or oligodendroglial tumors who did not receive radiotherapy or chemotherapy after surgery (16).

Mutations of the *IDH1/2* genes are common events in gliomas (17), especially among grade II gliomas, where *IDH1* mutations are observed in 70-80% of cases (11,17,18). Glioblastomas and anaplastic astrocytomas (WHO grade III) with IDH1/2 mutations have more favorable prognoses than those with a wild-type phenotype (17). Several studies have indicated that *IDH1/2* mutations are significantly associated with positive prognosis and chemosensitivity in low-grade gliomas (19,20), whereas others have reported that *IDH1/2* mutations were not associated with prognosis (21,22). Thus, the prognostic or predictive values of these genetic markers in grade II gliomas remain controversial.

In the present study, the clinicopathological factors, including age, Karnofsky performance status (KPS), histology, extent of resection, radiotherapy, chemoradiotherapy, largest tumor diameter, and MIB-1 staining index, as well as *IDH1/2* mutations and 1p/19q codeletion, were analyzed in grade II gliomas and correlated with the clinical course of the patients. Oligodendroglial tumors, age <40 years, initial KPS ≥80, and *IDH1/2* mutations were favorable prognostic factors for PFS and OS. The IDH1/2 mutation was a predictive factor of response to chemoradiotherapy in grade II gliomas.

Materials and methods

Patients and tissue collections. The data were collected from 72 patients who were found with WHO grade II gliomas at the first surgery. These included 49 diffuse astrocytomas and 23 oligodendroglial tumors, including 4 oligodendrogliomas and 19 oligoastrocytomas (male-female, 40:32; median age, 39.0 years). These consecutive cases were diagnosed and treated between 1991 and 2010 at the National Cancer Center Hospital in Japan. The clinical records of the patients were reviewed, and the data on the extent of tumor resection were obtained from the surgical report. Total or subtotal resection was defined as the removal of 90% or more of the tumor based on the surgeon's clinical report. Fifty-eight patients (80.6%) underwent initial surgeries followed by radiotherapy (22.2%) or chemoradiotherapy with ACNU (58.3%). Three patients with total or subtotal removal and 11 with partial resection or biopsy (19.4%) were followed-up without radiotherapy until progressive disease. Of the remaining patients, those who underwent initial treatment between 1991 and 2006 were treated with chemoradiotherapy and those treated between 2007 and 2010 underwent radiotherapy alone based on our treatment protocols. The radiation doses were 60 Gy before 2006 and 54 Gy after 2007. The chemotherapy in the diffuse astrocytoma cases consisted of ACNU administered twice during radiotherapy and 3 additional doses every 2 months after radiotherapy. The patients with oligodendroglial tumors received ACNU + VCR (vincristine) twice during radiotherapy, and thereafter, PAV [ACNU + VCR + PCZ (procarbazine)] was administered in 3 cycles every 2 months after radiotherapy. Each patient was worked up by MRI every 3-4 months until 2 years from the initial treatment and then every 6 months after 2 years. Progression was determined when the MRI showed a new enhancing lesion with Gd-DTPA, a new high intensity lesion or an obvious increased lesion (at least 20% larger than previous MRI in diameter) on T2/FLAIR images. Clinical deterioration of a patient was also determined as progression.

The formalin-fixed paraffin-embedded tumor samples and frozen specimens, when available, were collected from the primary resection for all the patients who underwent surgery in the National Cancer Center and whenever possible for those operated at other hospitals. The samples were examined for *IDH1/2* mutations and 1p/19q codeletion only when sufficient material for DNA extraction was available at either the primary or secondary resection. The study was approved by the internal review board of the National Cancer Center. The detailed information for all the 72 patients is listed in Table I.

Hematoxylin and eosin staining and immunohistochemical staining for MIB-1 and IDH1. The surgical specimens were fixed in 10% formalin and embedded in paraffin. The hematoxylin and eosin-stained specimens were examined to determine the histological tumor type. The multiple serial sections were subjected to immunohistochemical analyses (IHC) to visualize local staining. Antigen retrieval was carried out by exposing the tissue sections to 15 min of microwave heating in 0.1-mol/l sodium citrate (pH 6.0). This was followed with immunostaining of the specimens with the streptavidin-biotin-peroxidase complex method (Vectastain, Vector Laboratories, Inc., Burlingame, CA, USA). The samples were incubated in human monoclonal antibodies against MIB-1 (Dako, Tokyo, Japan). Positive immunostaining results were detected with the diaminobenzidine reaction, and the slides were subsequently counterstained with hematoxylin, dehydrated, cleared, and mounted.

Cell counting was performed with the aid of a light microscope (Olympus Corp., Tokyo, Japan). Cell counting was done at a magnification of x400. At least 200 tumor cells were counted, and the results were expressed as the mean of the counts obtained from 3 different locations within each specimen. The MIB-1-stained cells were also counted, and the percentage of the MIB-1-stained cells was calculated within the observed field and expressed as the MIB-1 index.

Human monoclonal antibodies specific against IDH1-R132H and IDH1-R132S were used to identify these 2 types of IDH1 mutations (Medical & Biological Laboratories, Tokyo, Japan). Positive immunostaining results were detected with the diaminobenzidine reaction, and the slides were subsequently counterstained with hematoxylin, dehydrated, cleared, and mounted. The positive granular cytoplasmic staining of the tumor cells was evaluated for mutant *IDH1* (23).

Extraction of nucleic acids. The tumor samples were immediately frozen in liquid nitrogen and stored at -80˚C. A peripheral blood sample was drawn from each patient and stored at -80˚C. Total DNA was extracted from either frozen tissue samples or paraffin-embedded specimens and from the patients' blood with a DNeasy Blood & Tissue kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions.

Sequencing of IDH1/2. A 129-base pair (bp) fragment of *IDH1* containing codon 132 or a 150-bp fragment of *IDH2* containing codon 172 was PCR amplified using the forward primer IDH1f (CGGTCTTCAGAGAAGCCATT) and reverse primer IDH1r (GCAAAATCACATTATTGCCAAC) for *IDH1* and the forward primer IDH2f (AGCCCATCATCTGCAAAAAC) and

reverse primer IDH2r (CTAGGCGAGGAGCTCCAGT) for *IDH2* (18). The thermocycling conditions consisted of 5 min at 95°C, 35 cycles for 30 sec at 95°C, 40 sec at 56°C, and 50 sec at 72°C, followed by 10 min at 72°C. For confirmation, the forward primer IDH1fc (ACCAAATGGCACCATACGA) and reverse primer IDH1rc (TTCATACCTTGCTTAATGGGTGT) generating a 254-bp fragment and the forward primer IDH2fc (GCTGCAGTGGGACCACTATT) and reverse primer IDH2rc (TGTGGCCTTGTACTGCAGAG) generating a 293-bp fragment were used for amplification with the same thermocycling conditions (24). After the purification of the PCR products using the QIAquick PCR Purification kit (Qiagen), DNA sequencing for the *IDH1/2* gene was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using the same primers as for PCR.

1p and 19q status by fluorescence in situ hybridization. For fluorescence *in situ* hybridization (FISH), the tumor sections were deparaffinized in Hemo-De (Falma, Tokyo, Japan), dehydrated with 100% ethanol, and digested using a Paraffin Pretreatment kit (Vysis-Abbott, Tokyo, Japan) according to the manufacturer's protocol. Each section was hybridized with LSI 1p36/1q25 and LSI 19q13/19p13 probes (Vysis-Abbott). The probes and target DNA were denatured individually at 72°C for 5 min, followed by 2 overnight incubations at 37°C. Posthybridization washes were carried out in standard saline solution twice, and the sections were air-dried. The nuclei were counterstained with 4,6-diamidino-2-phenylindole. The sections were analyzed using a fluorescence microscope (Biorevo BZ-9000, Keyence, Japan).

The 1p or 19q deletions were considered present when the population of the cells with single 1p36 or single 19q13 was <50% of the cells with double 1p36 or double 19p13, respectively. At least 100 non-overlapping nuclei were counted per hybridization.

1p and 19q status by multiplex ligation-dependent probe amplification analysis. We used the SALSA P088 kit (MRC Amsterdam, The Netherlands) containing 16 1p probes (6 probe at 1p36), 8 19q probes, and 21 control probes specific to other chromosomes, including 2 probes for 19p. Information regarding the probe sequences and ligation sites can be found at http://www.mlpa.com. Multiplex ligation-dependent probe amplification (MLPA) analysis was performed as described previously (25,26). The 1p36 or 19q deletions were considered present when 5 of 6 markers for 1p36 and 5 of 8 markers for 19q in each chromosome arm had normalized ratios <0.75.

Statistical analysis. All the statistical analyses, including the Kaplan-Meier survival analysis, were performed using the JMP ver. 8 software (Tokyo, Japan). The multivariate analysis with Cox regression, which was used to assess the independent prognostic factors for all the 72 cases, was performed only for the variables with $p<0.1$ and which included the data obtained in the univariate analysis for all the patients. A similar analysis was performed for 58 cases with radiotherapy or chemoradiotherapy.

Results

Progression-free and OS. The PFS and median OS times for all the 72 grade II glioma patients were 5.8 and 10.3 years, respectively (male-female, 40:32; median age, 39.0 years; Table I).

Table I. Characteristics of patients with grade II gliomas.

