Identification of glioma-specific RFX4-E and -F isoforms and humoral immune response in patients

Hirokazu Matsushita,[1,2] Akiko Uenaka,[1] Toshiro Ono,[3] Kosei Hasegawa,[1] Shuichiro Sato,[1] Fumihito Koizumi,[1] Kazuhiko Nakagawa,[1] Masahiro Toda,[4] Tetsuro Shingo,[2] Tomotsugu Ichikawa,[2] Yuji Noguchi,[1] Takashi Tamiya,[5] Tomohisa Furuta,[6] Takeshi Kawase,[4] Isao Date[2] and Eiichi Nakayama[1,7]

[1]Departments of Immunology and [2]Neurological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan; [3]Department of Radiation Research, Advanced Science Research Center, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8558, Japan; [4]Department of Neurological Surgery, Keio University School of Medicine, 35 Shinano-cho, Shinjuku, Tokyo 160-8582, Japan; [5]Department of Neurological Surgery, Faculty of Medicine, Kagawa University, 1750-1 Ikedo, Miki-cho, Kida-gun Kagawa 761-0793, Japan; and [6]Department of Clinical Psychology, School of Social Welfare, Kibi International University, 8 Iga-cho, Takahashi, Okayama 716-0018, Japan

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For *regulatory factor X4* (*RFX4*), two alternatively spliced variants, *RFX4-A* and *-B*, were reported in the testis. In this study, we identified transcript variants *RFX4-C*, *-D*, *-E*, and *-F*, and demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) that *RFX4-A*, *-B* and *-C* mRNAs were expressed only in the testis, and *RFX4-D* mRNA was expressed only in normal brain tissues. In tumors, *RFX4-E* and *-F* in addition to *RFX4-D* mRNA were expressed in gliomas by rapid amplification of cDNA ends and RT-PCR analyses. Expression of *RFX4* mRNA was not observed in other tumors, such as lung, esophageal, stomach, colon or liver cancers. Quantitative real-time RT-PCR using common primer pairs detecting all of the variant transcripts showed high expression in normal testis, low expression in the brain (1% compared to the expression in testis), and overexpression in 17 of 61 gliomas (28%). Western blot analysis using DC28 monoclonal antibody (mAb) produced against recombinant RFX4-D C-terminus protein showed expression of RFX4-A and -C proteins, but not RFX4-B protein, in the testis, and expression of RFX4-D protein in the brain. Moreover, expression of RFX4-E and -F proteins, but not RFX4-D protein, was observed in gliomas. Immunohistochemistry analysis using DC28 mAb showed positive staining in the nuclei of spermatocytes in the testis and glioma cells. Antibody against RFX4 was detected in the sera of 3 of 58 (5%) glioma patients by enzyme-linked immunosorbent assay, suggesting the immunogenicity of RFX4-E and -F proteins in glioma patients. (*Cancer Sci* 2005; 96: 801–809)

The prognosis for malignant glioma patients remains poor despite advances in surgery, radiation therapy, and chemotherapy.(1,2) Therefore, it is necessary to seek alternative treatments. Interest in immunotherapy for gliomas has grown recently.(3–5) An important prerequisite for successful tumor-specific immunotherapy is the identification of tumor antigens that are exclusively or preferentially expressed in gliomas and induce humoral and cellular immune responses.(6,7)

Regulatory factor X4 (*RFX4*) is a member of the *RFX* family of genes, which share a highly conserved winged-helix DNA-binding domain (DBD).(8–10) *RFX4* was initially isolated from breast cancer as a partial DBD fused to the estrogen receptor.(11) Two alternatively spliced variants of *RFX4*, *RFX4-A* and *-B*, were reported in the testis and recently, a brain-specific variant has been described.(12,13) In this study, we identified additional *RFX4* variants, *RFX4-C*, *-D*, *-E*, and *-F*, and investigated their mRNA and protein expression in normal tissues and tumors. We showed that the isoform RFX4-D was expressed weakly in the normal brain. However, in gliomas, RFX4-E and -F isoforms were expressed. No expression of RFX4-E or -F was observed in normal tissues. Antibody against RFX4-E and -F proteins was detected in sera from glioma patients, suggesting the immunogenicity of these proteins in glioma patients.

Materials and Methods

Tissues and sera

Sixty-one glioma tissue specimens were surgically obtained from patients at Okayama University Hospital, Okayama, Japan and classified according to the criteria of the World Health Organization (WHO).(14) They consisted of 50 astrocytic, 9 ependymal and 2 oligodendroglial tumors from 38 males and 23 females with a mean age at diagnosis of 41 years (range 2–84 years). Normal brain tissues were obtained from seven patients with brain tumors, which were accidentally included in brain tumor tissues. Sera were obtained from 58 glioma patients and 70 healthy volunteers including 50 adults and 20 children (< 18 years old) at Okayama University Hospital. Written informed consent was obtained from healthy volunteers, each patient, and the parent when the patient was younger than 18 years old.

[7]To whom correspondence should be addressed.
E-mail: nakayama@md.okayama-u.ac.jp

Abbreviations: DBD, DNA-binding domain; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; IHC, immunohistochemistry; mAb, monoclonal antibody; RACE, rapid amplification of cDNA ends; RFX4, regulatory factor X4; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SEZ, subependymal zone; UTR, untranslated region.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from frozen tumor tissues using an RNeasy kit (Qiagen, Hilden, Germany). Total RNA of the cerebrum, cerebellum, and fetal brain was obtained from Human Total RNA Panel IV (Clontech, Palo Alto, CA). Total RNA (2 µg) was reverse transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; Amersham Pharmacia, Piscataway, NJ, USA), and oligo $(dT)_{15}$ as a primer. The cDNA was tested for integrity by amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts for over 30 cycles.

