a median survival of 18.3 months (95% CI=14.6–22.5 months). When the 60 patients who were 18–70 years old on this trial were compared with the EORTC (RT+TMZ) data, the median survival (20.3 vs. 14.6 months) and percent surviving at 24 months (41.7% vs. 26.5%, $p=0.02$) appeared superior. Data on MGMT methylation and postprogression treatment with VEGF-targeted therapies for this population will be available for presentation. *Conclusion*: Talampanel was well tolerated and did not appear to increase the known hematologic or nonhematologic toxicities of TMZ. Talampanel can be added to RT+TMZ without significant added toxicity. These encouraging survival results in this study suggest that blocking AMPA receptors may be a useful strategy in glioblastoma.

O66. ONGOING CLINICAL TRIALS AND THE FUTURE DIRECTION OF GLIOMA TREATMENT

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Because of the proposed sensitivity to chemotherapy of oligodendroglial tumors both the RTOG and EORTC had investigated if these tumors benefit from adjuvant PCV chemotherapy. These studies, EORTC study 26951 and RTOG 9402, both showed that the addition of PCV chemotherapy (consisting of procarbazine, CCNU, and vincristine) to 59.4 Gy radiotherapy does increase progression-free survival without improving overall survival in anaplastic oligodendroglioma and anaplastic oligoastrocytoma. A major finding of both studies is the large difference in prognosis of patients with and without combined 1p/19q loss. Based on these differences in survival and the clear different outcome in anaplastic oligodendroglioma with 1p/19q loss, EORTC and the collaborative groups felt that it was no longer rational to treat these patients according to histology without taking the genotype of these tumors into account. For studies in anaplastic gliomas it was therefore proposed to classify into anaplastic glioma without 1p/19q loss and anaplastic oligodendroglial tumors with 1p and 19q codeletion. Another challenge is the definition of a proper end point for these trials. Overall survival seems to be the most relevant outcome parameter, even at progression. The outline and initiatives in grade III gliomas (EORTC 26053/22054, CATNON plus the codeleted trial) are presented. Standard therapy for glioblastoma is surgical resection aimed to be as complete as possible, respecting neurological function followed by chemoirradiation with temozolomide. TMZ given as concomitant and adjuvant therapy to RT has shown to increase progression-free survival (PFS) (rate at 6 months, 53.9% vs. 36.4%) and median survival (14.6 vs. 12.1 months) compared to adjuvant treatment with RT therapy only (EORTC 26981/22981 NCIC CE.3 trial). Still, many patients do not respond to therapy. The resistance of cells against DNA damage caused by nitrosoureas and temozolomide is at least in part mediated by the DNA-repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). Epigenetic silencing of the MGMT gene by promoter methylation compromises this DNA repair and has been associated with longer survival in (glioblastoma) patients who are treated with alkylating or methylating agents. An analysis of the EORTC 26981/22981 NCIC CE.3 trial showed, that indeed patients with glioblastoma containing a hypermethylated MGMT promoter benefited from TMZ (overall survival [OS] rate at 24 months, 46% vs. 23%), whereas those who did not have a methylated MGMT promoter did have a significantly worse survival rate and less benefit from the addition of temozolomide to RT (OS rate at 24 months, 14% vs.<2%). This raises the question if the small benefit from chemoirradiation observed in this group outweighs the toxicity and costs of the temozolomide treatment, and calls for the development of more effective drug regimens for this specific group of patients. Although there may be small numbers of patients with an unmethylated MGMT promoter that do benefit from combined chemoirradiation, for the entire subgroup of these molecularly defined GBM patients the overall benefit is questionable. Most interestingly, the phase II trial with the integrin inhibitor cilengitide also demonstrated a marked benefit mainly in the patients with glioblastoma containing a methylated MGMT promoter. Consequently, the current Merck/EORTC phase III trial is designated to delineate the role for cilengitide in glioblastoma with methylated MGMT. Even earlier, Eli Lilly took the approach to examine the protein kinase C-β inhibitor, enzastaurin, together with radiotherapy but without TMZ in patients with glioblastoma containing an unmethylated MGMT promoter. This raises the general question whether treatment in glioblastoma trials should not only be stratified according to MGMT but entry into those trials limited by MGMT status. This would call for different approaches of GBM patients, depending on the MGMT promoter gene status. The primary question to address in GBM with unmethylated MGMT promoter gene is the identification of drugs that provide more survival benefit compared to TMZ. The current EORTC trial initiatives are presented.