Characteristic	No. of patients	Years	Percentage (%)
Sex			
Male	40		55.6
Female	32		44.4
Age (years)			
Median		39	
Range		21-75	
Histology			
Astrocytoma	49		68
Oligodendroglioma	4		6
Oligoastrocytoma	19		26
Extent of removal			
Total removal	12		16.7
Subtotal removal	2		2.8
Partial removal	27		37.5
Biopsy	31		43.1
Largest diameter of initial tumor (cm)			
<6	40		55.6
≥6	32		44.4
Initial KPS			
<80	4		5.6
≥80	68		94.4
MIB-1 index (%)			
Median	3		
IDH mutation			
Mutation	42		58.3
Wild-type	30		41.7
Loss of 1p/19q			
1p/19q codeletion	15		25.0
1p deletion	24		40.0
19q deletion	23		38.3
Initial radiotherapy			
+	58		80.6
-	14		19.4
Initial chemotherapy			
ACNU	44		61
TMZ	2		3
None	26		36
PFS (years)			
Median		5.8	
Overall survival (years)			
Median		10.3	

Table II. Univariate analyses of progression-free survival time and overall survival time of patients with grade II gliomas.

Variable	No.of cases	PFS (95% CI)	p-value (log-rank)	OS (95% CI)	p-value (log-rank)
Histology					
Astrocytoma	49	3.6 (2.1-7.7)	0.08	8.3 (4.2-NR)	0.04
Oligodendroglioma/ oligoastrocytoma	23	8.3 (4.3-14.4)		11.7 (8.1-18.2)	
Age					
<40 years	38	7.0 (3.6-9.3)	0.2	NR (8.0-NR)	0.02
≥40 years	34	3.1 (1.8-8.9)		4.3 (3.9-16.3)	
IDH mutation					
Mutation	42	8.4 (3.2-10.2)	0.04	16.3 (9.6-18.2)	0.004
Wild-type	30	3.3 (1.7-7.0)		4.5 (3.9-10.0)	
Extent of removal					
Total and subtotal removal	14	10.4 (2.5-14.4)	0.1	18.3 (4.1-18.3)	0.08
Partial removal and biopsy	58	4.3 (2.3-8.3)		10.0 (5.2-16.3)	
Largest diameter of initial tumor (cm)					
<6	40	7.7 (2.3-10.4)	0.2	10.0 (8.0-NR)	0.7
≥6	32	4.3 (2.1-8.3)		10.3 (5.1-16.3)	
Initial KPS					
<80	4	0.6 (0.4-8.4)	0.01	1.7 (0.5-10.3)	0.0006
≥80	68	6.8 (3.1-8.9)		11.7 (8.0-18.2)	
MIB-1 index					
<4%	33	8.1 (2.3-8.9)	0.6	9.6 (5.1-NR)	0.6
≥4%	21	4.3 (1.8-NR)		NR (3.9-NR)	
1p/19q					
1p/19q codeletion (+)	15	6.8 (2.2-NR)	0.4	11.7 (4.3-11.7)	0.2
1p/19q codeletion (-)	45	3.6 (2.3-8.4)		8.3 (4.4-NR)	
1p					
1p deletion	24	5.8 (2.5-9.3)	0.96	11.7 (4.2-11.7)	0.9
Intact	36	4.2 (2.1-10.2)		9.6 (4.4-NR)	
19q					
19q deletion	23	7.0 (4.2-9.3)	0.5	11.7 (4.5-11.7)	0.5
Intact	37	3.1 (1.9-10.2)		8.3 (3.9-NR)	
Initial radiotherapy					
+	58	4.3 (2.9-8.9)	0.98	8.3 (5.1-18.2)	0.2
-	14	7.7 (2.5-9.1)		11.7 (4.2-16.3)	
Initial treatment					
Radiotherapy alone	16	2.9 (0.7-4.3)	0.01	4.2 (2.7-5.1)	0.0002
Chemoradiotherapy	42	8.1 (3.2-10.2)		18.2 (8.1-18.2)	

NR, PFS or median survival time is not reached; CI, confident interval.

These patients were initially treated with surgery followed by radiotherapy (22.2%) or chemoradiotherapy (58.3%). The median follow-up time for all the 72 patients was 6.4 years, and it was 7.6 years for the patients treated with chemoradiotherapy (n=42) and 4.0 years for those who underwent radiotherapy alone (n=16).

Progression-free and OS times according to clinical factors. The univariate analysis (Table II) showed that the patients with oligodendroglial tumors (n=23) had longer OS than those with diffuse astrocytoma (n=49; p=0.04). The PFS and OS were 3.6 and 8.3 years, respectively, in the patients with diffuse astrocytoma, and 8.3 and 11.7 years, respectively, in the patients with oligodendroglioma or oligoastrocytoma (Fig. 1A and B). The patients younger than 40 years (n=38) had longer OS than those who were 40 years or older (n=34; p=0.02). The PFS and median survival time of the patients in the younger age groups were 7.0 years and still not reached, respectively, whereas the PFS and OS of the patients in the older age groups were 3.1 and 4.3 years, respectively. The patients with an initial KPS score ≥80 (n=68) had significantly longer OS (p=0.0006) and PFS (p=0.01) than those with a KPS score <80 (n=4). The PFS and OS of the patients with a KPS score ≥80 were 6.8 and 11.7 years, respectively, and those of the patients with a KPS score <80 were 0.6 and 1.7 years, respectively. The patients in the total or subtotal resection (≥90% removal) groups (n=14; median age, 34.0 years) tended to have longer OS than those in the partial (<90%) removal or biopsy groups (n=58; median age, 41.0; p=0.08). The PFS and OS were 10.4 and 18.3 years, respectively, in the patients in the total or subtotal resection groups and 4.3 and 10.0 years, respectively, in the patients in the partial resection or biopsy groups. The patients who were initially treated with chemoradiotherapy after surgery showed significantly longer PFS (p=0.01) and OS (p=0.0002) than those treated with radiotherapy alone (Fig. 1C and D). The PFS and OS of the patients who were initially treated with radiotherapy after surgery (n=16) were 2.9 and 4.2 years, respectively, and the PFS and OS of the patients who were initially treated with chemoradiotherapy after surgery (n=42) were 8.1 and 18.2 years, respectively. According to MIB-1 staining index, there was no significant difference of survival between groups with cut-off point at 4, 8 and 15% in our study.

Presence of 1p/19q codeletion, 1p deletion, and 19q deletion and survival. The presence of 1p/19q deletions was determined for 25 or 26 primary resections and for 7 or 2 secondary resection samples by MLPA or FISH, respectively. The 1p/19q codeletion was observed in 15.9% (7/44) of the astrocytomas and 50% (8/16) of the oligodendroglial tumors. The OS of the patients with 1p/19q codeletion was 11.7 years, and the OS of those without 1p/19q codeletion was 8.3 years (p=0.2; Fig. 1E and F). In the patients with astrocytic tumors, the median survival time of those with 1p/19q codeletion was not reached and the OS of those without 1p/19q codeletion was 6.3 years (p=0.5). The OS of the patients with 1p/19q codeletion was 11.7 years, and the OS of those without 1p/19q codeletion was 10.3 years in the oligodendroglial tumors (p=0.5). The presence of 1p/19q codeletion, 1p deletion, or 19q deletion was not correlated with the PFS or OS time (Table II).

Table III. Mutation of *IDH1/2*.

	Diffuse astrocytoma (%)	Oligodendroglioma (%)	Oligoastrocytoma (%)
IDH1/2 mutation by sequence			
IDH1 R132H	13 (26.5)	2 (50.0)	5 (26.3)
IDH1 R132S	1 (2.0)	0 (0.0)	0 (0.0)
IDH2 R172K	1 (2.0)	0 (0.0)	0 (0.0)
Wild-type	15 (30.6)	1 (25.0)	2 (10.5)
IDH mutation by IHC			
IDH1 R132H	8 (16.3)	1 (25.0)	11 (57.9)
IDH1 R132S	0 (0.0)	0 (0.0)	0 (0.0)
Mutation (-)	11 (22.4)	0 (0.0)	1 (5.3)
Total	49 (100)	4 (100)	19 (100)
Mutation	23 (46.9)	3 (75.0)	16 (84.2)
Wild-type	26 (53.1)	1 (25.0)	3 (15.8)

IHC, immunohistochemical staining.

IDH1/2 mutations and survival in the whole series. *IDH1/2* mutations were determined in 55 samples at the primary resection and 17 at the secondary resection by IHC alone for 32 cases (44.4%) and by direct sequencing in 40 cases (55.6%). IDH1/2 mutations were found in 46.9% (23/49) of the astrocytomas, 84.2% (16/19) of the oligoastrocytomas, and 75.0% (3/4) of the oligodendrogliomas (Table III).

The patients with *IDH1/2* mutations (n=42) had longer PFS (p=0.04) and OS (p=0.004) than those without IDH1/2 mutations (n=30; Table II). The PFS and OS of the patients with *IDH1/2* mutations were 8.4 and 16.3 years, respectively, and the PFS and OS of the patients without *IDH1/2* mutations were 3.3 and 4.5 years, respectively (Fig. 1G and H). The diffuse astrocytoma patients with *IDH1/2* mutations (n=23) tended to have longer survival times than those without *IDH1/2* mutations (n=26), although the difference was not significant (p=0.08). The median survival time of the diffuse astrocytoma patients with *IDH1/2* mutations was not reached and that of the diffuse astrocytoma patients without *IDH1/2* mutations was 4.4 years. The oligodendroglial tumor patients with *IDH1/2* mutations also tended to have longer, though not significant, survival times (p=0.1).

The survival of the patients with *IDH1/2* mutations and 1p/19q codeletion was longer than that of the patients with neither *IDH1/2* mutations nor 1p/19q codeletion (11.7 vs. 4.4 years, respectively), although the difference did not reach statistical significance (p=0.1). Furthermore, a combined *IDH1/2* and 1p/19q status did not correlate with the PFS and OS of the patients who were initially treated with chemoradiotherapy after surgery regardless of the histological tumor type.

