

# Downregulation of fibroblast growth factor receptor 2 and its isoforms correlates with a high proliferation rate and poor prognosis in high-grade glioma

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**Abstract.** Fibroblast growth factor receptor 2 (FGFR-2) contributes to the progression of numerous types of cancers; however, its role in glioma has yet to be determined. We investigated the expression of FGFR-2 and its predominant isoforms, FGFR-2 IIIb and FGFR-2 IIIc, in gliomas of all histological grades. Using immunohistochemistry, we demonstrated that FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc were expressed in the astrocytes of normal human brains. The percentages of cells expressing FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc and the intensities of their staining in glioblastomas (grade IV) were significantly reduced when compared to these parameters in the low-grade tumors (grade I, II and III;  $P < 0.05$ ). A high MIB-1 index, indicated by Ki-67 expression in  $>20\%$  of the cells, was also associated with low expression of each FGFR-2 protein. Lower expression of FGFR-2 and FGFR-2 IIIc was correlated with a reduced survival rate ( $P = 0.02$  and  $0.0253$ , respectively). Quantitative PCR analysis confirmed that the mRNA levels of FGFR-2 IIIb and FGFR-2 IIIc in a high-grade glioma-derived cell line (YKG-1) were lower than levels in a low-grade glioma-derived cell line (KG-1-C). These findings suggest that the decrease or loss of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in high-grade gliomas correlates with poor prognosis, which we attribute to the high proliferation rate of the tumor.

## Introduction

Malignant brain tumors, gliomas in particular, are the most commonly diagnosed adult primary tumors of the central nervous system. Gliomas can be classified into 3

main types: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas, which are typically distinguished by their histological features. The division of gliomas into World Health Organization (WHO) grades I and II (low-grade), and WHO grades III and IV (high-grade or malignant) is based on the presence of nuclear atypia, mitoses, microvascular proliferation and necrosis (1). The treatment of high-grade gliomas has posed a challenge to clinicians due to the lack of effective therapeutic options. In fact, the 5-year survival rate for patients with glioblastoma, a grade IV neoplasm, is approximately 3% (2). Despite significant advances in neurosurgical techniques, in radiation therapy, and in our understanding of the molecular underpinnings of tumorigenesis, the prognosis for patients with malignant glioma, and glioblastoma in particular, remains poor (2,3).

Fibroblast growth factors (FGFs), FGF-1 to -23, are heparin-binding ligands that exert their biological activities through binding high-affinity tyrosine kinase FGF receptors (FGFRs) on the surface of cells (4,5). FGFRs comprise four members, FGFR-1 to -4. FGFRs 1-3 have two isoforms, IIIb and IIIc. In normal human tissues, FGFR-2 IIIb, also known as keratinocyte growth factor receptor, and FGFR-2 IIIc are primarily expressed in epithelial and mesenchymal cells, respectively (6). FGFR-2 gene amplification, abnormal activation, or single nucleotide polymorphisms (SNPs) reportedly play important roles in the progression of cancers including those of the breast, endometrium, colon and pancreas (7-12). Studies have reported that FGFR-2 levels in human gliomas gradually diminish or are lost, whereas FGFR-1 expression increases as the tumor progresses from benign to malignant (13,14). However, no studies have further evaluated the expression of FGFR-2 in gliomas and its clinicopathological significance.

In the present study, we evaluated the expression of FGFR-2 and its major isoforms, FGFR-2 IIIb and FGFR-2 IIIc, in gliomas of all histological grades. In addition, we assessed the correlation between FGFR-2 expression levels, clinical features and survival rates. We report here that FGFR-2 is expressed at lower levels in high histological grade gliomas, and that decreased FGFR-2 expression may be related to lower survival rates.