O67. THE RESULT OF A CLINICAL TRIAL FOR MALIGNANT GLIOMAS BY JCOG BRAIN TUMOR STUDY GROUP (JCOG 0305)

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Purpose: Japan Clinical Oncology Group (JCOG) Brain Tumor Study Group conducted a multiinstitutional randomized controlled trial on malignant gliomas entitled, a randomized controlled phase II study of chemoradiotherapy using ACNU versus procarbazine and ACNU for astrocytoma grade 3 and 4, with the support of the Health and Labour Sciences Research Grants of the Ministry of Health, Labour, and Welfare in order to establish a standard therapy for malignant gliomas in Japan. *Method*: The patients with newly diagnosed supratentorial astrocytoma grade 3 or 4 were enrolled and randomized into two groups. The patients in group A were treated with ACNU (80 mg/m^2 iv) during the postoperative radiotherapy (RT, 60 Gy local), while those in group B received procarbazine (80 mg/m^2 for 10 days per os) preceding administration of ACNU. Each regimen was continued every 8 weeks for 2 years if it was tolerable for the patients and their disease did not progress. The primary end point was the overall survival rate and the secondary end points were the response rate on the MRI and the frequency of the adverse events. Procarbazine is expected to reduce O^6-methylguanine-DNA methyltransferase (MGMT) and enhance the anticancer activity of nitrosoureas. The protocol was activated in April 2004 and 111 patients were registered by the end of August 2006 from 19 collaborating neurosurgical institutes of JCOG-BTSG. *Results*: The overall survival of the patients treated with ACNU+RT was 16.2 months and that of procarbazine+ACNU+RT was 18.7 months, while PFS of both groups were 6 months. CTCAE grade 3/4 was observed in 40–60% of the patients. *Conclusion*: ACNU-based chemoradiotherapy was an effective but toxic treatment.

O68. CURRENT CLINICAL TRIALS OF GLIOMA THERAPY AND SITUATIONS OF NEURO-ONCOLOGY PRACTICE IN KOREA

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There has been no qualified sponsor-investigator clinical trial program, and the standard therapies have been all we could do for the treatment of malignant glioma patients in the Korean Brain Tumor Society. We have just started to join two international clinical trials since 2008. In this article the past and current status of the neuro-oncology field in Korea as well as eastern and northern Asian countries will be introduced, and clinical outcomes of concurrent radiotherapy and temozolomide chemotherapy for 100 patients of four university hospitals of Korea (Advisory Board of S-P Korea) will be presented.

O69. HISTOGRAM ANALYSIS OF PERFUSION MRI DATA FOR THE ASSESSMENT OF TUMOR RESPONSE DURING GLIOMA THERAPY

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Purpose: A recently developed histogram analysis of relative cerebral blood volume (rCBV) from the entire tumor has been reported to offer excellent interobserver agreement for quantitative analysis and demonstrate the heterogeneous morphologic features of glioma vascularity. We aimed to determine whether histogram analysis can be adopted in the assessment of tumor response during glioma therapy. *Methods*: We retrospectively studied 51 dynamic susceptibility contrast 3-T MR imaging data of 29 patients (mean age 50.5 years, range, 18–76) with histologically confirmed gliomas (9 low grade, 20 high grade). rCBV maps were created and normalized to unaffected white matter. Histogram width (HW), peak height position (PHP), and maximum value (MV) of the entire tumor were measured from normalized histogram distribution. *Results*: The values (mean±SD) of HW, PHP, and MV were 4.64±2.03, 4.58±2.63, and 6.29±2.79 for the preoperative imaging of high-grade gliomas ($n=8$), and 3.83±1.96, 2.66±1.66, and 4.73±1.96 for the final imaging, which showed definite radiological tumor progression or confirmed tumor recurrence by biopsy ($n=8$). Thirty-two imaging data obtained during the median imaging follow-up of 3.7 months were divided into two groups (progression vs. stable/radiation necrosis) according to the follow-up result, and three parameters were compared. All three parameters were positively correlated with tumor progression (HW, 3.05±2.18 vs. 1.02±0.50; PHP, 2.39±1.71 vs. 0.94±0.28; MV, 4.13±2.83 vs. 1.56±0.52) and MV was the most predictive with multivariate analysis. *Conclusion*: Our results suggest that histogram analysis of rCBV can be a more objective and useful diagnostic