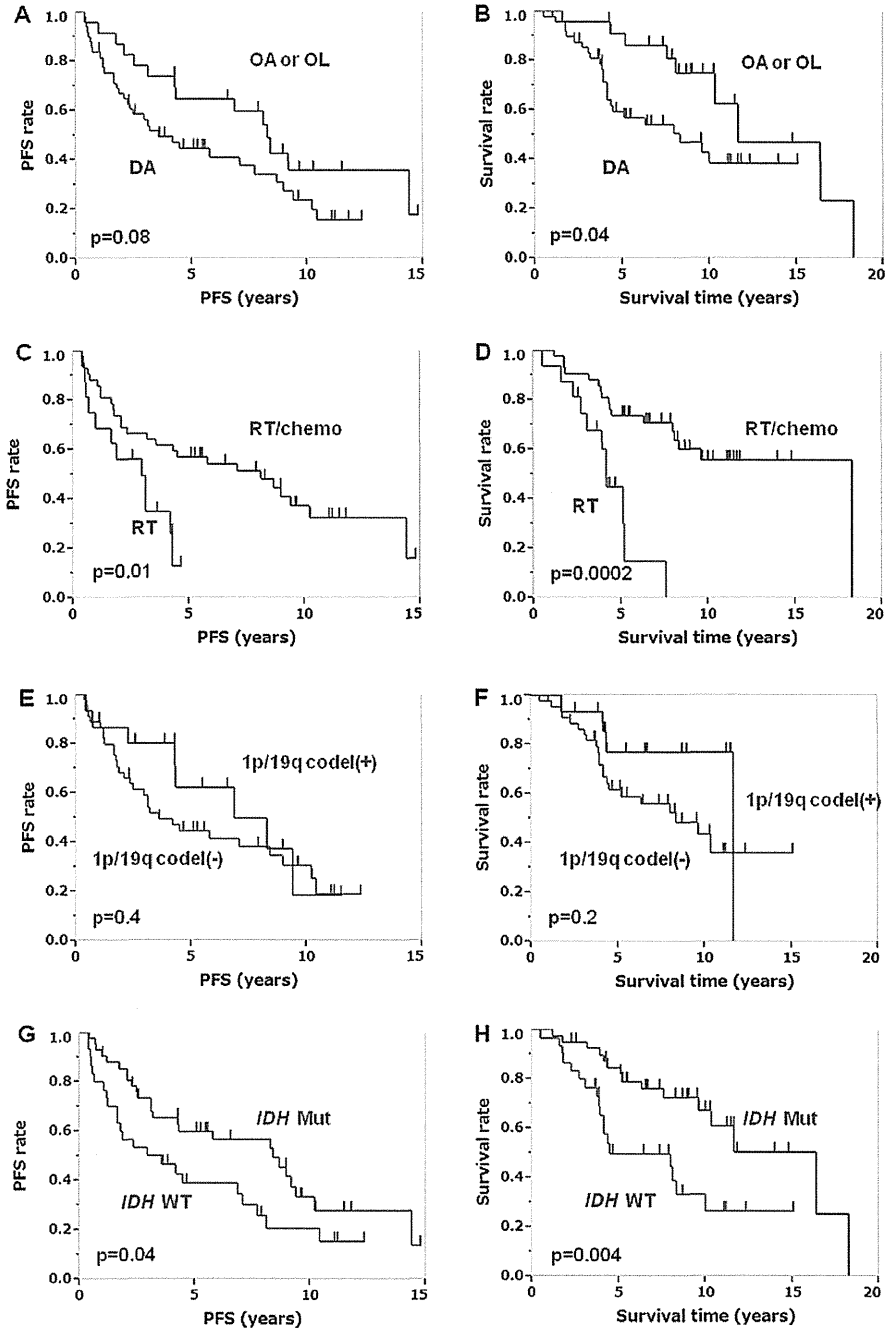

Figure 1. Kaplan-Meier survival curves of the patients with WHO grade II gliomas grouped according to genetic and clinical factors associated with overall survival (OS) and progression-free survival (PFS) by univariate analysis. The survival estimates were calculated according to the following variables: (A) PFS, diffuse astrocytoma (DA) versus oligodendroglial tumors (OA or OL); (B) OS, diffuse astrocytoma (DA) versus oligodendroglial tumors (OA or OL); (C) PFS, radiotherapy (RT) versus chemoradiotherapy (RT/chemo); (D) OS, radiotherapy (RT) versus chemoradiotherapy (RT/chemo); (E) PFS, 1p/19q codeletion (codel) (+) or (-); (F) OS, 1p/19q codeletion (codel) (+) or (-); (G) PFS, IDH1/2 mutation (mut) or wild-type (WT); and (H) OS, IDH1/2 mutation (mut) or wild-type (WT).

In the total or subtotal resection group, the patients with *IDH1/2* mutations had longer OS than those without *IDH1/2* mutations (p=0.04; Fig. 2A). The OS of the patients with *IDH1/2* mutations (n=6, 2 diffuse astrocytomas, 3 oligoastrocytomas, and 1 oligodendrogliomas) was 18.2 years; to date, 5 are still alive and 1 is dead. The OS of the patients without *IDH1/2* mutations (n=8, 7 astrocytomas and 1 oligoastrocytoma) was 8.0 years. In the partial resection or biopsy group, the patients with *IDH1/2* mutations had longer OS than those without *IDH1/2* mutations in the partial resection or biopsy group (p=0.01; Fig. 2B). The OS of the patients with *IDH1/2* mutations (n=36, 21 diffuse astrocytomas, 13 oligoastrocytomas, and 2 oligodendrogliomas)

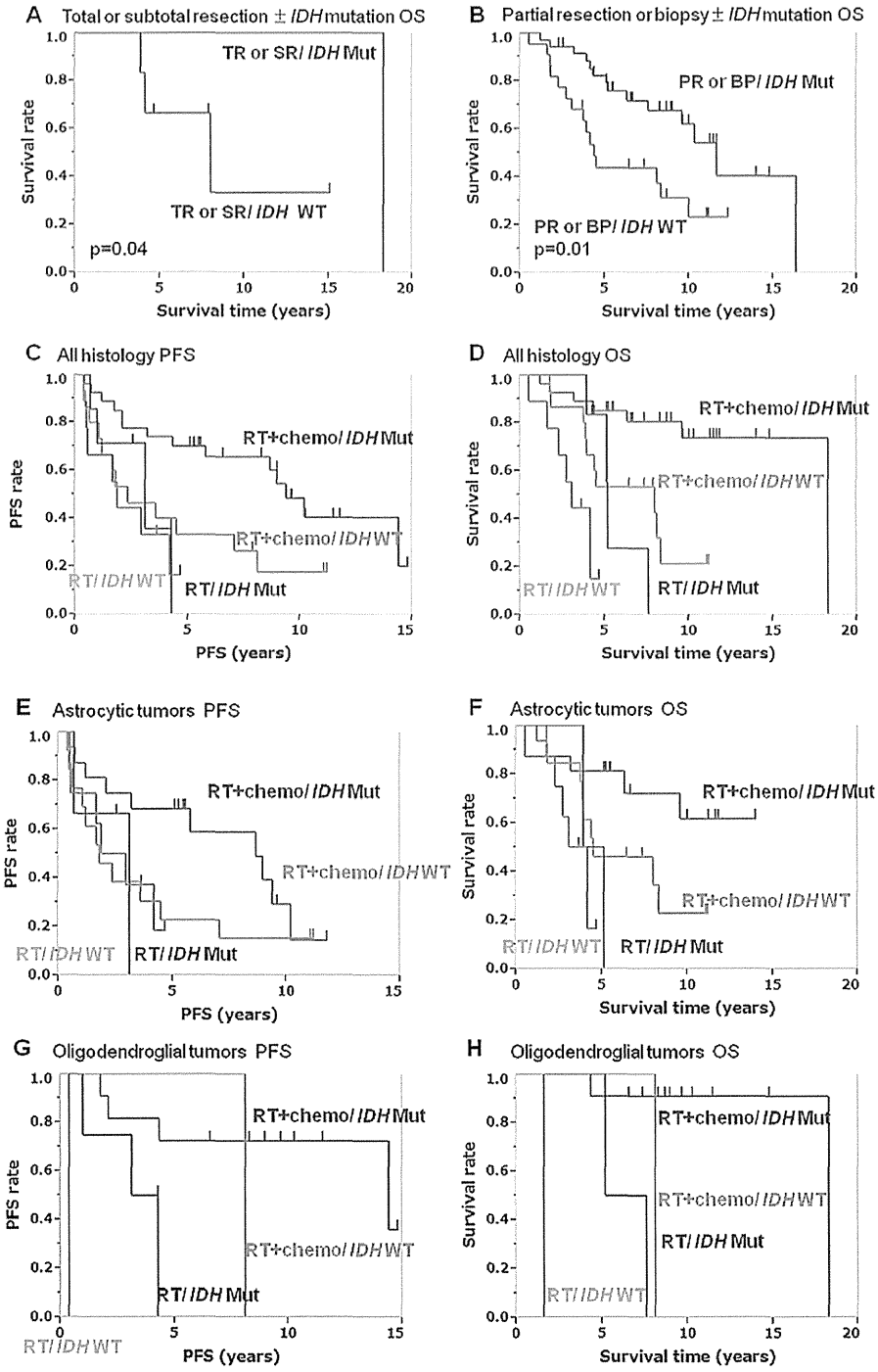

Figure 2. (A and B) Kaplan-Meier survival curves of the patients in the total or subtotal tumor resection (TR or SR) (A) and partial resection or biopsy (PR or BP) (B) groups according to the *IDH1/2* status. (C-H) Kaplan-Meier survival curves of the patients who were initially treated with radiotherapy (RT) and chemotherapy (chemo) or radiotherapy alone according to *IDH1/2* status associated with the overall survival (OS) and progression-free survival (PFS) by univariate analysis: (C) PFS of all the WHO grade II gliomas, (D) OS of all the WHO grade II gliomas, (E) PFS of the diffuse astrocytomas, (F) OS of the diffuse astrocytomas, (G) PFS of the oligodendroglial tumors, (H) OS of the oligodendroglial tumors.

was 11.7 years, and that of the patients without *IDH1/2* mutations in these groups (n=22, 19 diffuse astrocytomas, 2 oligoastrocytomas, and 1 oligodendroglioma) was 4.4 years.