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## Materials and methods

**Patients and samples.** Tissues from 56 glioma patients who received treatment at Nippon Medical School Hospital (Bunkyo-ku, Tokyo, Japan) from 2007 to 2011 were utilized in this study: 7 patients with grade I (pilocytic astrocytoma), 16 patients with grade II (14 diffuse astrocytomas and 2 oligodendrogliomas), 10 patients with grade III (anaplastic astrocytoma), and 23 patients with grade IV glioma (glioblastoma; Fig. 1). Paraffin-embedded specimens were prepared for immunohistochemical analysis as described previously (15). Normal brain tissues were obtained from autopsy cases without brain disease (n=2). Non-neoplastic brain tissues were obtained from the surgical margins of the glioblastoma patients. This study was conducted in accordance with the principles embodied in the Declaration of Helsinki (2008), and informed consent was obtained from each patient for the use of their tissues.

**Immunohistochemistry.** To investigate the protein levels of FGFR-2 IIIb and FGFR-2 IIIc in gliomas, isoform-specific antibodies were prepared as we described previously (12,16). The expression of FGFR-2 was detected using goat polyclonal anti-human FGFR2 antibody (N20) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), which recognizes both IIIb and IIIc isoforms (11). Mouse monoclonal anti-Ki-67 antibody was purchased from Dako, Japan (Tokyo, Japan). Paraffin-embedded sections (3- $\mu$ m) were immunostained using the Histofine Simple Stain Max Po (R) kit (Nichirei, Tokyo, Japan) for FGFR-2 IIIb and FGFR-2 IIIc, the Max Po (G) Kit for FGFR-2, and the Max Po (M) Kit for Ki-67. After deparaffinization, endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were then incubated with the anti-FGFR-2 (1:400), anti-FGFR-2 IIIb (1:1,000), anti-FGFR-2 IIIc (1:200), or anti-Ki-67 antibody (1:100) diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 16 h at 4°C. Bound antibodies were detected with the Histofine Simple Stain Max Po (R), (G), or (M) reagents, using diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained using Mayer's hematoxylin. Negative control tissue sections were prepared by omitting the primary antibody from the staining procedure.

**Evaluation of immunohistochemical variables.** To evaluate the expression of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in glioma tissues, we analyzed both the percentage of positive cells and the intensity of staining for each antibody in whole tumor lesions at x200 magnification as described elsewhere (11). The following scale was employed to evaluate the intensity of staining: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, intense staining. The percentage of positive cells in the entire field of the specimen was determined at x200 magnification. Blinded cell counts were independently performed by three investigators (R.O., T.I. and Y.M.). The mean values were used in subsequent analyses.

**Glioma cell lines.** KG-1-C and YKG-1 cell lines established from human low-grade glioma and high-grade glioblastoma,

respectively, were obtained from Riken BioResource Center (Ibaraki, Japan). KG-1-C and YKG-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% and 10% fetal bovine serum (FBS), respectively, at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

**Quantitative PCR.** Glioma cells were grown in DMEM supplemented with 10 or 20% FBS for 48 h. Total-RNA was extracted using the FastPure RNA kit (Takara Bio Inc., Shiga, Japan). Next, cDNA synthesis was performed using a High-Capacity cDNA reverse transcription kit according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the ABI StepOnePlus System (Life Technologies) as previously described (12).

The quantitative PCR primers nt 1693-1716 (5'-GGA TAT CCT TTC ACT CTG CAT GGT-3') and nt 1770-1794 (5'-TGG AGT AAA TGG CTA TCT CCA GGTA-3') were used to amplify a 102-bp fragment of human FGFR-2 IIIc (accession no. NM\_000141.4). The TaqMan probe 5'-CAG TTC TGC CAG CGC CTG GAA GA-3' was used for FGFR-2 IIIc. PCR primers nt 1587-1606 (5'-CAC TCG GGG ATA AAT AGT TC-3') and nt 1719-1736 (5'-CGC TTG CTG TTT TGG CAG-3') were used to amplify a 150-bp fragment of human FGFR-2 IIIb (accession no. NM\_022970). The TaqMan probe 5'-TGC CAA AAC AGC AAG CGC CTG G-3' was used for FGFR2IIIb. The PCR reaction mixture containing 2  $\mu$ l of template cDNA, 10  $\mu$ l of TaqMan Fast Universal PCR Master Mix, and 1  $\mu$ l of each of TaqMan gene expression assay was placed in a 96-well reaction plate. 18S rRNA, amplified using a TaqMan gene expression assay, was used as an internal positive control. The optimized program for FGFR-2 IIIb, FGFR-2 IIIc and 18S rRNA involved an initial denaturation at 95°C for 20 sec, followed by 50 cycles of amplification (95°C for 1 sec and 60°C for 20 sec). The internal standard concentration ratio (target/18S rRNA) was calculated for each gene. Gene expression measurements were performed in triplicate.