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ORIGINAL ARTICLE

O^6-Methylguanine DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression is correlated with progression-free survival in patients with glioblastoma

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Abstract
Objective The prognostic significance of O^6-methylguanine DNA methyltransferase (MGMT) was evaluated by analysis of both *MGMT* promoter methylation and protein expression in a series of patients with newly diagnosed glioblastoma.
Methods Seventy-three patients with glioblastomas treated with alkylating agents were analyzed for MGMT expression by immunohistochemistry. Genomic DNA was isolated from frozen surgical specimens obtained from 62 of 73 patients. *MGMT* promoter methylation was determined by methylation-specific polymerase chain reaction. The prognostic significance of MGMT was evaluated together with other well-known prognostic factors.
Results *MGMT* promoter hypermethylation was detected in 35 of 62 patients (56.4%). MGMT immunoreactivity was low in 26 (35.6%) tumors, moderate in 24 (32.9%), and high in 23 (31.5%). Significant correlation was observed between MGMT expression and *MGMT* promoter methylation ($P < 0.001$). Both *MGMT* promoter methylation and low MGMT expression were independently associated with better progression-free survival but not with longer overall survival. However, in the subgroup analysis, *MGMT* promoter hypermethylation was significantly associated with longer overall survival in patients treated with temozolomide (TMZ) after nimustine hydrochloride (ACNU) treatment.
Conclusions Low MGMT expression and *MGMT* promoter methylation are both predictive markers for slower tumor progression in patients with glioblastoma.

Keywords Glioblastoma · O^6-Methylguanine DNA methyltransferase · Methylation-specific polymerase chain reaction · Immunohistochemistry

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Introduction

Glioblastoma is the most common primary malignant tumor in adults, and the median survival continues to be approximately 12 months despite therapeutic advances. Survival is related to age, preoperative Karnofsky performance status (KPS), more extensive tumor resection, radiotherapy, and adjuvant chemotherapies [1–8]. Our understanding of the genetic alterations in glioblastoma has progressed, but clinically useful molecular markers predictive of the therapeutic response and prognosis are still rare. Chloroethylnitrosourea such as nimustine hydrochloride (ACNU) was commonly used as the standard chemotherapeutic drug for glioblastomas in Japan. The mechanism of cytotoxic effect by ACNU is thought to be alkylation at the O^6 position of guanine, an important site of alkylation in DNA, resulting in the formation of DNA lethal cross-links. [9] More recently, temozolomide (TMZ) has been to shown to significantly prolong survival in patients with glioblastoma [10]. TMZ converts the cytotoxic methylating agent at physiologic pH, which forms methyl adducts at the O^6-position of guanine in DNA. The formation of O^6-methylguanine then results in GT

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mismatches during subsequent cycles of DNA replication, followed by DNA strand-break formation and eventually cell death [10, 11]. Although the mechanisms of antitumor effects by these drugs are not the same, expression of O^6-methylguanine DNA methyltransferase (MGMT) is critical to their effectiveness, as it removes alkyl adducts from the O^6 position. *MGMT* promoter methylation results in transcriptional silencing and inhibition of MGMT expression [12, 13]. *MGMT* promoter methylation is strongly associated with survival in patients treated with either ACNU or TMZ [8, 9, 11, 14–16].