Reverse transcription-polymerase chain reaction (RT-PCR)

To amplify cDNA segments from normal tissues and tumors, gene-specific primers were designed. The primer pairs used in this study are shown in Fig. 1a. RT-PCR was performed using 35 amplification cycles followed by a 10-min elongation step at 72°C. The PCR products were analyzed by electrophoresis in conventional agarose gels. The primer pairs were as follows: A, 5′-GCAGAAATATCACGGAATGGT-3′ (forward), 5′-AGCCACTTTTAGCCACTCATC-3′ (reverse); BC, 5′-GGGCTTCTCCAAACTCCTGT-3′ (forward), 5′-AGCCACTTTTAGCCACTCATC-3′ (reverse); D, 5′-ATGCATTGTGGGTTACTGGAG-3′ (forward), 5′-TGAATATGCCACTGTCTGTTTG-3′ (reverse).

Rapid amplification of cDNA ends (RACE)

5′ and 3′ RACE were performed using a GeneRacer kit (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated from specimens using an RNeasy kit (Qiagen) and used as the template. First-strand cDNA was synthesized using the GeneRacer Oligo dT primer following the manufacturer's directions. The primers used for 5′ and 3′ RACE were R1 and R2, and F1, respectively (Fig. 1a). The primer sequences were as follows: R1, 5′-TGAATATGCCACTGTCTGTTTGC-3′; R2, 5′-TTAGGTGAAAAGACCCGAAGCT-3′; F1, 5′-GCCACTCCACTATGCCCCTTACCA-3′. The RACE products were cloned into pCI 2.1 vector (Invitrogen) and sequenced using an ABI PRISM automated sequencer (PerkinElmer, Foster City, CA, USA).

Quantitative real-time RT-PCR

Two-step real-time RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The gene-specific primers and TaqMan probe for *RFX4* were designed using Primer Express software (version 1.5) (Applied Biosystems). To avoid the possible amplification of contaminating genomic DNA, the primers were designed so that the PCR product encompassed one intron. The setting of primers for the quantitative real-time RT-PCR is indicated in Fig. 1a. For *RFX4*, the forward TaqMan primer was 5′-TTTCGGCACAAGGGTGATC-3′ and the reverse primer was 5′-TTAGGTGAAAAGACCCGAAGCT-3′. The TaqMan probe was FAM-CATGACCTTGCACAGCGCCCC-TAMRA. PCR was performed using TaqMan PCR Master Mix (Applied Biosystems) and the thermal cycling conditions comprised an initial denaturation at 95°C for 10 min, then 50 cycles at 95°C for 15 s and 60°C for 1 min. The parameter Ct was defined as the threshold cycle number at which the fluorescence generated by cleavage of the probe passed above the baseline. The *RFX4* target message was quantified by measuring the Ct value, and transcripts of *GAPDH* were quantified as an endogenous RNA control using TaqMan human *GAPDH* control reagents (Applied Biosystems). For each sample, the amount of target and endogenous reference was determined from a standard curve made from serially diluted plasmid DNA templates encoding *RFX4* and *GAPDH*. The amount of target *RFX4* in each sample was normalized on the basis of the endogenous *GAPDH* content.

Production of recombinant RFX4 protein

RFX4-D N- and C-terminal proteins were expressed in *Escherichia coli* using the histidine-tag-containing vector pQE30 (Qiagen). cDNA amplification primers were designed to encompass the partial coding sequence of the gene corresponding to amino acid positions 1–126 and 323–735 for the N- and C-termini of RFX4-D, respectively. The induction of recombinant protein synthesis and subsequent purification on a Ni^{2+}-NTA column were performed according to the manufacturer's instructions.

Production of monoclonal anti-RFX4 antibody

BALB/c mice were immunized intramuscularly twice with 100 µg of pCI-neo vector encoding full-length RFX4-D using *in vivo* electroporation with a 2-week interval and boosted intraperitoneally once with 100 µg of recombinant C-terminal (323–735 amino acids) protein (Fig. 1b). Spleen cells from immunized mice were fused with NS-1 myeloma cells. The selection and cloning of hybridomas was performed using ClonaCell-HY Medium D (StemCell Technologies, Vancouver, BC, Canada). The supernatants from the cloned populations were screened for binding to the immunizing protein by enzyme-linked immunosorbent assay (ELISA) and Western blotting. A clone DC28 positive for the RFX4-D C-terminus was obtained and purified with an MAbTrap kit (Amersham Biosciences, Uppsala, Sweden).

Immunohistochemistry (IHC)

IHC was done as described elsewhere.[17] DC28 monoclonal antibody (mAb; 2 µg/mL) was used as the primary antibody. NS-1 culture supernatant was used as the negative control.

Elisa

Recombinant RFX4 protein (1 µg/mL) in 0.05 M carbonate buffer (pH 9.6) was adsorbed onto 96-well plates (Nunc, Roskilde, Denmark) at 4°C overnight. ELISA was done using goat anti-human IgG (100 µL/mL) labeled with horseradish peroxidase (MBL, Nagoya, Japan) as the second antibody.[17] A positive reaction was defined as an optic density (OD) value for 1:400 diluted serum that exceeded the mean OD value of sera from healthy donors by three standard deviations.