**Statistical analysis.** All data are shown as mean values  $\pm$  standard error of the mean (SEM). The data between two groups were compared using the Student's t-test. The  $\chi^2$  and Fisher's exact tests were used to analyze the correlation between FGFR-2 isotype expression levels and clinicopathological features. The cumulative survival rate was calculated using the Kaplan-Meier method and the significance of the survival rate differences was analyzed by the log-rank test. P<0.05 was considered to indicate a statistically significant difference in all analyses.

## Results

**Clinicopathological characteristics of the glioma patients.** In total, 34 male and 22 female patients with a mean age of 53 $\pm$ 23 years (range 5-79 years) were enrolled in the study (Table I). The tumors were localized to the frontal (n=22), temporal (n=16), parietal (n=4) and other brain regions (n=14). Follow-up data were available for 46 patients. The mean follow-up duration was 694.2 $\pm$ 632 days (range 30-2,180 days). At the last follow-up, 30 patients were confirmed to be alive and 16 patients were deceased.

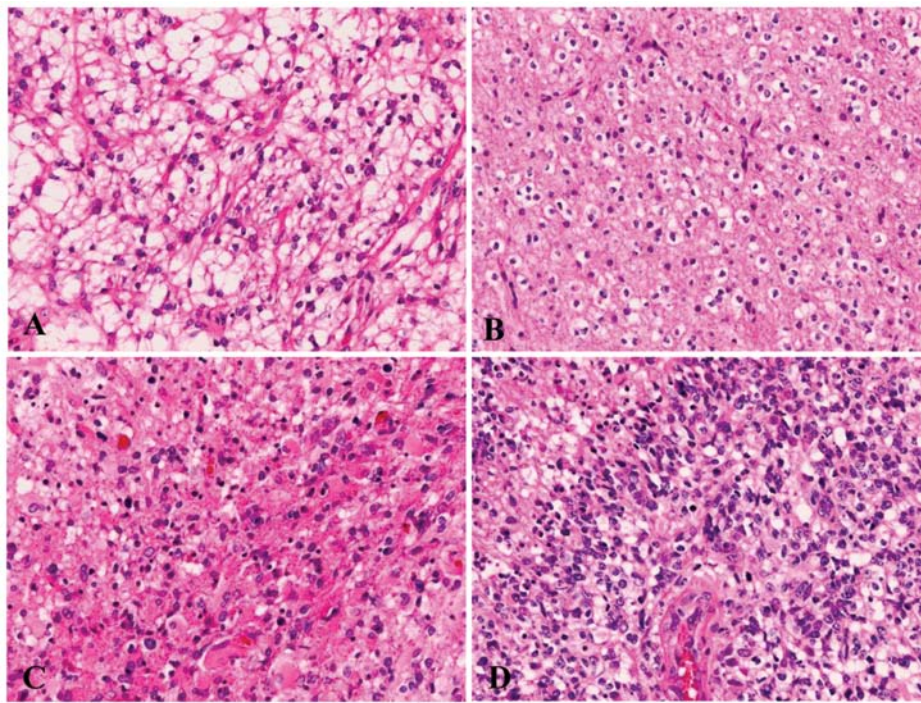


Figure 1. Histological features of the gliomas. (A) Pilocytic astrocytomas (grade I) exhibit low cellularity and rare mitoses, and are composed of varying proportions of compacted bipolar cells and loose-texture multipolar cells. (B) Oligodendrogliomas (grade II) exhibit moderate cellularity and are composed of monomorphic cells with uniform round nuclei and perinuclear halos. (C) Anaplastic astrocytomas (grade III) are hypercellular and possess hyperchromatic irregular 'naked' nuclei and high mitotic activity. (D) Glioblastomas (grade IV) are composed of poorly differentiated pleomorphic astrocytes and show rapid mitotic activity and focal necrotic changes.

Table I. Clinicopathological features of the glioma cases with correlation to FGFR-2 isoform expression.

	FGFR2		P-value	FGFR2 IIIb		P-value	FGFR2 IIIc		P-value
	>30%	<29%		>40%	<39%		>40%	<39%	
Sample size, n	21	35		23	33		26	30	
Gender									
Male	12	22	0.67	13	21	0.59	13	21	0.13
Female	9	13		10	12		13	9	
Age (years)									
≥55	12	22	0.67	12	22	0.36	15	19	0.66
<55	9	13		11	11		13	9	
Tumor location									
Frontal	8	14	0.91	9	13	0.051	11	11	0.5
Parietal	1	3		2	2		1	3	
Temporal	7	9		4	12		7	9	
Other site	5	9		10	4		8	6	
Tumor grade									
High (III, IV)	5	28	<0.01	5	28	<0.01	5	28	<0.01
Low (I, II)	16	7		18	5		21	2	
Ki-67 expression									
Positive (>20%)	3	17	0.011	4	16	0.0169	4	16	<0.01
Negative (<19%)	18	18		19	17		22	14	

*Immunohistochemical analysis of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in non-neoplastic brain tissues and gliomas.*

Immunohistochemical analyses showed that FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc were evenly distributed at low