MGMT promoter methylation status is commonly assessed by methylation-specific polymerase chain reaction (PCR) [11, 14]. However, methylation-specific PCR is a relatively complicated method not often available in local treatment centers. Immunohistochemistry is a widely used and reliable method in diagnostic histopathology and is available in most laboratories. In addition, immunohistochemistry allows evaluation of both staining degree and target factor localization in individual cells. MGMT protein can be visualized immunohistochemically, and commercial anti-MGMT antibodies are available. Several studies have reported significant associations of immunohistochemically assessed MGMT expression with outcome in patients with glioma [17–22]. However, the correlation between *MGMT* promoter methylation and MGMT protein expression in gliomas remains unclear, as contradictory findings have been reported [23–28].

This study evaluated the prognostic significance of MGMT by analyzing both *MGMT* promoter methylation and protein expression in a series of patients with newly diagnosed glioblastoma managed according to a common diagnostic and therapeutic protocol.

Patients and methods

Patients and tissue preparation

Seventy-three patients with glioblastoma, including 44 males (60.3%) aged 3–76 years (median 53 years), were admitted to the Department of Neurosurgery, Tohoku University Hospital. Tumor samples were obtained during the surgical procedure, formalin-fixed and paraffin-embedded for histological studies, quick-frozen in liquid nitrogen, and kept at −80°C until nucleic acid extraction. Sixty-seven patients (91.8%) underwent surgery (49 gross total resection, 18 partial or subtotal resection) and 6 (8.2%) underwent biopsy. Resection rate was estimated by postoperative magnetic resonance imaging (MRI) within 3 days after surgery. The Ethics Committee of Tohoku University Hospital approved this study. Informed consent for use of their tissues was obtained from all study participants. All patients received adjuvant radiotherapy (total dose of 60 Gy) and chemotherapy consisting of ACNU for 43 patients and TMZ for 30 patients. Twenty-three patients who received ACNU at initial therapy subsequently received TMZ at relapse. Twenty-nine patients underwent second surgery or radiosurgery at first or second relapse.

MGMT methylation analysis

Genomic DNA was isolated from 62 frozen surgical specimens with the Qiagen kit (Qiagen, Valencia, CA, USA). *MGMT* promoter methylation was analyzed by methylation-specific PCR, as described previously [16]. Tumor DNA (2 μg) was treated with sodium bisulfite using the CpG genome DNA modification kit (Qiagen). Primer sequences for the nonmethylated reaction were 5′TTTGTG TTTTGATGTTTGTAGGTTTTTGT3′ (forward) and 5′A ACTCCACACTCTTCCAAAAACAAAACA3′ (reverse), and for the methylated reaction 5′TTTCGACGTTCG TAGGTTTTCGC3′ (forward) and 5′GCACTCTTCCGAA AACGAAACG3′ (reverse). Annealing temperature was 60°C. PCR products were separated on 4% agarose gel. The investigators who selected and analyzed the glioblastoma samples were unaware of all clinical information.

Table 1 Association of O^6-methylguanine DNA methyltransferase (*MGMT*) promotor methylation and MGMT expression in human glioblastomas

	Total	Methylated	Unmethylated	Not done
3 groups				
Low (<20%)	26	18	1	7
Intermediate (20–50%)	24	12	10	2
High (≥50%)	23	5	16	2
2 groups				
Negative (<20%)	26	18	1	7
Positive (≥20%)	47	17	26	4

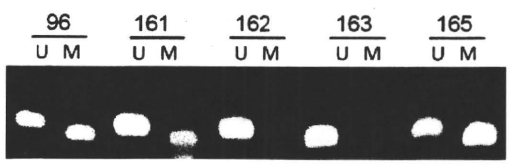

Fig. 1 Methylation-specific polymerase chain reaction (PCR) of O^6-methylguanine DNA methyltransferase (MGMT) promoter in glioblastomas. The presence of a PCR band under *lanes M* or *U* indicates methylated or nonmethylated genes, respectively. Cases 92, 161, and 165 are methylated, whereas cases 162 and 163 are nonmethylated

Immunohistochemistry of MGMT

Immunohistochemical procedures were routine, as previously described. Mouse monoclonal antibody (MT3.1; Chemicon, Temecula, CA, USA) was diluted 1:20. Sections were counterstained with hematoxylin. At least 1000 tumor-cell nuclei were individually reviewed and scored on the sections showing the highest density of immunopositive nuclei by two observers (MW, MY). Endothelial cells and perivascular lymphocytes were excluded from the positive cell count. MGMT protein immunoreactivity was evaluated semiquantitatively by estimating the fraction of positive