Western blot analysis

Normal and tumor tissue extracts were prepared in radioimmunoprecipitation assay buffer supplemented with protease inhibitor, complete Mini (Roche, Penzberg, Germany). For normal brain tissue, trichloroacetic acid was used for

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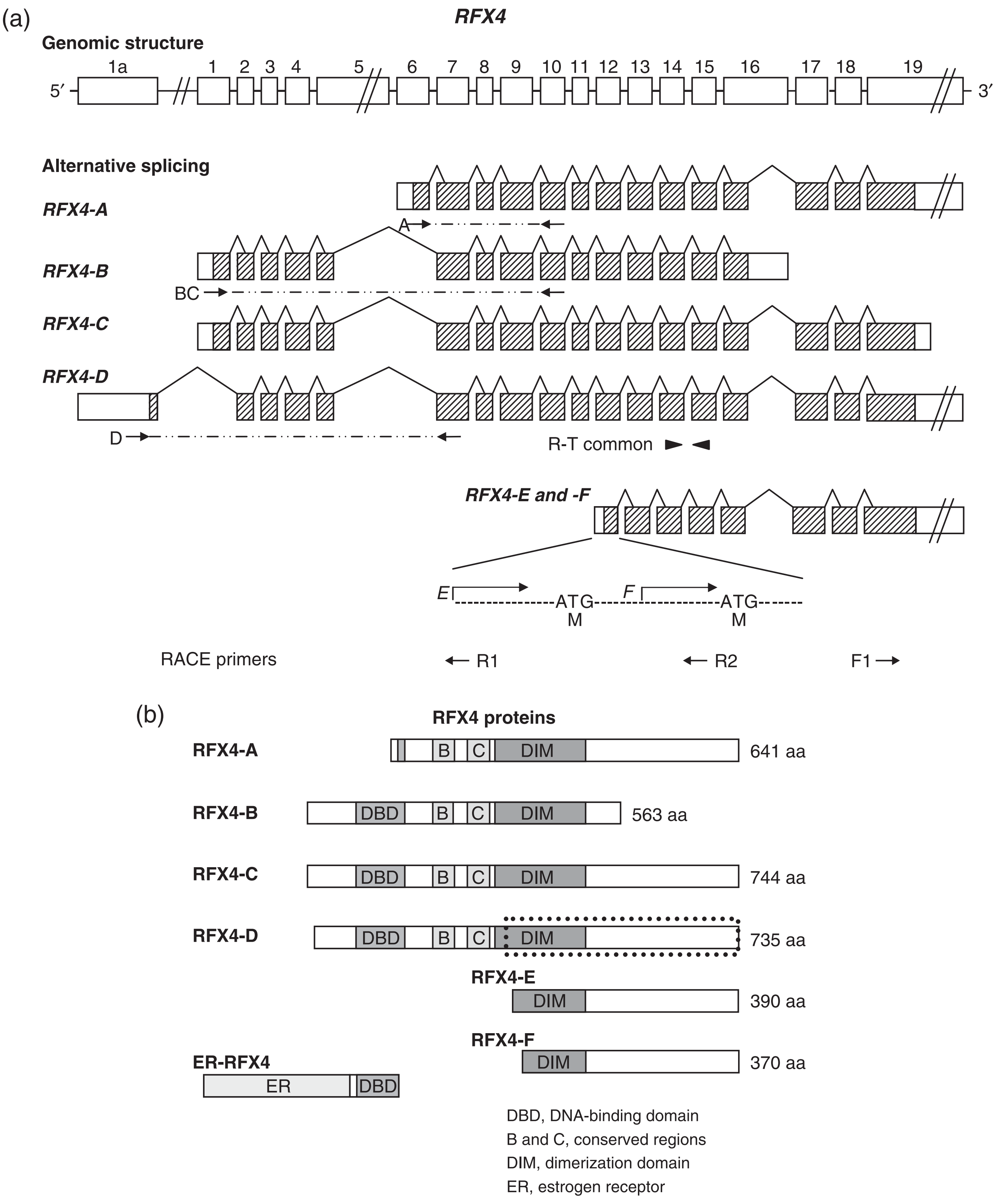

Fig. 1. (a) Schematic representation of the genomic structure of *RFX4* and alternatively spliced variants. The exons are shown in boxes and the open reading frame is shown in hatched boxes. The primer pairs used in this study for conventional reverse transcription polymerase chain reaction (RT-PCR) and TaqMan real-time RT-PCR are indicated by arrows and arrowheads, respectively. Different transcription start sites of *RFX4-E* and *-F* are shown in exon 12. *RFX4-E* starts from the initial base in exon 12 and *RFX4-F* starts from 78 bp downstream of the site. ATG, start codon; M, methionine; R-T common, the primers for the quantitative real-time RT-PCR. (b) Schematic representation of RFX4 proteins. Six RFX4 isoforms and the ER-RFX4 protein (11) are shown. The C-terminal portion of the RFX4-D protein used for the production of monoclonal antibody specific for RFX4 is shown in the dotted box.

precipitation to obtain a more concentrated preparation. Protein (50 µg) was separated by 7.5% or 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane, and incubated with DC28 mAb (4 µg/mL), sera from glioma patients or healthy donors (1:100), or goat anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse or -human, or rabbit anti-goat secondary antibody (1:3000; Jackson Immuno-Research, West Grove, PA, USA) using a Bio-Rad substrate kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The *RFX4* mRNA expression level in normal brain tissues and gliomas was analyzed with the Mann–Whitney *U*-test. The difference in frequency of RFX4 expression was analyzed by Fisher's exact probability test. $P < 0.05$ was considered to be significant.