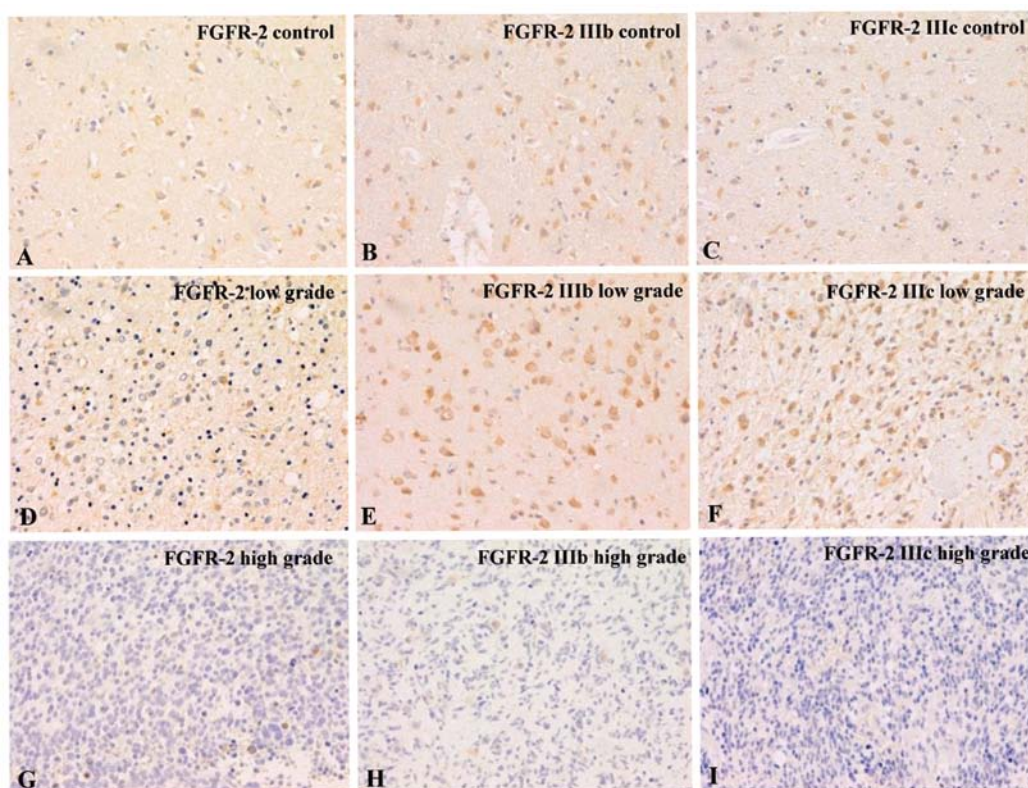


Figure 2. Immunohistochemical analysis of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in normal brain tissues and gliomas. Fibroblast growth factor receptor (FGFR)-2 (A), FGFR-2 IIIb (B) and FGFR-2 IIIc (C) were expressed ubiquitously in astrocytes in all the regions of the normal brain tissue. Low-grade astrocytomas (grade I) exhibited moderate expression of FGFR-2 (D), FGFR-2 IIIb (E), and FGFR-2 IIIc (F), while that of FGFR-2 (G), FGFR-2 IIIb (H) and FGFR-2 IIIc (I) in high-grade astrocytomas (grade IV) was undetectable or absent.

levels in all the astrocytes present in the normal brain tissues collected from autopsy cases (Fig. 2A-C). In the low-grade astrocytomas (grade I or II), the expression of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc did not differ from that in the healthy brain tissues (Fig. 2D-F). In contrast, high-grade astrocytomas (grade III or IV) showed a significant decrease or loss of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc expression. Notably, the expression of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc was virtually absent in some of the glioblastoma cases (Fig. 2G-I). To confirm the histopathological grade of gliomas, we performed immunostaining for Ki-67. High-grade astrocytomas demonstrated a marked increase in Ki-67-positive cells relative to low-grade astrocytomas.

The percentages of cells positive for FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in grade IV astrocytomas were significantly lower than these percentages in the grade I, II and III tumor groups (Fig. 3A, C and E;  $P < 0.001$ ). The percentages of FGFR-2 IIIb- and FGFR-2 IIIc-positive cells in the grade III gliomas were lower than these percentages in the grade II tumors (Fig. 3C and E;  $P < 0.001$ ). Likewise, the staining intensities for FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in the grade IV astrocytomas were also lower than the intensities in the other lower grade tumors (Fig. 3B, D and F;  $P < 0.001$  or  $P < 0.05$ ). No significant differences were observed in grade I and II with respect to any of the proteins examined.

*Correlation between FGFR-2 isoform expression and the clinicopathological features of the gliomas.* To evaluate the relationship between FGFR-2 and FGFR-2 isoform expression

in gliomas and the clinicopathological features, we separated the patients into two groups: high expression ( $>$ cutoff value) and low expression ( $<$ cutoff value) (Table I). To select an appropriate cutoff value, we conducted statistical analyses using different percentages of positive cells (20, 30, 40 and 50%) as the cutoff. For each protein, the cutoff value that yielded the most statistically significant difference was used in the final analysis. A cutoff value of 30% was used for FGFR-2, whereas 40% was used for FGFR-2 IIIb and FGFR-2 IIIc. For FGFR-2 and both of its isoforms, the degree of protein expression was negatively correlated with the tumor grade (Table I;  $P < 0.01$  for each protein). A high MIB-1 index, indicated by Ki-67 expression in  $>20\%$  of the cells, was also associated with low expression of each protein (Table I;  $P = 0.011$  for FGFR-2,  $P = 0.0169$  for FGFR-2 IIIb and  $P < 0.01$  for FGFR-2 IIIc). Gender, age and tumor location did not show any significant difference between the groups expressing low and high levels of FGFR-2 or its isoforms. The FGFR-2 and FGFR-2 IIIc low expression groups were associated with a low survival rate (Fig. 4A and C;  $P = 0.020$  and  $0.0253$ , respectively). The FGFR-2 IIIb low expression group showed a tendency towards a reduced survival rate (Fig. 4B;  $P = 0.085$ ).