IDH1/2 mutations and survival in the patients who underwent chemoradiotherapy after surgery. Among the grade II glioma patients who were initially treated with chemoradiotherapy

Table IV. PFS and OS in patients with radiotherapy or chemoradiotherapy according to IDH1/2 status.

Variable		No. of cases	PFS (95% CI)	OS (95% CI)
All grade II gliomas				
RT+chemo	Mut (+)	27	9.3 (4.3-NA)[a,b]	18.2 (9.6-18.2)[c,d]
RT+chemo	Mut (-)	15	2.3 (0.6-7.0)[a-,b]	8.0 (3.8-8.3)[c,d]
RT only	Mut (+)	7	3.1 (0.7-4.3)[b]	5.1 (3.9-7.5)[b,d,e]
RT only	Mut (-)	9	1.9 (0.4-4.2)	3.1 (0.5-4.2)[b,e]
Diffuse astrocytoma				
RT+chemo	Mut (+)	16	8.6 (2.1-10.2)[f]	NR (6.3-NR)[g,h]
RT+chemo	Mut (-)	13	1.8 (0.6-4.5)[f]	4.5 (3.8-NR)[g,h]
RT only	Mut (+)	3	3.1 (0.7-3.1)[f]	4.5 (3.9-5.1)[h]
RT only	Mut (-)	8	2.4 (0.5-NR)	3.6 (0.5-NR)
Oligodendroglioma/oligoastrocytoma				
RT+chemo	Mut (+)	11	14.4 (2.1-NR)[e]	18.2 (NR)[a]
RT+chemo	Mut (-)	2	8.1 (NR)[e]	8.1 (NR)[a]
RT only	Mut (+)	4	3.7 (1.0-4.3)[e,i]	6.3 (5.1-7.5)[a,i]
RT only	Mut (-)	1	0.4 (NR)[i]	1.6 (NR)[i]

NR, PFS or median survival time is not reached; CI, confident interval; RT, radiotherapy; chemo, chemotherapy; mut, mutation. [a]p=0.02; [b]p=0.01; [c]p=0.004; [d]p=0.008; [e]p=0.03; [f]p=0.1; [g]p=0.06; [h]p=0.07; [i]p=0.05.

Table V. Multivariate analyses of PFS and OS of patients with all grade II gliomas.

Variable	No. of cases	PFS hazard ratio (95% CI)	PFS p-value (Cox)	OS hazard ratio (95% CI)	OS p-value (Cox)
Histology					
Diffuse astrocytoma	49	1	0.1	1	0.02
Oligodendroglioma/ oligoastrocytoma	23	0.576 (0.262-1.186)		0.290 (0.086-0.815)	
IDH mutation					
Wild-type	31	1	0.08	1	0.01
Mutation	41	0.558 (0.289-1.068)		0.365 (0.155-0.819)	
Age (years)					
≥40	34	1	0.5	1	0.02
<40	38	0.802 (0.440-1.460)		0.400 (0.175-0.877)	
Extent of removal					
Partial removal and biopsy	58	1	0.1	1	0.2
Total and subtotal removal	14	0.556 (0.222-1.217)		0.463 (0.107-1.403)	
Initial KPS					
<80	4	1	0.01	1	0.0002
≥80	68	0.179 (0.063-0.640)		0.045 (0.011-0.198)	

CI, confident interval.

after surgery, those with *IDH1/2* mutations had significantly longer PFS and OS than those without *IDH1/2* mutations (PFS: p=0.02, OS: p=0.004; Fig. 2C and D; Table IV).

An important finding is that the patients who were initially treated with chemoradiotherapy after surgery and had *IDH1/2* mutations showed significantly longer PFS and OS than those

Table VI. Multivariate analyses of PFS and OS of patients with all grade II gliomas with radiotherapy ± chemotherapy.

Variable	No. of cases	PFS hazard ratio (95% CI)	PFS p-value (Cox)	OS hazard ratio (95% CI)	OS p-value (Cox)
Histology					
Diffuse astrocytoma	40	1	0.2	1	0.2
Oligodendroglioma/ Oligoastrocytoma	18	0.549 (0.209-1.290)		0.490 (0.133-1.445)	
IDH mutation					
Wild-type	24	1	0.05	1	0.01
Mutation	34	0.467 (0.215-0.999)		0.316 (0.117-0.793)	
Age (years)					
≥40	28	1	0.5	1	0.5
<40	30	0.758 (0.362-1.559)		0.745 (0.300-1.808)	
Extent of removal					
Partial removal and biopsy	47	1	0.03	1	0.08
Total and subtotal removal	11	0.364 (0.118-0.918)		0.356 (0.080-1.120)	
Initial treatment					
Radiotherapy alone	16	1	0.04	1	0.002
Chemoradiotherapy	42	0.408 (0.182-0.948)		0.198 (0.073-0.529)	

CI, confident interval.

treated with radiotherapy alone with *IDH1/2* mutations. The PFS and OS of the patients with *IDH1/2* mutations who were initially treated with chemoradiotherapy after surgery (n=27) were 9.3 and 18.2 years, respectively, and the PFS and OS of those treated with radiotherapy alone with *IDH1/2* mutations (n=7) were 3.1 and 5.1 years, respectively (PFS, p=0.01; OS, p=0.008). In the oligodendroglial tumors, the PFS and OS of the patients with *IDH1/2* mutations who were initially treated with chemoradiotherapy (n=11) were 14.4 and 18.2 years, respectively, and the PFS and OS of those treated with radiotherapy alone with *IDH1/2* mutations (n=4) were 3.7 and 6.3 years, respectively (PFS: p=0.03, OS: p=0.02; Fig. 2G and H). Similar tendencies, although not reaching statistical significance, were observed in the astrocytic tumors (PFS: p=0.1, OS: p=0.07; Fig. 2E and F).

The *IDH1/2* status had no impact on the PFS of all the grade II glioma or diffuse astrocytoma patients who underwent radiotherapy alone. No significant difference in PFS was observed between the radiotherapy and chemoradiotherapy groups in the grade II glioma patients without *IDH1/2* mutations. Chemoradiotherapy did not prolong the PFS of the patients without *IDH1/2* mutations in the astrocytic and oligodendroglial tumors.

Multivariate analysis. Oligodendroglial tumors (hazard ratio (HR)=0.29, p=0.02), age <40 years (HR=0.40, p=0.02), initial KPS ≥80 (HR=0.045, p=0.0002), and *IDH1/2* mutations (HR=0.37, p=0.01) were favorable prognostic factors for OS time, as determined by the multivariate analysis, of the 72 patients included in the study (Table V). The *IDH1/2* mutation status was not a prognostic factor for PFS when all the patients were considered, including those who did not undergo initial radiotherapy or chemotherapy (p=0.08). In contrast, total or subtotal tumor resection (HR=0.36, p=0.03), chemoradiotherapy (HR=0.41, p=0.04), and *IDH1/2* mutations (HR=0.47, p=0.05) were favorable prognostic factors for PFS, as determined by the multivariate analysis, of the patients who were initially treated with radiotherapy or chemoradiotherapy (Table VI). Histological appearance was not a prognostic marker for PFS in this series (p=0.2) compared with *IDH1/2* mutations (p=0.05).

Discussion

WHO grade III and IV astrocytomas with *IDH1/2* mutations have more favorable prognoses than those with wild-type *IDH1/2* (17). *IDH1/2* mutations, 1p/19q codeletion, and *MGMT* promoter methylation are pivotal prognostic factors in anaplastic oligodendroglial tumors treated with radiotherapy or chemoradiotherapy (EORTC 26951) (27). However, the impact of *IDH1/2* mutations and/or 1p/19q codeletion as biomarkers in grade II gliomas remains controversial. The present study was therefore aimed at identifying prognostic and/or predictive factors in grade II gliomas.

The presence of IDH1/2 mutations is a favorable prognostic marker for OS. The results of the univariate analysis revealed that the presence of *IDH1/2* mutations was a prognostic factor of longer OS (p=0.004) and PFS (p=0.04) in the entire patient cohort and among the patients who underwent with or without radiation therapy after initial surgery with or without chemotherapy. The multivariate analysis revealed that the presence of

IDH1/2 mutations was associated with prolonged PFS (p=0.05) and OS (p=0.01) in the patients who initially underwent radiotherapy with or without chemotherapy. Our results suggest that *IDH1/2* mutations may be involved in the response to genotoxic therapy, such as radiotherapy or chemotherapy, and may act as a prognostic factor for chemotherapy or radiotherapy in grade II gliomas. There are currently increasing numbers of reports showing that *IDH1/2* mutations are prognostic markers for several malignancies, including grade II gliomas. Houillier *et al* (19) reported that the presence of *IDH1/2* mutations is a significant prognostic marker for OS and chemosensitivity in low-grade glioma patients who were initially treated with temozolomide (TMZ) before any other treatment except surgery. Hartmann *et al* (16) reported that the *IDH1* mutation was a prognostic factor for PFS and OS in grade II glioma patients who underwent radiotherapy or chemotherapy after surgery. In our study, the presence of *IDH1/2* mutations was demonstrated by multivariate analysis to be a favorable prognostic factor (p=0.01) for OS but not a prognostic marker for PFS (p=0.08) in whole cohort, which included 14 patients who did not receive initial radiotherapy. Our finding that *IDH1/2* status did not affect PFS was in line with the findings reported by Hartmann *et al* (16) or Houillier *et al* (16,19), who showed that *IDH1* mutations did not affect the PFS in grade II glioma patients who did not receive radiotherapy or chemotherapy alone after surgery. Kim *et al* (21) and Mukasa *et al* (22) reported that the presence of *IDH1/2* mutations was not a prognostic factor for the survival of patients with low-grade glioma in univariate or multivariate analyses. The treatment of those patients was not fully described in their reports.