Results

Identification of new RFX4 variants in testis, brain and glioma

The *RFX4* gene contains 19 exons (Fig. 1a). Two alternatively spliced variants have been described in the testis. The *RFX4-A* transcript, which was reported as *NYD-sp10* (GenBank accession number AF332192), is composed of exons 6–19 and lacks DBD encoded by exons 3, 4, 5 and 7 (Fig. 1a,b). *RFX4-B* (GenBank accession number AB044245) is composed of exons 1–16 except exon 6 and contains DBD.[10] Both products share the evolutionarily conserved B and C regions and a dimerization domain.

We identified four new *RFX4* transcript variants from testis, brain and glioma by RACE analysis. The 5′ and 3′ RACE primers are described in the Materials and Methods section and shown in Fig. 1a. Using primers R1 and F1, we identified *RFX4-C* and *RFX4-D* in the testis and brain, respectively. The *RFX4-C* cDNA spanned 18 exons without exon 6 from the 5′ end of *RFX4-B* to the 3′ end of *RFX4-A* with a shorter 3′ untranslated region (UTR) (Fig. 1a), 2560 bp in length, and encoded a putative protein 744 amino acids in size (Fig. 1b). The *RFX4-D* cDNA contained exon 1a instead of the exon 1 present in *RFX4-C*, had a longer 3′ UTR (Fig. 1a), was 3955 bp in length, and encoded a putative protein of 735 amino acids (Fig. 1b). The *RFX4_v3* cDNA (GenBank accession number AY102009), which was recently reported,[12] is almost identical to *RFX4-D*, but differs at its 5′ end, being approximately 350 bp shorter and having methionine instead of valine at amino acid position 522. The difference between RFX4-C and RFX4-D proteins was the initial 23 and 14 N-terminal amino acids corresponding to exons 1 and 1a, respectively (Fig. 1a).

Transcript variants *RFX4-E*, and *-F* were identified from glioma using primers R2 and F1. *RFX4-E* and *-F* were 2548 and 2470 bp in length, respectively, and they had different transcription start sites within the same exon 12, resulting in two different putative proteins of 390 and 370 amino acids (Fig. 1a,b). These four new sequences are available from GenBank/EMBL/DDBJ under the accession numbers AB095365, AB095366, AB195784 and AB195785 for *RFX4-C*, *-D*, *-E*, and *-F*, respectively.

RT-PCR analysis of RFX4 mRNA expression in normal tissues and glioma

To investigate the expression of the transcript variants *RFX4-A*, *-B*, *-C* and *-D*, which were identified from testis and brain, in normal tissues (Multiple Tissue cDNA panels; Clontech), we performed RT-PCR using variant-specific primer pairs A, BC, and D (Fig. 1a). No variant *B*- or *C*-specific primer pair was available because of low efficiency of amplification. As shown in Figure 2a, *RFX4-A*, *-B* and *-C* were expressed only in the testis. *RFX4-D* was expressed only in the brain. In gliomas, no *RFX4-A*, *-B* or *-C* mRNA was detected (Fig. 2b). On the other hand, *RFX4-D* mRNA was detected. *RFX4-D* mRNA expression was confirmed by long PCR with primer pairs in exon 1a and 19 using a TaKaRa LA PCR Kit (TaKaRa Bio, Ohtsu, Japan) (data not shown). *RFX4-E* and *-F* mRNAs were detected only in gliomas, but not in the normal tissues, by RACE analysis (data not shown). No specific primer pairs were available to detect those mRNAs by RT-PCR.

No *RFX4* mRNA expression was observed in other malignancies including 16 lung cancers, 16 esophageal cancers, 10 stomach cancers, 20 colon cancers, and 15 liver cancers.

Quantitative real-time RT-PCR analysis of RFX4 mRNA in normal tissues and glioma

To investigate *RFX4* mRNA expression quantitatively, we performed a real-time RT-PCR analysis. The primer pair R-T common used in this study (Fig. 1a) can amplify *RFX4-A*,

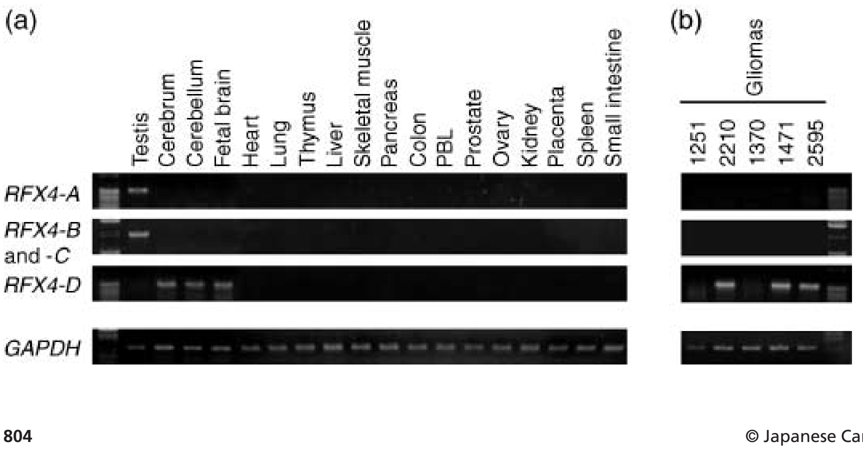

Fig. 2. Reverse transcription-polymerase chain reaction analysis of *RFX4* mRNA expression using variant-specific primer pairs in normal tissues (a) and gliomas (b). 1251 and 2210, diffuse astrocytoma; 1370, anaplastic astrocytoma; 1471, anaplastic ependymoma; 2595, glioblastoma.