*Expression of FGFR-2 IIIb and FGFR-2 IIIc in the glioma cell lines.* To examine the mRNA levels of FGFR-2 IIIb and FGFR-2 IIIc in human glioma cell lines, qPCR analysis was performed using KG-1-C and YKG-1 cells that were derived from low-grade and high-grade astrocytomas, respectively. FGFR-2 IIIb and FGFR-2 IIIc mRNA was present in the

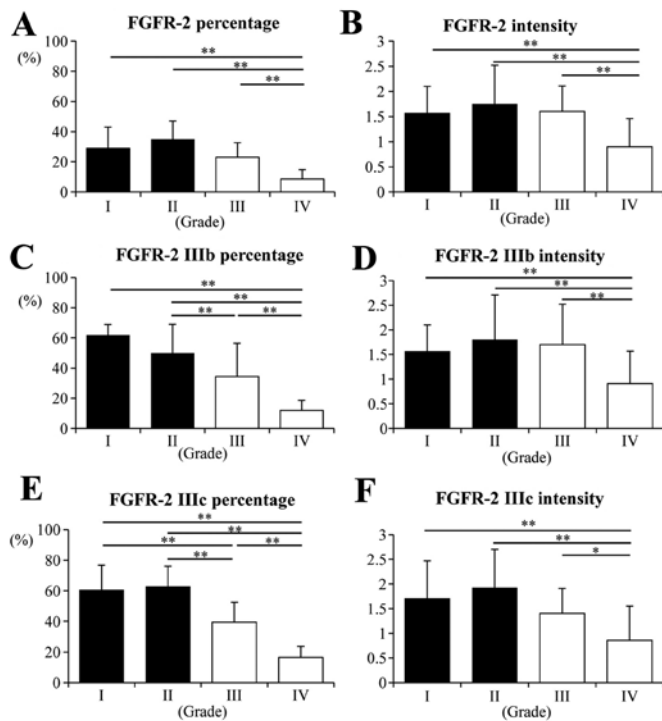


Figure 3. Semi-quantitative analysis of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc expression in grade I, grade II, grade III and grade IV astrocytomas. The percentages of cells positive for FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in grade IV astrocytomas were significantly lower than these percentages in the grade I, II and III tumor groups (A, C and E). The percentages of FGFR-2 IIIb- and IIIc-positive cells in grade III gliomas were lower than those in grade II tumors (C and E). Likewise, the intensity of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc staining in grade IV astrocytomas was lower than that in the lower grade tumors (B, D and F). There was no significant difference in intensity between grade I and II gliomas for any of the proteins examined. \*\*P<0.001; \*P<0.05.

KG-1-C cells. Notably, their expression levels in the KG-1-C cells were lower than levels in the fetal brain tissues. We attribute this to the high level of expression and activity of FGF/FGFR signaling components in fetal brain tissues (17) (Fig. 5). The levels of FGFR-2 IIIb and FGFR-2 IIIc mRNA were considerably lower in the YKG-1 cells when compared with these levels in the KG-1-C cells. The low expression levels of FGFR-2 isoforms in the YKG-1 glioma cell line derived from high-grade glioma were consistent with the results of our immunohistochemical analysis.