The presence of IDH1/2 mutations is a predictive marker for PFS in the grade II glioma patients treated with chemoradiotherapy. The patients who were initially treated with chemoradiotherapy after surgery showed significantly longer OS (p=0.0002) and PFS (p=0.01) than those treated with radiotherapy alone in our study. Chemoradiotherapy significantly prolonged PFS and OS compared with radiotherapy alone in all the grade II gliomas with *IDH1/2* mutations (p=0.01 and 0.0008, respectively), diffuse astrocytoma (p=0.1 and 0.07, respectively), and oligodendroglial tumors (p=0.03 and 0.02, respectively) in the univariate analysis. Chemoradiotherapy was shown by multivariate analysis (p=0.04) to significantly prolong the PFS of grade II glioma patients carrying *IDH1/2* mutations who underwent radiotherapy with or without concomitant chemotherapy (p=0.04). In contrast, there were no differences in PFS between the radiotherapy and chemoradiotherapy groups among the grade II glioma patients without *IDH1/2* mutations in the univariate analysis. PFS did not differ by *IDH1/2* status in the grade II glioma patients who underwent radiotherapy alone. However, the present study was limited by the small number of samples and the differences in the follow-up periods between the radiation and chemoradiotherapy groups (4 and 7.6 years, respectively). A prospective study including a larger patient cohort is required to obtain conclusive evidence that the presence of *IDH1/2* mutations is a predictive marker for chemoradiotherapy in grade II gliomas. Nonetheless, our results suggest that *IDH1/2* mutation is a predictive marker for chemoradiotherapy in grade II glioma patients and indicate that these patients may benefit from concurrent chemotherapy and radiotherapy compared with patients who do not carry *IDH1/2* mutations.

Mutations in *IDH1/2* result in the acquisition of new enzymatic activity that enables the NADPH-dependent reduction of α-ketoglutarate to 2-hydroxyglutarate, and the mutation confers oncogenic properties (28). *IDH1* mutations are early events in the development of astrocytomas and oligodendrogliomas (11). Another possible function of *IDH1/2* mutations is the dominant-negative inhibition of the oxidative decarboxylation of isocitrate as a result of the formation of a wild-type/mutant heterodimer (29). Cellular IDH1 levels are associated with the protection from apoptosis and cell death after exposure to reactive oxygen species or ultraviolet B-induced phototoxicity and *IDH1/2* functions in cellular defense reactions (30). Glioma cells with *IDH1/2* mutations may be vulnerable to irradiation and chemotherapeutic agents, which might explain why *IDH1/2* mutations could be a predictive and prognostic marker for grade II gliomas in patients receiving chemoradiotherapy. Our findings warrant a prospective large-scale clinical study addressing the efficacy of chemoradiotherapy in grade II glioma patients in association with *IDH1/2* status.

Grade II glioma patients with wild-type IDH1/2 have poor prognoses even after total resection. The extent of resection of tumors has been reported to be significantly associated with survival and recurrence of disease in low-grade glioma patients (9,31). In our study, the patients in the total or subtotal resection (≥90% removal) group tended to have longer survival times than the patients in the partial (<90% removal) or biopsy group (p=0.08). The patients without *IDH1/2* mutations had shorter OS than those with *IDH1/2* mutations in the total and subtotal resection groups (p=0.04) and in the partial and biopsy groups (p=0.01). Although the number of patients examined was small, we believe that this is a very important finding and that it indicates that patients without *IDH1/2* mutations may require more intensive treatment, such as chemoradiotherapy, even after total resection of the tumor.

1p/19q codeletion is not a prognostic factor. In our study, the OS and PFS in the diffuse astrocytomas with 1p/19q codeletion tended to be longer than those in the patients without 1p/19q codeletion, but the difference did not reach statistical significance. Furthermore, no significant differences were observed between the grade II glioma patients with regard to 1p/19q status. Prior studies reported that the presence of the 1p/19q codeletion was significantly associated with longer OS in low-grade gliomas (12,13,15,21,32). On the other hand, Houillier *et al* and Mukasa *et al* (19,22) reported that loss of 1p/19q was not a sensitive prognostic biomarker. Ichimura *et al* and Vogazianou *et al* reported that total 1p/19q loss is rare and that when present, it is associated with longer survival than other 1p/19q changes in adult gliomas independent of pathological diagnosis (14,15). Deletion of 1p or 19q was determined mainly by FISH analysis in our study, and this technique cannot discriminate between total and partial 1p/19q deletion, which might explain the discrepancy in the results.

Clinicopathological factors in grade II gliomas. The multivariate analysis showed that age ≥40 years (p=0.02), astrocytic tumors (p=0.02), initial KPS <80 (p=0.0002), and wild-type *IDH1/2* (p=0.01) were unfavorable prognostic factors in our series. These results are generally in line with previous reports

showing that older age, astrocytic histology, presence of neurologic deficits before surgery, largest tumor diameter, and tumors crossing the midline were important unfavorable prognostic factors for survival in adult patients with low-grade gliomas (5-9).

In conclusion, the multivariate analysis showed that age <40 years, oligodendroglial tumors, initial KPS ≥80, and *IDH1/2* mutations were favorable prognostic factors for survival of the grade II glioma patients. The presence of *IDH1/2* mutations was a prognostic factor for grade II glioma patients with radiotherapy. Furthermore, it is a predictive factor of response to chemoradiotherapy in grade II gliomas. Patients carrying *IDH1/2* mutations may benefit more from concurrent chemotherapy and radiotherapy compared with those without *IDH1/2* mutations.

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ORIGINAL ARTICLE

Pathological findings and prognostic factors in recurrent glioblastomas

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Abstract Glioblastomas, which are the most common primary intracranial tumor, are associated with the poorest survival time, which is typically 1–2 years. Age at initial diagnosis, Karnofsky performance score, and O^6-methylguanine DNA-methyltransferase (*MGMT*) promoter methylation status are the most well-documented predictors of survival in patients with newly diagnosed glioblastoma. Few studies have examined prognostic factors in patients with recurrent glioblastomas. At relapse, the pathological features of glioblastomas are affected by tumor regrowth and the influence of chemoradiotherapy during the initial treatment. Morphological transformations at recurrence include quantitative changes in tumor cells, such as the presence of giant cells and gemistocytic cell formation, radiation necrosis, and vascular structural changes. Therefore, we should carefully examine pathological findings at recurrence. In this report, we analyzed *MGMT* promoter status, the MIB-1 index, and the pathology of tumor samples at the first (primary tumor) and second (recurrent tumor) surgeries and clarified prognostic factors in patients with recurrent cases. In the multivariate analysis, we showed that MIB-1 indexes at the time of the second surgery ($p = 0.004$) persisted as a significant independent prognostic factor in survival of patients with recurrent glioblastoma.

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Introduction

Temozolomide (TMZ) is the standard therapy for patients with glioblastomas [1]. A recent study showed an improvement in median survival time (MST) from 12.1 to 14.6 months with the addition of concurrent TMZ to the previous standard therapy of surgery and radiotherapy in patients with glioblastomas [1]. Age at diagnosis, Karnofsky performance score (KPS), extent of surgical resection, and *MGMT* promoter methylation status have been well-documented prognostic factors of survival in patients with newly diagnosed glioblastomas [2–6]. Only a few studies have reported prognostic factors in patients with recurrent glioblastomas. The initial histology of the glioblastoma, increased patient age, KPS <80, and corticosteroid use have been reported to be poor prognostic factors for survival in patients with recurrent gliomas [7]. However, the prognostic factors of recurrent glioblastomas remain unclear. Higher MIB-1 indexes of gliomas have been demonstrated to correlate well with poorer survival time [4, 8, 9]. However, MIB-1 indexes of glioblastomas at the first surgery do not predict overall survival or the response to adjuvant therapy as an independent risk factor [10], and the significance of MIB-1 indexes in glioblastomas remains unclear. Methylated O^6-methylguanine DNA-methyltransferase (*MGMT*) promoter status could serve as a good prognostic factor for glioblastomas [11]. Variations in *MGMT* promoter methylation can occur within the same tumor after treatment [12, 13]. However, the prognostic value of variations in *MGMT* promoter methylation before and after chemoradiotherapy has been estimated in only a

few reports [14]. Giant cells, gemistocytic cell formation, and coagulation necrosis are often found in recurrent gliomas, and these findings suggest the presence of degenerative changes in tumor cells that are caused by hypoxia, irradiation, and chemotherapy [15]. Therefore, we also estimated the degenerative changes of tumor cells that are influenced by chemoradiotherapy in order to determine whether these degenerative changes may be a prognostic factor. Pathological features of glioblastomas at recurrence are affected by tumor regrowth and the influences of irradiation and chemotherapy during the initial treatment. Morphological transformations at recurrence include quantitative changes of tumor cells, radiation necrosis, and vascular structural changes [15–17]. Therefore, it is very difficult to estimate pathological findings at recurrence. In this study, we analyzed a number of prognostic factors, including MIB-1 indexes, methylation statuses of the *MGMT* promoter, and pathological findings in patients with recurrent glioblastomas.