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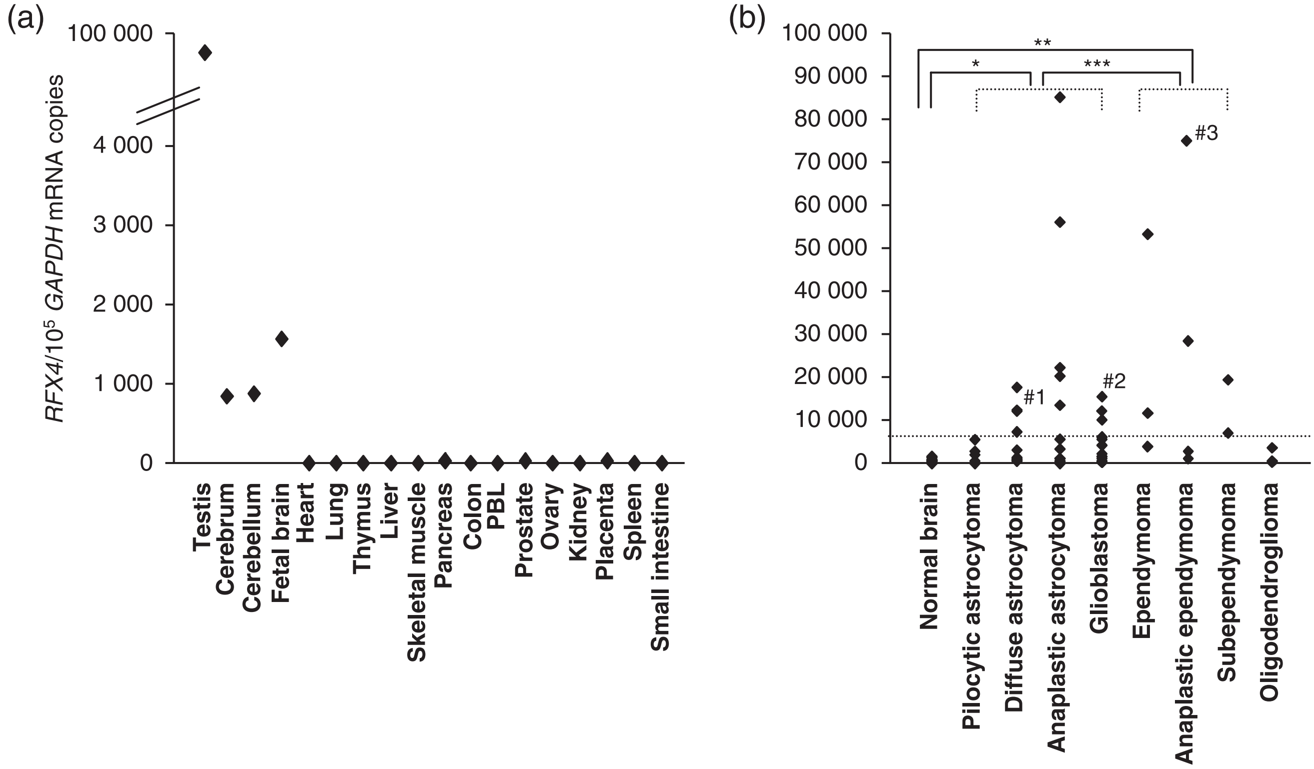

Fig. 3. Quantitative real-time reverse transcription-polymerase chain reaction analysis of *RFX4* mRNA expression in normal tissues (a) and gliomas (b). The numbers of *RFX4* mRNA copies per 10^5 glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA copies for each sample are plotted in the panels. Normal brain samples in (b) consisted of three normal brain cDNAs purchased from Clontech and seven normal peritumoral tissues from brain tumors. *, $P = 0.019$; **, $P = 0.0004$; ***, $P = 0.0056$; #1, seropositive patient 2210; #2, seropositive patient 2595; #3, seropositive patient 1471.

-B and *-C* in the testis and *RFX4-D* mRNA in the brain. Approximately 100 000 *RFX4* mRNA copies/10^5 *GAPDH* mRNA copies were detected in the testis (Fig. 3a). Approximately 1000 *RFX4* mRNA copies/10^5 *GAPDH* mRNA copies were detected in the brain. The copy number was less than 20 in other normal tissues, indicating that the expression was restricted to the testis and brain in normal tissues. The level of *RFX4-D* mRNA expression in the brain appeared to be approximately 1% of the levels of *RFX4-A*, *-B* and *-C* mRNA expression in the testis.

Next we examined *RFX4* mRNA expression in gliomas by real-time RT-PCR using the primer pair R-T common. No specific primer pairs for *RFX4-E* or *-F* were available. High *RFX4* mRNA copy numbers were detected in some specimens. *RFX4* mRNA expression differed significantly between the normal brain and astrocytic tumors ($P = 0.019$) and between the normal brain and ependymal tumors ($P = 0.0004$) according to the Mann–Whitney *U*-test (Fig. 3b). *RFX4* mRNA copy numbers were significantly higher in ependymal tumors than astrocytic tumors ($P = 0.0056$). No significant difference in expression was observed among the various grades (WHO grade I–IV) of gliomas. Marginal levels of *RFX4* mRNA expression were observed in two oligodendroglioma specimens. The number of tissue samples that expressed *RFX4* mRNA at more than 10 times the mean value for normal brain is shown in Table 1. The difference in frequency of *RFX4* mRNA expression between ependymal (6 of 9) and astrocytic (15 of 50) tumors was significant ($P < 0.05$) (Table 1). No correlation was observed between the frequency of *RFX4* mRNA expression and age, sex or WHO grade (data not shown).