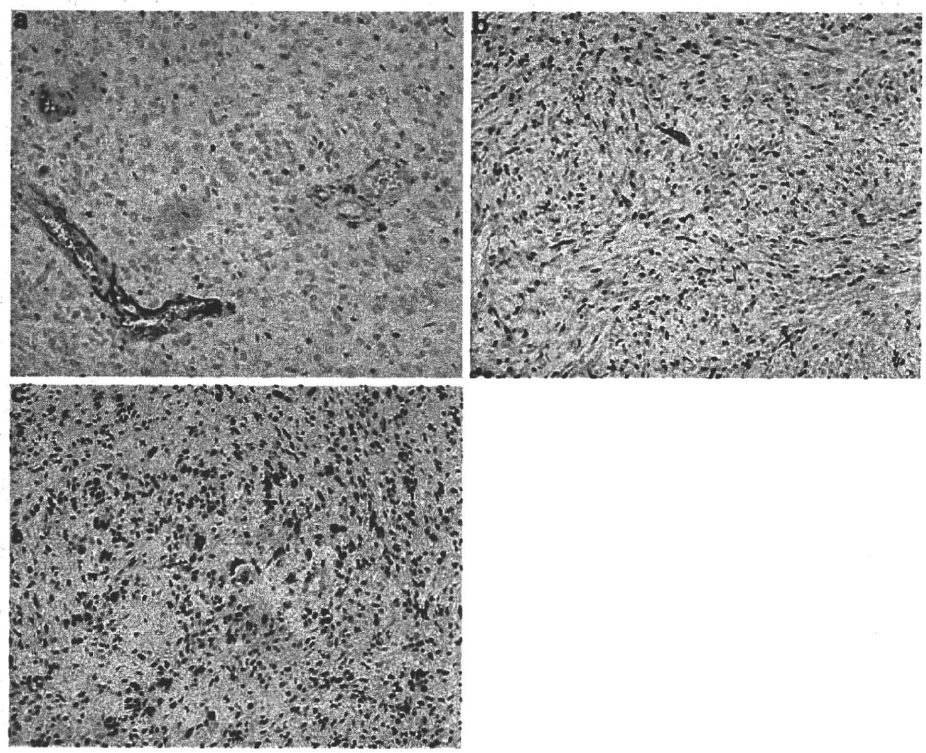

Fig. 2 Representative photomicrographs illustrating low (**a**), moderate (**b**), and high (**c**) immunoreactivity for O^6-methylguanine DNA methyltransferase (MGMT) in tumor samples (×200)