Materials and methods

Patient and tissue collections

One hundred eighty-nine patients with glioblastoma were treated from 1996 to 2010 at our institute. Thirty-two patients (16.9%) were diagnosed initially with glioblastomas from 1996 to March 2010 and underwent second surgical resections for recurrence in the National Cancer Center Hospital. The recurrent surgical cases did not include any case of glioblastoma with oligodendroglial component (GBMO). Those patients underwent surgery twice or more during the treatment period of 1996–2010. They underwent initial surgeries, followed by chemoradiotherapy with nimustine hydrochloride (ACNU) or TMZ. Tumor samples were analyzed from primary and recurrent resected tumors; however, not all primary tumor samples resected in other hospitals were obtained. We only evaluated tumor samples with sufficient specimens for immunohistochemistry and DNA extraction. The MIB-1 index and *MGMT* promoter methylation status of the tumor samples from the first (primary tumor) and second (recurrent tumor) surgeries were determined. The presence of degenerative changes in the tumors, including pseudopalisading necrosis, coagulation necrosis, gemistocytic cells, and giant cells, was observed. The internal review board of the National Cancer Center approved this study. We defined the first progression-free survival (PFS) time as the time from the first operation to the first recurrence, and the second PFS was defined as the time from the second operation to the second recurrence. Detailed information on all 32 patients is listed in Table 1.

Table 1 Characteristics of patients with recurrent glioblastoma

Characteristic	Number of patients	Percent
Sex		
Male	20	62.5
Female	12	37.5
Age (years)		
Median	57	
Range	19–71	
Extent of removal at the first surgery		
Total removal	11	34.4
Subtotal removal	5	15.6
Partial removal	11	34.4
Biopsy	5	15.6
Extent of removal at the second surgery		
Total removal	5	15.6
Subtotal removal	5	15.6
Partial removal	20	62.5
Biopsy	2	6.3
MIB-1 index at the first surgery (%)		
Median	22.5	
Range	6.8–90.0	
MIB-1 index at the second surgery (%)		
Median	13.2	
Range	0.6–85.7	
MGMT promoter status at the first surgery		
Methylated	6	31.6
Unmethylated	13	68.4
MGMT promoter status at the second surgery		
Methylated	5	21.7
Unmethylated	18	78.3
Initial chemotherapy		
ACNU	20	62.5
TMZ	12	37.5
First PFS (months)		
Median	6.2	
Range	2.7–47.1	
Second PFS (months)		
Median	6.9	
Range	0.6–68.3	
Overall survival (months)		
Median	19.6	
Range	7.8–72.2	

ACNU nimustine hydrochloride, *TMZ* temozolomide, *PFS* progression-free survival

Histopathological analysis

Surgical specimens were fixed in 10% formalin and embedded in paraffin. Hematoxylin-and-eosin (H&E)-stained specimens were examined to determine histological tumor type. Degenerative changes, including pseudopalisading necrosis,

coagulation necrosis, gemistocytic cells, and giant cells, were also examined in H&E-stained specimens from the second operation. Multiple serial sections were subjected to immunohistochemical analyses in order to determine local staining. Furthermore, tissue sections were subjected to 15 min of microwave heating to activate antigens in a retrieval solution composed of 0.1 mol/L sodium citrate (pH 6). This was followed with immunostaining of the specimens with the streptavidin–biotin–peroxidase complex method (Vectastain; Vector Laboratories, Inc., Burlingame, CA, USA). Human monoclonal antibodies were used that recognize MIB-1 (Dako, Tokyo, Japan). Positive immunostaining was demonstrated with diaminobenzidine reactions, and slides were subsequently counterstained with hematoxylin, dehydrated, cleared, and mounted. Cell counting was performed with the aid of a light microscope (Olympus Corporation, Tokyo, Japan) at a magnification of 400×. At least 200 tumor cells were counted, and data consisted of the mean of the counts from three different locations within the specimen. MIB-1-stained cells were also counted and the percentage calculated within the observed field as the MIB-1 index.

Extraction of nucleic acids

Tumor samples were immediately frozen in liquid nitrogen and stored at −80°C. From each patient, a peripheral blood sample was drawn and stored at −80°C. Total DNA was extracted from either frozen tissue samples or paraffin-embedded specimens and each patient's blood with a DNeasy Blood & Tissue Kit (QIAGEN Sciences, Germantown, MD, USA), according to the manufacturer's protocol.

MGMT promoter methylation analysis

MGMT promoter methylation status was determined by methylation-specific polymerase chain reaction (PCR) (MSP). Tumor DNA was subjected to bisulfite treatment overnight at 50°C and then purified using an EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA, USA), according to the manufacturer's protocol. Tumor DNA obtained from paraffin-embedded specimens was amplified with the use of the first PCR using the following primers: 5′-GGATATGTTGGGATAGTT-3′ and 5′-CCA AAAACCCCAAACCC-3′ and then subjected to MSP (two-step approach) [11, 18]. Thermocycling conditions consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 52°C, and 50 s at 72°C. Tumor DNA obtained from frozen tissues was directly subjected to MSP. Primer sequences used to amplify sequences from the methylated or unmethylated *MGMT* promoter were 5′-TTTCGACG TTCGTAGGTTTTCGC-3′ (M-MGMT-F) and 5′-GCAC TCTTCCGAAAACGAAACG-3′ (M-MGMT-R) or 5′-TT TGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (U-MGMT-F) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (U-MGMT-R). Thermocycling conditions for the methylated *MGMT* promoter consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 67°C, and 50 s at 72°C. Thermocycling conditions for the unmethylated *MGMT* promoter consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 64°C, and 50 s at 72°C. PCR products were separated on 2% agarose gels.

Statistical analysis

A multivariate analysis with the Cox model, which was used to assess truly independent prognostic factors, was performed only for variables for which p values <0.1 were obtained in the univariate analysis (JMP ver 8, Tokyo, Japan).

Results

Overall and progression-free survival of glioblastoma patients

Overall survival and PFS of 189 patients with newly diagnosed glioblastomas who were treated from 1996 to 2010 at our institute were 15.1 and 7.7 months, respectively (M:F = 121:68; median age 60.0 years). The MST of patients treated with initial chemoradiotherapy with ACNU ($n = 79$), TMZ ($n = 91$), and radiation only ($n = 19$) were 14.8, 16.2, and 6.6 months, respectively. There was no significant difference in MST after initial chemoradiotherapy with ACNU and TMZ ($p = 0.32$). The PFS of patients treated with initial chemoradiotherapy with TMZ is significantly longer than those treated with ACNU (10.0 and 5.3 months, $p < 0.01$). Patients who had undergone surgery twice or more ($n = 35$; MST 21.0 months) showed longer overall survival time than those who had undergone surgery only once ($n = 154$; MST 12.9 months; $p = 0.008$). The MST of patients in the total or subtotal resection group ($n = 69$; median age 62.0 years) and that of patients in the partial or biopsy group ($n = 120$; median age 60.0 years) who underwent a first surgery was 17.6 and 13.4 months, respectively ($p = 0.03$). Thirty-two patients with glioblastoma relapsed and underwent a second surgery at the first recurrence. Eight of 32 patients underwent a third operation at the second recurrence. Only one patient underwent a fourth operation at the third recurrence. The first median PFS was 6.2 months and the second 6.9 months. In the univariate analysis (Table 2), patients ≤50 years had a longer survival time than those who were 50 years old ($p = 0.05$). MST of patients ≤50 years was 31.2 months and of those who were 50 years was 16.6 months.

Table 2 Univariate analyses of overall survival time of patients with recurrent glioblastoma

Variable	Number of cases	MST (95% confident interval)	*p* value (log-rank)
Age			
≤50 years	12	31.2 (21.0–44.7)	0.05
>50 years	20	16.6 (12.7–19.6)	
Operation time			
Twice	24	17.1 (13.6–30.8)	0.09
≥3 times	8	33.3 (21.0–64.9)	
MIB-1 index at the first surgery			
≤30%	15	22.4 (13.2–55.5)	0.097
>30%	11	17.9 (12.3–23.7)	
MIB-1 index at the second surgery			
≤10%	13	42.9 (17.3–64.9)	0.005
>10%	19	16.9 (12.7–22.8)	
MGMT promoter status at the first surgery			
Methylated	6	30.6 (NA)	0.6
Unmethylated	13	19.6 (14.5–41.3)	
MGMT promoter status at the second surgery			
Methylated	5	16.9 (NA)	0.7
Unmethylated	18	19.6 (14.5–41.3)	

NA not applicable

Patients with three or more surgical resections tended to have longer survival times than those with two operations ($p = 0.09$). The MST of patients with three or more surgical resections was 33.3 months and of those with two operations was 17.1 months (Table 2). The MST of the patients in the total or subtotal resection (≥90% removal) group ($n = 16$; median age 60.0) and of patients in the partial (<90% removal) removal or biopsy ($n = 16$; median age 54.0) group who underwent a first surgery were 18.0 and 22.4 months, respectively ($p = 0.2$) in recurrent surgical cases. However, the overall survival time of patients in the total or subtotal resection group ($n = 10$; median age 57.0) and the partial or biopsy group ($n = 22$; median age 57.0) who underwent a second surgery were 42.9 and 18.0 months, respectively ($p = 0.03$). The MST after the second surgery for each group was 16.4 and 11.1 months, respectively ($p = 0.02$).

MIB-1 index at the second operation showed prognostic value in relapsed glioblastoma patients

The MIB-1 indexes of primary tumors were obtained from 26 patients and those of recurrent tumors from all patients. The median MIB-1 index of primary tumors was 22.5% (range 6.8–90.0%) and of recurrent tumors was 13.2% (range 0.6–85.7%). MIB-1 indexes were smaller for recurrent than for primary tumors ($p = 0.03$, Mann–Whitney U test) (Fig. 1).