Table 1. Expression of RFX4 in gliomas

Tissue	*n*	†Real-time RT-PCR	IHC
Astrocytic tumor	50	11 (22%)	10 (20%)
Pilocytic astrocytoma	5	0 (0%)	0 (0%)
Diffuse astrocytoma	11	3 (27%)	3 (27%)
Anaplastic astrocytoma	14	5 (36%)	4 (29%)
Glioblastoma	20	3 (15%)	3 (15%)
Ependymal tumor	9	‡6 (67%)	‡5 (56%)
Ependymoma	3	2 (67%)	1 (33%)
Anaplastic ependymoma	4	2 (50%)	2 (50%)
Subependymoma	2	2 (100%)	2 (100%)
Oligodendroglial tumor	2	0 (0%)	0 (0%)
Oligodendroglioma	2	0 (0%)	0 (0%)

†The numbers of tissue specimens that expressed *RFX4* at more than 10 times the mean value for the normal brain are shown. ‡The difference in frequency of RFX4 expression between ependymal and astrocytic tumors was significant ($P < 0.05$). Statistical analysis was performed by Fisher's exact probability test. IHC, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction.

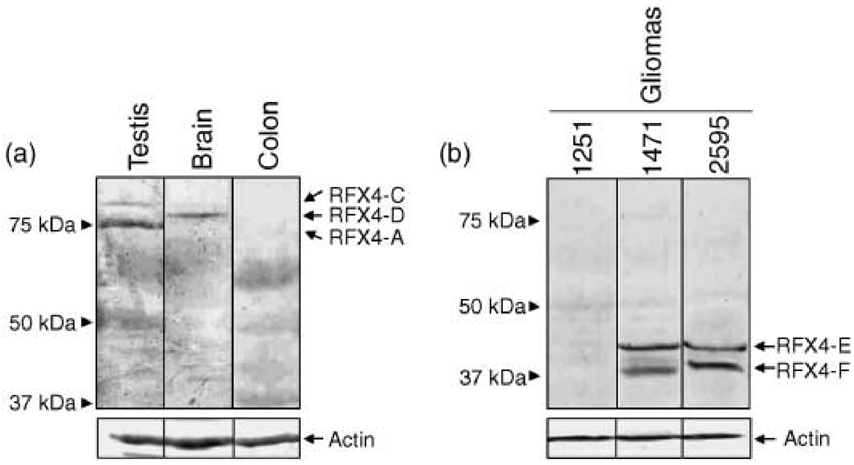

Fig. 4. Western blot analysis in normal tissues (a) and gliomas (b) using DC28 mAb. We used 7.5% and 10% gels in A and B, respectively, to separate protein lysates in sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Western blot analysis

To investigate RFX4 protein expression in the testis and brain, we performed Western blot analysis using DC28 mAb produced against RFX4-D C-terminal half protein. DC28 mAb recognized approximately 75-kDa and 85-kDa bands corresponding to RFX4-A and -C, respectively, in the testis and an approximately 80-kDa band corresponding to RFX4-D in the brain (Fig. 4a). No RFX4-B was detected.

In gliomas, DC28 mAb recognized approximately 42-kDa and 38-kDa bands corresponding to RFX4-E and -F isoforms, respectively (Fig. 4b). Those bands were observed only in gliomas, not in the normal testis or brain. The isoform RFX4-D was undetectable irrespective of its mRNA expression in glioma specimens (Fig. 2b).

IHC

In the IHC analysis using DC28 mAb, positive staining was observed in the nuclei of spermatocytes in the testis and some cells in the subependymal zone (SEZ) of two peritumoral normal brain tissue samples (Fig. 5a).

In glioma, positive staining was observed in 15 of 61 (25%) specimens that overexpressed *RFX4* mRNA, and staining was seen predominantly in the nuclei of tumor cells (Fig. 5b and Table 1). A high frequency of RFX4 protein expression was observed in ependymal tumors (Table 1).

RFX4 antibody response in glioma patients

Sera from 58 glioma patients and 70 healthy donors were analyzed for RFX4 antibody by ELISA using recombinant RFX4-D N- and C-terminal proteins. Figure 6a shows the titration curves for RFX4 antibody-positive and -negative sera, and Table 2 summarizes the results. IgG antibody against the RFX4-D C-terminus was detected in 2 of 52 (4%) astrocytic and 1 of 6 (17%) ependymal tumor patients. *RFX4* mRNA overexpression was observed by real-time RT-PCR (Fig. 3b) and protein expression was observed by IHC (Fig. 5b) in tumor specimens from three seropositive patients. The antibody response was observed against RFX4-E and -F in lysate extracted from glioma specimens by Western blot analysis in those three patients (Fig. 6b). No antibody was found against the RFX4-D N-terminus in the sera from gliomas. No antibody response to the RFX4-D N- or C-terminus was observed in 70 healthy donors. The results of real-time RT-PCR, IHC and antibody production are summarized in Table 3.

Table 2. RFX4 antibody response in glioma patients

Sera	Positive/examined
Healthy donors	0/70 (0%)
Astrocytic tumor	2/52 (4%)
Pilocytic astrocytoma	0/4 (0%)
Diffuse astrocytoma	1/10 (10%)
Anaplastic astrocytoma	0/15 (0%)
Glioblastoma	1/23 (4%)
Ependymal tumor	1/6 (17%)
Ependymoma	0/4 (0%)
Anaplastic ependymoma	1/2 (50%)

Table 3. Summary of RFX4 expression in gliomas and antibody production in patients

RFX4 expression		*n*	Antibody positive/ Sera examined
mRNA	*Protein		
+	+	14	3/9
+	−	3	0/3
−	+	1	0/0
−	−	43	0/22
ND	ND	24	0/24

*RFX4 protein expression was examined by immunohistochemistry. ND, not done.