## Discussion

FGFRs are single transmembrane receptors that contain extracellular, transmembrane and intracellular domains (5). The extracellular domains of FGFRs are typically composed of 3 immunoglobulin (Ig)-like domains (I-III). Alternative splicing in the C-terminal half of the third Ig-like domain of FGFRs 1-3 generates their IIIb and IIIc isoforms. FGFR4 does not possess these alternatively spliced exons. The FGF/FGFR pathway plays important roles in development and differentiation in normal human tissues. Moreover, FGF/FGFR is tightly linked to carcinogenesis and cancer progression in various organs. We previously reported that FGFR-2 is overexpressed in the invasive front of colorectal cancer (11). However, the

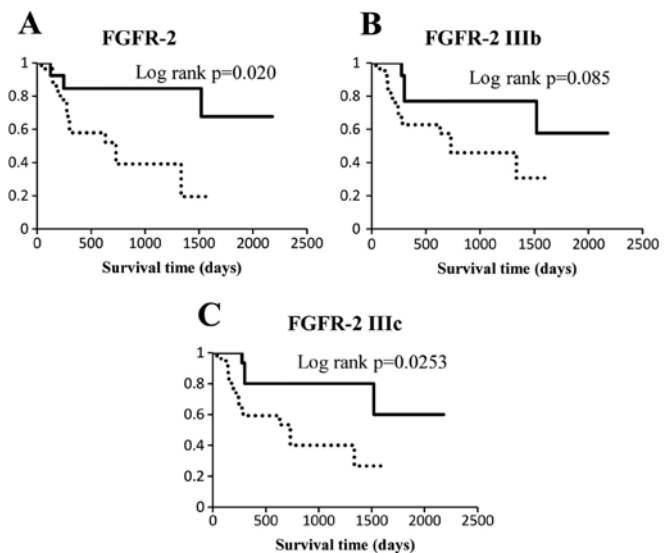


Figure 4. Survival curves for the glioma groups expressing high and low FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc. The survival curves indicate a worse prognosis in the FGFR-2 and FGFR-2 IIIc low-expression groups (A, C, respectively, dotted lines) compared to the FGFR-2 and FGFR-2 IIIc high-expression groups (solid lines; P=0.020 and 0.0253, respectively). There was a trend towards poor prognosis in the FGFR-2 IIIb low-expression group (B, dotted line) compared to the FGFR-2 IIIb high-expression group (solid line; P=0.085).

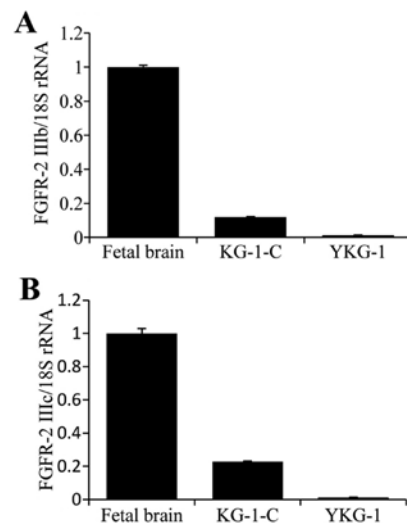


Figure 5. Levels of FGFR-2 IIIb and FGFR-2 IIIc mRNA in the glioma cell lines. FGFR-2 IIIb (A) and IIIc (B) mRNAs were present in KG-1-C cells derived from low-grade astrocytoma, but at levels lower than in fetal brain tissues. In the YKG-1 cells derived from high-grade astrocytoma, the levels of both FGFR-2 IIIb and FGFR-2 IIIc mRNAs were considerably lower when compared to levels in the KG-1-C cells.

expression of FGFRs and their IIIb and IIIc isoforms have not been thoroughly examined in the brain. In addition, their clinical significance in gliomas remains unclear. In the present study, we examined the presence of FGFR-2, FGFR-2 IIIb and FGFR-2 IIIc in normal human brain tissues and gliomas using immunohistochemical staining. To our knowledge, this is the first study reporting the correlation between the expression levels of FGFR-2 and its two isoforms and clinicopathological

features in glioma. We demonstrated that the decrease or loss of these molecules is highly related to the progression of gliomas and a poor prognosis.