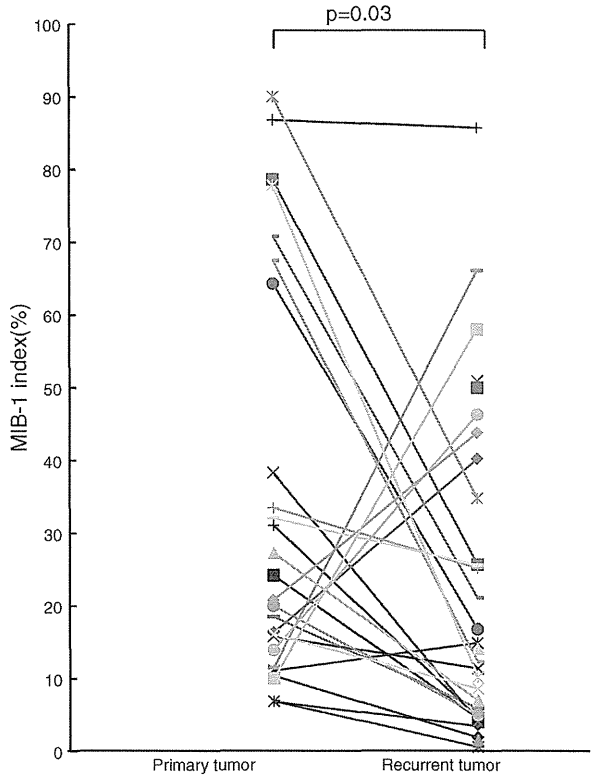

Fig. 1 Changes in MIB-1 indexes of primary and recurrent tumors. MIB-1 indexes were smaller in recurrent tumors than in primary tumors ($p = 0.03$, Mann–Whitney U test)

The MST of patients with MIB-1 indexes ≤30% was 22.4 months and of those with MIB-1 indexes >30% 17.9 months in primary tumors. Patients with MIB-1 indexes ≤30% in primary tumors tended to survive longer ($p = 0.097$) (Table 2). The MST of patients with MIB-1 indexes ≤10% was 42.9 months and of patients with indexes >10% 16.9 months in recurrent tumors. The MIB-1 indexes in recurrent tumors significantly correlated with MST ($p = 0.005$) (Table 2; Fig. 2a). The survival time of patients with MIB-1 indexes ≤10% after the second surgery was 21.6 months and of patients with indexes >10% was 6.0 months ($p = 0.0007$) (Table 3; Fig. 2b). The MIB-1 indexes in recurrent tumors significantly correlated with overall survival ($p = 0.004$), even in the multivariate analysis (Table 4). This analysis resulted in a hazard ratio (HR) of 5.252 [95% confidence interval (CI) 1.666–20.587).

Status of methylated *MGMT* promoter did not show prognostic value in relapsed glioblastoma patients

We obtained the *MGMT* promoter status in 19 cases with primary and 23 with recurrent tumors. The methylated *MGMT* promoter was found in six patients (31.6%) with primary and in five (21.7%) with recurrent tumors

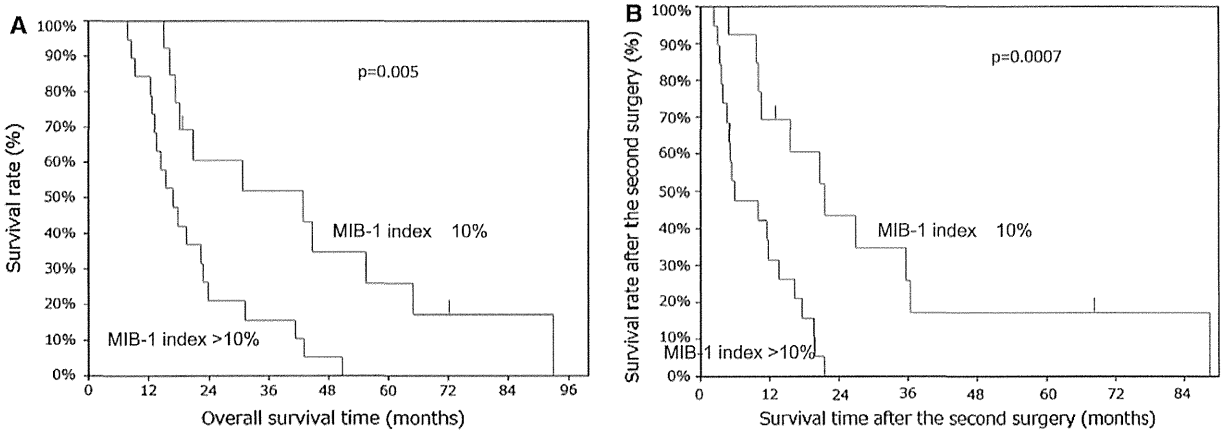

Fig. 2 **a** Kaplan–Meier survival curve comparing high and low MIB-1 indexes in recurrent tumors. The estimated overall survival rate of patients with MIB-1 indexes ≤10% was significantly higher than that of patients with indexes >10% ($p = 0.005$). **b** Kaplan–Meier survival curve after the second operation comparing high and low MIB-1 indexes in recurrent tumors. The estimated survival rate after the second operation of patients with MIB-1 indexes ≤10% was significantly higher than that of patients with indexes >10% ($p = 0.0007$)

Table 3 Univariate analyses of survival time after the second surgery of patients with recurrent glioblastoma

Variable	Number of cases	MST after second surgery (95% confident interval)	*p* value (log-rank)
MGMT promoter status at the second surgery			
Methylated	5	13.7 (NA)	0.8
Unmethylated	18	11.1 (5.0–19.9)	
MIB-1 index at the second surgery			
≤10%	13	21.6 (10.0–36.4)	0.0007
>10%	19	6.0 (3.9–13.7)	
Pseudopalisading necrosis			
Positive	8	10.9 (NA)	0.8
Negative	24	11.6 (6.0–19.7)	
Coagulation necrosis			
Positive	18	13.7 (5.4–19.7)	0.4
Negative	14	10.8 (3.7–35.7)	
Gemistocytic cell			
Positive	16	16.4 (9.7–21.6)	0.2
Negative	16	8.0 (3.9–13.7)	
Giant cell			
Positive	16	10.2 (4.7–16.4)	0.1
Negative	16	13.7 (5.0–21.6)	

NA not applicable

Table 4 Multivariate analyses of overall survival time of patients with recurrent glioblastoma

Variable	Number of cases	Hazard ratio (95% confident interval)	*p* value (Cox)
Age			
≤50 years	12	2.008 (0.811–5.506)	0.1
>50 years	20		
Operation time			
Twice	24	0.403 (0.119–1.146)	0.09
≥3 times	8		
MIB-1 index at the first surgery			
≤30%	15	1.073 (0.402–2.927)	0.9
>30%	11		
MIB-1 index at the second surgery			
≤10%	13	5.252 (1.666–20.587)	0.004
>10%	19		

(Table 1). The MST of patients with methylated or unmethylated *MGMT* promoters in primary tumors was 30.6 and 19.6 months ($p = 0.6$), respectively (Table 2). The MST of patients with methylated or unmethylated *MGMT* promoters in recurrent tumors was 16.9 and 19.6 months ($p = 0.7$), respectively (Table 2). No methylated *MGMT* promoter statuses in primary and recurrent tumors correlated with survival time (Table 2). Similarly, the methylated *MGMT* promoter in primary tumors did not correlate with the first PFS and in recurrent tumors did not correlate with the second PFS (Table 5).

Twenty patients underwent initial chemoradiotherapy with ACNU, and 12 underwent initial chemoradiotherapy with TMZ. Nine patients among 20 with initial chemoradiotherapy with ACNU were finally treated with TMZ. The MST of each of these groups was 23.3 and 14.0 months, respectively ($p = 0.02$). We then analyzed the correlation of survival time with initial chemotherapy regimen and *MGMT* promoter status in 18 patients whose *MGMT* promoter status was determined in both primary and recurrent tumors. The initial PFS of patients who received

Table 5 Univariate analyses of first and second progression-free survival (PFS) and mean survival time (MST) in 18 recurrent glioblastoma patients with *MGMT* promoter methylation status in both primary and recurrent tumors

Variable		Number of cases	First median PFS (95% confident interval)	*p* value (log-rank)	Second median PFS (95% confident interval)	*p* value (log-rank)	MST (95% confident interval)	*p* value (log-rank)
Initial treatment and the methylated *MGMT* promoter status in primary tumors								
Initial treatment	*MGMT* (primary)							
ACNU	Methylated	4	12.6 (9.1–18.3)	0.9	21.2 (2.1–NA)	0.3	49.3 (18.2–NA)	0.5
ACNU	Unmethylated	8	8.1 (3.3–26.3)		7.7 (1.6–13.6)		27.5 (15.1–64.9)	
TMZ	Methylated	1	5.2 (NA)	0.2	0.6 (NA)	0.03	9.2 (NA)	0.2
TMZ	Unmethylated	5	8.8 (2.7–22.9)		4.4 (1.0–16.4)		14.5 (7.8–41.3)	
Initial treatment and the methylated *MGMT* promoter status in recurrent tumors								
Initial treatment	*MGMT* (recurrent)							
ACNU	Methylated	3	18.1 (12.1–24.1)	0.8	13.4 (1.6–NA)	0.4	44.7 (15.5–NA)	0.6
ACNU	Unmethylated	9	9.1 (3.3–26.3)		6.6 (2.1–35.7)		31.2 (15.1–64.9)	
TMZ	Methylated	0						
TMZ	Unmethylated	6	7.6 (2.7–22.9)		3.8 (0.6–16.4)		13.4 (7.8–41.3)	
Changes of the methylated *MGMT* promoter status								
First surgery	Second surgery			0.9		0.3		0.5
Methylated	Methylated	1	3.3 (NA)		68.3 (NA)		72.2 (NA)	
Methylated	Unmethylated	4	10.9 (5.2–18.3)		4.4 (0.6–35.7)		30.6 (9.2–55.5)	
Unmethylated	Methylated	2	18.1 (12.1–24.1)		7.5 (1.6–13.4)		30.1 (15.5–44.7)	
Unmethylated	Unmethylated	11	6.4 (3.3–22.9)		5.0 (3.2–13.6)		19.6 (12.3–41.3)	