Discussion

For *RFX4*, two alternatively spliced variants *RFX4-A* and *-B* were reported in the testis, and recently, a brain-specific variant, *RFX4_v3*, has been described.[10–12] In this study, we identified transcript variants *RFX4-C*, *-D*, *-E*, and *-F* by RACE analysis, and demonstrated that *RFX4-A*, *-B* and *-C* mRNAs were expressed only in the testis, and *RFX4-D* mRNA, which is homologous to *RFX4_v3*, was expressed only in the brain in normal tissues, as determined by RT-PCR. In tumors, both *RFX4-E* and *-F* mRNAs, in addition to

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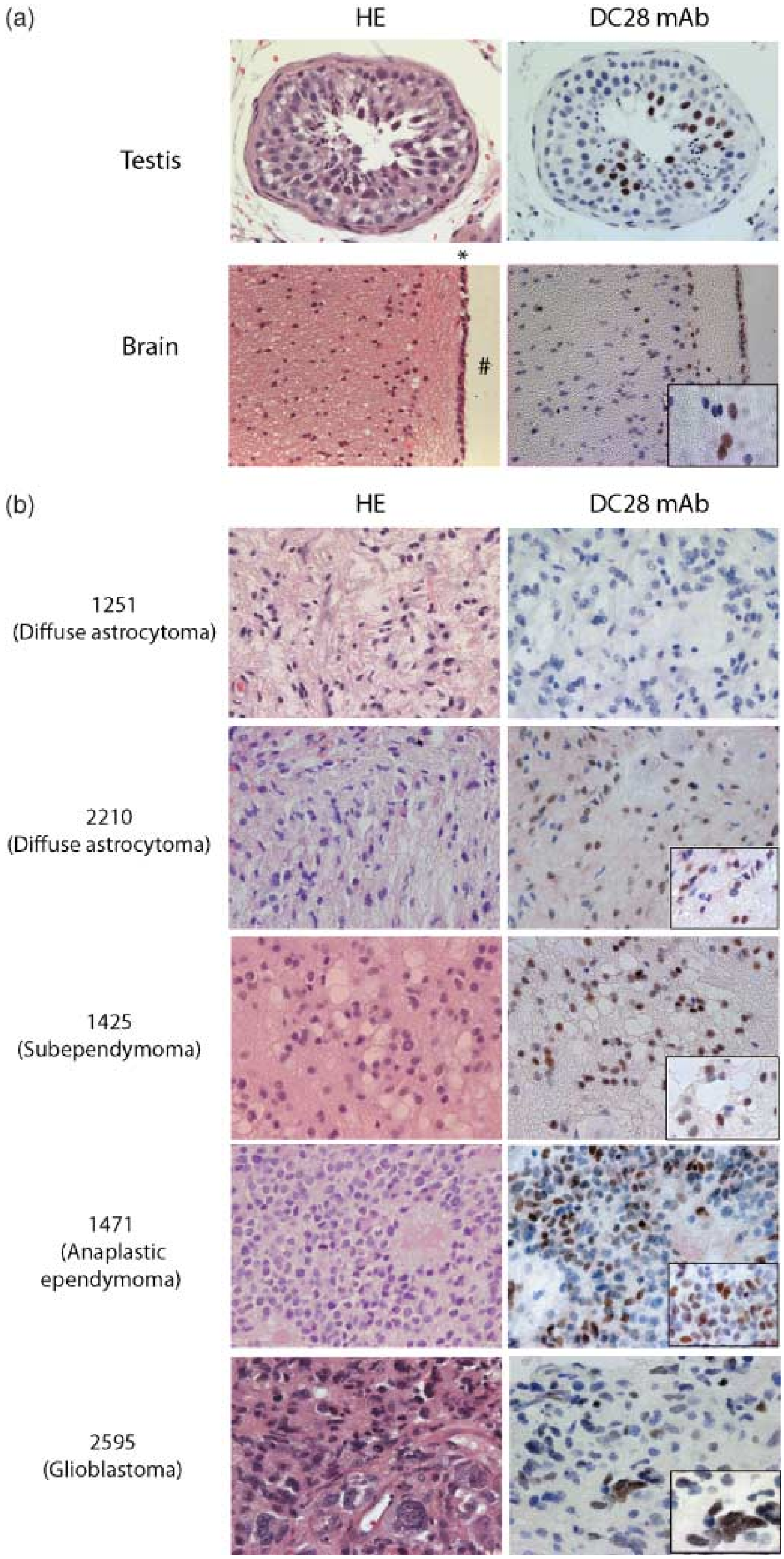

Fig. 5. (a) Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) of RFX4 using DC28 monoclonal antibody (mAb) in normal testis and brain. Original magnification, ×200; inset, × 1000. *, ependymal cell layer; #, inferior horn of lateral ventricle. (b) H&E and IHC of RFX4 using DC28 mAb in gliomas. Original magnification, × 400. Inset, original magnification, × 1000 for RFX4 protein-positive specimens.