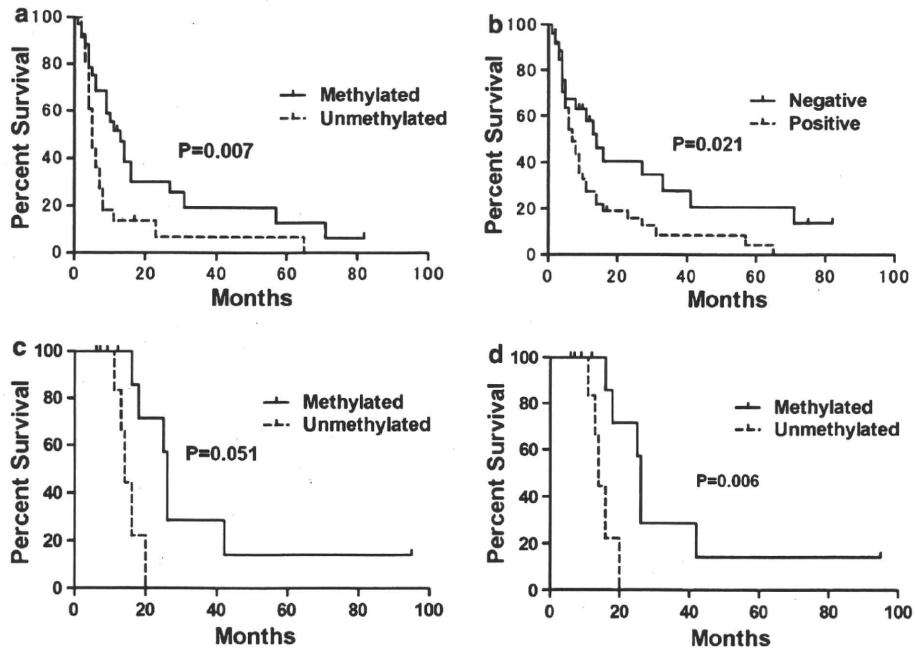

Fig. 3 Progression-free survival curves of patients with glioblastoma according to O^6-methylguanine DNA methyltransferase (MGMT) methylation status (**a**) or MGMT protein expression (**b**). Progression-free survival curves of patients treated with nimustine hydrochloride (ACNU) according to MGMT methylation status (**c**). Overall survival curves of patients treated with temozolomide (TMZ) after ACNU according to MGMT methylation status (**d**)

cells and defining <20% as low reactivity, 20–50% as moderate, and >50% as high.

Statistical methods

The relationship between methylation-specific PCR findings and MGMT expression was evaluated by the χ^2 and Fisher's exact tests. Probabilities of overall and progression survival were calculated according to the Kaplan–Meier method and compared with the log-rank test. *MGMT* promoter methylation status and MGMT expression, together with demographic (age and sex), clinical (preoperative KPS), and therapeutic (extent of resection and initial chemotherapy) variables achieving $P < 0.1$ in the univariate analysis, were subsequently introduced in a backward stepwise proportional hazard analysis (Cox model) as independent predictors of survival. MGMT immunohistochemistry was reclassified as negative (low staining) and positive (moderate and high staining) for statistical purposes. All statistical methods adopted a significance level of $P = 0.05$ using statistical package software (SPSS, Inc., Chicago, IL, USA).

Results

MGMT protein expression and *MGMT* promoter methylation status

Results are summarized in Table 1. *MGMT* promoter hypermethylation was detected in 35 of 62 patients (56.4%) (Fig. 1). MGMT immunoreactivity was low in 26 tumors, moderate in 24, and high in 23 (Fig. 2). Heterogeneous immunostaining was observed in all positive tumor samples. Methylated *MGMT* promoter was associated with low MGMT protein expression in 18 tumors, moderate in 12, and high in 5. In contrast, only one case with low MGMT expression showed nonmethylated *MGMT* promoter. Therefore, significant correlation between *MGMT* promoter methylation and MGMT protein expression was observed ($P < 0.0001$).

Progression-free survival

At the end of the follow-up period, 18 patients (24.6%) remained progression free. Progression-free survival (PFS) was 1.7–96.7 (median 9.2) months. Univariate analysis showed significant prognostic factors were extent of resection, *MGMT* promoter methylation status, and MGMT protein expression (Fig. 3a, b; Table 2). Cox's regression model revealed that gross total resection, *MGMT* promoter methylation status, and MGMT protein expression were independent factors for longer PFS (Tables 3, 4). For subgroup analysis, we divided our patients into 2 groups based on the initial chemotherapy (ACNU or TMZ). In patients treated with ACNU, *MGMT* promoter methylation showed nearly significantly improved PFS compared with those with *MGMT* promotor nonmethylation (log-rank, $P = 0.051$; Fig. 3c), whereas those treated with TMZ showed no statistical difference of PFS between *MGMT* methylation status, as there were too few cases initially treated with TMZ (log-rank, $P = 0.077$).