The most notable finding in our study was that FGFR-2, which is normally expressed in brain tissue, was significantly diminished or nearly abolished as gliomas progressed from low to high grade. To date, few studies have examined the expression of FGFR-2 in the brain. Yamaguchi *et al* examined the expression of FGFR-1 and FGFR-2 in normal human brain tissues and astrocytomas (13). They showed that FGFR-2 was abundantly expressed in normal brain tissues, whereas FGFR-1 expression was minimal. Conversely, FGFR-2 expression was low or undetectable in high-grade astrocytomas, such as glioblastomas, in which FGFR-1 was highly upregulated. They speculated that the loss of FGFR-2 and gain of FGFR-1 are associated with the malignant progression of astrocytomas from low- to high-grade tumors. Although we did not assess FGFR-1 expression in this study, the decreased FGFR-2 expression we observed in high-grade astrocytomas is consistent with their findings. We speculated that FGFR-1 and FGFR-2 play reciprocal roles in the development of astrocytomas. FGFR-1 and FGFR-2 have different ligand specificity. Therefore, examining the expression of the FGFR-1 and FGFR-2 ligands in human brain specimens in future studies may reveal a functional relationship between these two molecules.

The mechanism underlying the loss of FGFR-2 expression in high-grade astrocytomas is unclear. The *FGFR-2* locus is on the long arm of chromosome 10q26. Interestingly, approximately 80% of all glioblastomas are associated with the loss of an entire copy of chromosome 10 (18,19). Therefore, the production of FGFR-2 is likely impaired as a result of losing chromosome 10 during the astrocyte transformation process. Alternatively, the FGFR-2 gene may still be present in glioblastomas, but silent following translocation to another chromosome. As it is lost in glioblastomas, chromosome 10 is the proposed location of a tumor-suppressor gene. Since decreased FGFR-2 expression was observed in higher-grade astrocytomas, we postulate that FGFR-2 has a tumor-suppressing effect. On the other hand, one may argue that the decrease in FGFR-2 expression is not directly related to tumorigenesis. Instead, it may simply reflect the loss of one copy of chromosome 10. We were unable to test alterations of the chromosomes in our study, leaving the underlying mechanism of FGFR-2 loss in gliomas unexplored. Additional studies are required to elucidate the mechanism of FGFR-2 loss in the future.

We present evidence that the levels of FGFR-2 isoforms, FGFR-2 IIIb and FGFR-2 IIIc, are altered in gliomas. FGFR-2 IIIc is widely expressed in cancer cells including adenocarcinomas, squamous cell carcinomas and urothelial cell carcinomas (20-25). In the prostate, a switch from FGFR-2 IIIb to FGFR-2 IIIc expression is related to the progression of cancer (26). FGFR-2 IIIc expression in prostate cancer cells induces epithelial-mesenchymal transition and alters splicing, which may contribute to cancer metastasis (27). We recently demonstrated that enhanced expression of FGFR-2 IIIc promotes pancreatic cancer cell proliferation (12). In contrast to FGFR-2 IIIc, the role of the FGFR-2 IIIb isoform is controversial. Overexpression of the FGFR-2 IIIb isoform has been reported in breast, endometrial, gastric, pancreatic

and colorectal cancers; however, several of these studies presented conflicting results regarding its clinical roles (28-32). Higher expression of FGFR-2 IIIb in pancreatic cancers was correlated with a poor prognosis (32). In contrast, expression of FGFR-2 IIIb in gastric cancer cells was associated with a better prognosis (31). Here, we showed that the expression of the FGFR-2 IIIb and FGFR-2 IIIc isoforms decreased with the progression of gliomas from low to high grade. Lower expression of the FGFR-2 isoforms was associated with a worse prognosis. These findings indicate that these two isoforms are synchronized in the development of glioma. Therefore, it is unlikely that they have conflicting roles in gliomas. We anticipate that the decrease or loss of FGFR-2 isoform expression can be used as a marker to predict the malignant potential of gliomas. Additional studies are warranted to validate this hypothesis.

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