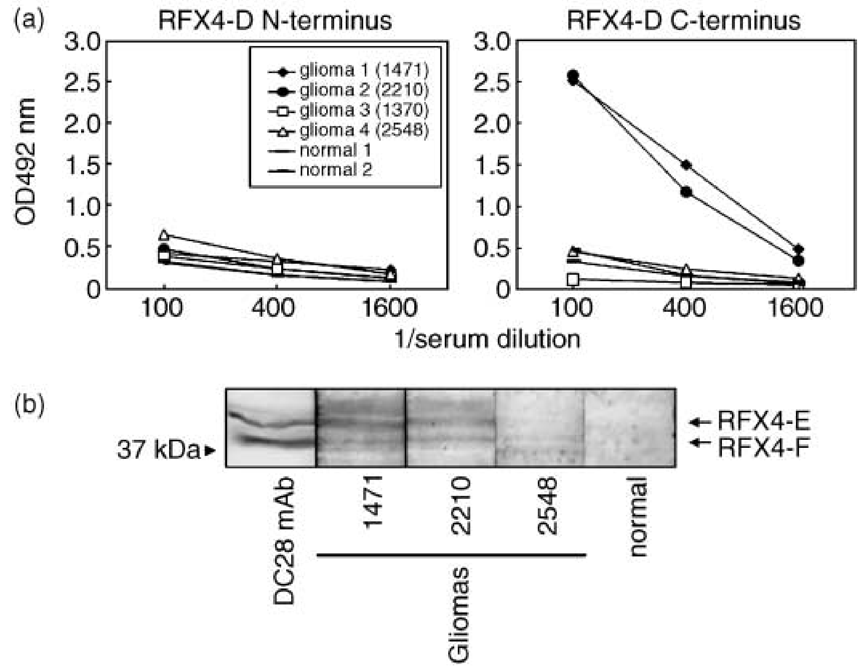

Fig. 6. (a) Antibody production against the recombinant RFX4-D N- and C-terminal proteins in glioma patients. Each line represents the titration curve of a serially diluted serum sample from four glioma patients and two healthy donors. (b) Antibody response in glioma patients against RFX4-E and -F proteins from glioma tissue lysate. Serum from patient 2548 was included as a negative control. *RFX4* mRNA overexpression was detected by real-time reverse transcription polymerase chain reaction (Fig. 3b) and protein expression by immunohistochemistry (Fig. 5C) in patients 1471 and 2210, but not patient 2548.

RFX4-D mRNA, were expressed in three glioma specimens, as determined by RACE and RT-PCR analyses. Expression of *RFX4* mRNA was not observed in other tumors, including lung, esophageal, stomach, colon and liver cancers. Quantitative real-time RT-PCR using common primer pairs detecting all of the variant transcripts showed that high expression in normal testis resulted from amplification of *RFX4-A*, *-B* and *-C*, low expression in the brain (1% compared to the expression in testis) resulted from amplification of *RFX4-D*, and extremely high-level expression in some gliomas resulted from amplification of *RFX4-D*, *-E* and *-F*. Western blot analysis using DC28 mAb produced against recombinant RFX4-D C-terminus protein showed expression of RFX4-A and -C proteins, but not RFX4-B protein in the testis, and expression of RFX4-D protein in the brain. Moreover, expression of RFX4-E and -F proteins, but not RFX4-D protein was observed in gliomas. IHC analysis using DC28 mAb showed positive staining in the nuclei of spermatocytes in the testis and glioma cells. A discrepancy between mRNA and protein expression was observed in some glioma specimens. It could be due to the heterogeneity of samples in the same tumor specimens taken for IHC and RT-PCR.

The lack of detection of RFX4-B using DC28 mAb by Western blot was confirmed with three independent tests. This could simply be due to a low level of expression of RFX4-B in the testis or a lack of DC28 mAb epitope in the isoform. RFX4-E and -F protein expression was observed at high frequency in gliomas. Its frequency was extremely high in ependymal tumors (56%) compared with astrocytic tumors (20%). We observed no RFX4 expression in three central neurocytomas, two gangliocytomas or five medulloblastomas. The finding that *RFX4-D* mRNA was expressed in some SEZ cells in normal brain and gliomas may imply that those cells in the normal brain could be the targets of a tumor initiation event in some gliomas. It is unknown whether the expression of RFX4-E and -F isoforms is causally related to gliomagenesis. Alternatively, those spliced forms could be the products of malignancy. RFX4 is a transcription factor that binds to DNA by way of a highly conserved winged helix DBD and exerts its effect by forming a dimer, similar to other RFX members.[8–10] Recently, brain-specific RFX4, which is homologous to RFX4-D, was reported to be expressed dynamically in the developing central nervous system from the neural plate stages and crucial for early brain development in the mouse.[12] *RFX4_v3* -/- mice could not form dorsal midline brain structure and resulted in perinatal death. In addition, the mouse RFX4 is expressed in the suprachiasmatic nucleus, which controls the circadian clock, and is induced by exposure to light.[13] The functions of RFX4 variants in the testis and brain, and their relevance to gliomagenesis should be investigated.

Antibody against the RFX4-D C-terminus protein, but not the N-terminus protein, was detected in the serum of 3 of 58 (5%) glioma patients by ELISA. Antibody production appeared to be induced by overexpressed RFX4-E and -F proteins, suggesting their immunogenicity in glioma patients. It has been reported that antibody against tumor antigens was rarely detected.[15] Approximately 5–10% of melanoma patients produced antibody against NY-ESO-1 antigen, which appears to be the most immunogenic cancer/testis antigen.[15–17] Almost no patients with tumors that expressed the cancer/testis antigens MAGE and SSX, or differentiation antigens MART-1 and gp100, showed an antibody response to these antigens.[15] However, it has been shown that glioma patients frequently produce antibodies against PHF3[18] and SOX6,[19] which were identified as glioma antigens by serological identification of antigens by recombinant expression cloning (SEREX). About half of NY-ESO-1-seropositive patients were shown to develop a CD8 T-cell response.[20]

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T-cell responses in RFX4-seropositive patients should be investigated.

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