamoto Y, et al (2011). Gene expression of mesenchyme forkhead 1 (FOXC2) significantly correlates with the degree of lymph node metastasis in colorectal cancer. *Int Surg*, **96**, 207-16.

Wu X, Liu NF (2011). FOXC2 transcription factor: a novel regulator of lymphangiogenesis. *Lymphology*, **44**, 35-41.

Zheng CH, Quan Y, Li YY, et al (2014). Expression of transcription factor FOXC2 in cervical cancer and effects of silencing on cervical cancer cell proliferation. *Asian Pac J Cancer Prev*, **15**, 1589-95.

Zhu JL, Song YX, Wang ZN, et al (2013). The clinical significance of mesenchyme forkhead 1 (FoxC2) in gastric carcinoma. *Histopathology*, **62**, 1038-48.