ACNU nimustine hydrochloride, *TMZ* Temozolomide, *NA* not applicable

chemoradiotherapy with ACNU and who had methylated ($n = 4$) and unmethylated ($n = 8$) MGMT promoters in primary tumors was 12.6 and 8.1 months, respectively ($p = 0.9$; Table 5). In contrast, the initial PFS of patients receiving chemoradiotherapy with TMZ and who had methylated ($n = 1$) and unmethylated ($n = 5$) *MGMT* promoters in primary tumors was 5.2 and 8.8 months, respectively ($p = 0.2$; Table 5). Three patients who initially underwent chemoradiotherapy with ACNU and who had methylated *MGMT* promoters, and five of nine patients who had unmethylated *MGMT* promoters in recurrent tumors were finally treated with TMZ. The MST of patients who underwent initial ACNU treatment followed by TMZ and who had methylated *MGMT* promoter status in recurrent tumors was longer than that of patients who had unmethylated *MGMT* promoter status (44.7 vs. 31.2 months, Table 5); however, this finding was not significant ($p = 0.6$). None of the patients who underwent the initial TMZ treatment received other alkylating agents. There was no significant difference in the PFS and MST of primary and recurrent tumors according to *MGMT* methylation status. Six patients showed changes in the methylated *MGMT* promoter in primary and recurrent tumors. Changes in the methylated *MGMT* promoter in primary and recurrent tumors did not correlate with the first and second PFS (Table 5).

Degenerative changes in tumor cells by chemoradiotherapy

We examined the degenerative changes in tumor cells, which included pseudopalisading necrosis, coagulation necrosis, gemistocytic cells, and giant cells, in H&E-stained specimens in recurrent tumors (Table 6). The MST of patients with pseudopalisading necrosis was 19.2 months and of patients without pseudopalisading necrosis 20.2 months ($p = 0.98$). The MST of patients with coagulation necrosis was 19.6 months and of patients without coagulation necrosis 25.4 months ($p = 0.16$). The MST of patients with gemistocytic cells was 22.4 months and of patients without gemistocytic cells 17.4 months ($p = 0.27$). The MST of patients with giant cells was 16.4 months and of patients without giant cells 22.4 months ($p = 0.08$). We found no correlations between morphological changes in tumor cells and survival time (Table 6).

Table 6 Univariate analyses of overall survival time of patients with recurrent glioblastoma with regard to degenerative changes of tumor at second surgery

Variable	Number of cases	MST (95% confident interval)	p value (log-rank)
Pseudopalisading necrosis			
Positive	8	19.2 (NA)	0.98
Negative	24	20.2 (16.2–31.2)	
Coagulation necrosis			
Positive	18	19.6 (13.6–23.7)	0.16
Negative	14	25.4 (15.5–44.7)	
Gemistocytic cell			
Positive	16	22.4 (16.2–50.8)	0.27
Negative	16	17.4 (13.2–30.8)	
Giant cell			
Positive	16	16.4 (13.2–30.8)	0.08
Negative	16	22.4 (16.9–44.7)	

MST mean survival time, *NA* not applicable

Discussion

We attempted to clarify the prognostic factors in recurrent glioblastomas. Clinically, patients ≤50 years had a longer survival time than patients who were >50 years old ($p = 0.05$). Glioblastoma patients who underwent surgery twice or more ($n = 35$; MST 21.0 months) showed increased overall survival compared with those who underwent only one surgery ($n = 154$; MST 12.9 months) at our institute from 1996 to 2010 ($p = 0.008$). Patients who had three or more surgical resections tended to survive longer than those who had two operations ($p = 0.09$). Whether surgical resections of recurring glioblastomas prolong survival of glioblastoma patients is unclear, but reoperations in recurrent patients have been reported to be beneficial for selected patients [19].

The extent of surgical resection in patients with newly diagnosed glioblastoma has been a well-documented prognostic factor for survival [20, 21]. In this study, we found a significant difference in survival time between the total and subtotal resection groups and the partial and biopsy groups of 189 newly diagnosed glioblastoma patients ($p = 0.03$). The extent of surgical resection during the first surgery had no correlation with the overall survival time in patients who underwent a second surgery in recurrent surgical cases; however, there was a significant difference between survival time and the extent of surgical resection during the second surgery. These data indicate that the extent of surgical resection is important, even in recurrent cases.

MIB-1 indexes that >30% in primary tumors tended to be poor prognostic factors, which were not significant. However, MIB-1 indexes in recurrent tumors had a definite correlation with overall survival time and survival time after the second surgery. Overall, survival of patients with MIB-1 indexes ≤10% at recurrence was 42.9 months and those with indexes >10% was 16.9 months, a significant difference. Schroder et al. [22] reported that MIB-1 indexes of glioblastomas at recurrence correlated with time to

recurrence. Kunishio et al. [23] reported that MIB-1 indexes of tumors with radiation necrosis after interstitial brachytherapy were 7.6 ± 5.5%, whereas that of primary tumors was significantly higher at 17.0 ± 11.2% ($p < 0.05$). These results were similar to ours. In contrast, Ralte et al. [24] reported that the difference in the MIB-1 indexes of initial (10.33 ± 7.98) and recurrent (13.8 ± 9.40) glioblastomas were not statistically significant ($p = 0.79$). Kodera et al. [25] reported that the MIB-1 indexes of recurrent glioblastomas after stereotactic radiosurgery were significantly lower than those before. In our study, MIB-1 indexes was also smaller in recurrent (13.2%) than in primary (22.5%) tumors ($p = 0.03$, Mann–Whitney U test). It has been postulated that MIB-1 indexes were smaller in recurrent than in primary tumors when tumor cells respond to initial chemoradiotherapy and degenerative changes of the tumor cells occur. Degenerative changes, such as giant cells, gemistocytic cell formation, and coagulation necrosis, are often found in recurrent gliomas [15], but these morphological changes did not correlate with survival time in our study.

It has been reported that the methylated *MGMT* promoter was found in 44.7–48.4% of newly diagnosed glioblastoma cases [11, 26, 27]. In our study, the methylated *MGMT* promoter was found in 31.6% of primary tumors. The first PFS was 6.2 months in patients treated with chemoradiotherapy with ACNU or TMZ, and the time was shorter than a previous report that found the first PFS to be 6.9 months with radiotherapy and TMZ treatment in newly diagnosed glioblastoma patients [1]. The rate of finding the methylated *MGMT* promoter seemed to be smaller in our series than in previous reports, which may be because our series could have included unfavorable cases with regrowth that did not respond to chemoradiotherapy and therefore needed a second surgery.

Brandes et al. estimated the correlation between *MGMT* promoter methylation status at first and relapse operation and survival time in 44 TMZ-treated paired tumors. They suggested that *MGMT* methylation status determined only at the first surgery appears to be of prognostic value. The MST of patients with methylated or unmethylated *MGMT* promoters in primary tumors was 30.6 and 19.6 months, respectively [14]. In contrast to their TMZ-treated series, in our series, 12 patients were initially treated with ACNU and six with TMZ. The *MGMT* promoter methylation status in primary tumors had no correlation with survival time and PFS. Brandes et al. also showed that overall survival and survival time after second surgery were not correlated with *MGMT* methylation status of recurrent tumors, even though most patients were treated with TMZ rechallenge and nitrosourea-based chemotherapy after the second surgery. The authors suggested that this may depend in part on the low activity of second-line therapies, especially those with alkylating agents, at the time of failure after chemoradiotherapy with TMZ [14].

Twenty patients in our study had initial chemoradiotherapy with ACNU and 12 patients had TMZ. The MST of each group was 23.3 and 14.0 months ($p = 0.02$), respectively. Nine patients among 20 patients with initial chemoradiotherapy with ACNU were finally treated with TMZ. The MST of patients with methylated *MGMT* promoter status in recurrent tumors treated with initial ACNU followed by TMZ tended to be longer than that with unmethylated *MGMT* (44.7 vs. 31.2 months, $p = 0.6$, Table 5). It is possibility that patients with recurrence who maintain the methylated *MGMT* status have sensitivity to TMZ even after the failure of initial ACNU. Nagane et al. [28] reported that protein expression of MGMT by Western blotting is an important prognostic factor for recurrent glioblastoma patients treated with TMZ after failure of initial ACNU-based chemotherapy. Methylated *MGMT* promoters were found in 31.6% of primary tumors and 21.7% of recurrent tumors. Six patients showed alterations in the methylation of the *MGMT* promoter between the primary and recurrent tumor. The *MGMT* promoter status of four of the five primary tumors with methylated *MGMT* promoters (80%) changed to unmethylated and the status of two of the 13 primary tumors with unmethylated *MGMT* promoters (15.4%) changed to methylated. Brandes et al. reported that *MGMT* status changed from methylated to unmethylated in eight of 13 (61.5%) tumors and from unmethylated to methylated in six of 25 (24%). Moreover, significant changes in *MGMT* methylation status occurred more frequently in *MGMT* methylated cases than unmethylated cases [14]. There are a number of potential explanations for these changes, including regional variation within the tumor, direct influence on methylation by treatment, selection of unmethylated cell populations by treatment, and further dedifferentiation of the tumor [12, 13].

In conclusion, we performed a multivariate analysis in relapsed glioblastoma patients and showed that only MIB-1 indexes in recurrent tumors persisted as significant independent prognostic factors in cases that had second surgeries. *MGMT* promoter status was frequently observed to change from methylated to unmethylated, but the *MGMT* promoter methylation status in primary and recurrent tumors had no correlation with survival time and PFS in recurrent surgical cases.

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