Overall survival

At the end of the follow-up period of 103.2 months, 37 patients (50.7%) remained alive. Survival was 6.0–103.2 (median

Table 2 Predictors of progression-free and overall survival in the patients with glioblastoma

Variables	*n*	Progression-free survival		Overall survival	
		Median (months)	*P* value	Median (months)	*P* value
Age (years)					
≤60	50	8		26	
>60	23	10	0.853	18	0.083
Sex					
Male	45	10		26	
Female	28	9	0.632	19	0.207
Preoperative KPS					
≥80	38	10		26	
<80	35	8	0.396	17	0.174
Total resection					
Yes	49	10		43	
No	24	6	0.010	16	0.001
Chemotherapy (initial)					
ACNU	43	8		26	
TMZ	30	9	0.749	19	0.056
MGMT promoter status					
Methylated	35	12		26	
Unmethylated	27	5	0.019	17	0.473
MGMT expression					
Negative	26	13		43	
Positive	47	7	0.045	19	0.398
Second surgery					
Yes	22			19	
No	51			42	0.920
Radiosurgery					
Yes	10			103	
No	63			20	0.189
Second-line TMZ					
Yes	20			20	
No	53			43	0.478

KPS Karnofsky performance status, *MGMT* O^6-methylguanine DNA methyltransferase, *ACNU* nimustine hydrochloride, *TMZ* temozolomide

Table 3 Multivariate analysis of factors associated with survival

	Progression-free survival			Overall survival		
	P value	HR	95% CI	*P* value	HR	95% CI
Total resection	0.007	2.339	1.267–4.316	0.001	3.597	1.745–7.416
Age	NS			NS		
Chemotherapy	NS			NS		
MGMT promoter methylation	0.011	2.113	1.183–3.773	NS		

NS not significant, *HR* hazard ratio, *CI* confidence interval

Table 4 Multivariate analysis of factors associated with survival

	Progression-free survival			Overall survival		
	P value	HR	95% CI	*P* value	HR	95% CI
Total resection	0.006	2.265	1.265–4.055	0.001	3.597	1.745–7.416
Age	NS			NS		
Chemotherapy	NS			NS		
MGMT negative expression	0.049	1.777	1.002–3.151	NS		

NS not significant, *HR* hazard ratio, *CI* confidence interval

22.5) months. Univariate analysis showed factors affecting overall survival were sex and extent of resection, whereas salvage treatments (second-line TMZ, re-resection, and radiosurgery), *MGMT* methylation and expression, and overall prognosis had no correlation (Table 2). Multivariate analysis showed that total resection was independently associated with longer overall survival (Tables 3, 4). *MGMT* methylation was also a significant prognostic factor in patients treated with second-line TMZ (log-rank, $P = 0.006$; Fig. 3d); however, MGMT expression was not significantly associated with longer overall survival in these patients (log-rank, $P = 0.22$).

Discussion

The proportion of tumors exhibiting either absence of MGMT protein immunoreactivity or *MGMT* promoter hypermethylation did not differ from those previously reported [9, 23, 29–34]. Several studies of the relationship between *MGMT* promoter hypermethylation and protein expression in gliomas have observed contradictory results [16, 23–28]. In our study, 17 tumors had methylated *MGMT* promoter, of which 12 showed moderate immunoreactivity and 5 showed high immunoreactivity for MGMT protein. As methylation-specific PCR is a highly sensitive method, a methylated band might be detected in a small portion of tumor cells with *MGMT* promoter methylation [9]. In our study, the MGMT immunostaining patterns were heterogeneous in different regions of the same tumor. Although we cannot rule out the presence of contaminating normal cells, other explanations for the observed variability include monoallelic promoter methylation, methylation of a small portion of malignant cells, and loss of heterozygosity in 10q26 [9, 32]. However, we did observe a significant correlation between *MGMT* promoter methylation and MGMT protein expression, as almost all tumors with nonmethylated *MGMT* promoter showed positive MGMT expression.

Some studies found that *MGMT* promoter methylation was associated with improved time to progression or overall survival [9, 20, 22, 29, 31, 33], whereas other studies found no association between *MGMT* promoter methylation and prognosis [30, 32]. Immunohistochemical analysis of MGMT has shown negative MGMT expression was significantly associated with patient survival [15, 17, 19, 21, 34, 35] but no correlation between MGMT expression and prognosis [26–28, 36, 37].

This study found both low tumor MGMT expression and aberrant *MGMT* promoter methylation were independently associated with longer progression-free survival in patients with glioblastomas but not with longer overall survival. One reason is considered to be the effect of various treatments at recurrence. Despite no survival benefit, 29 patients received surgical resection and/or radiosurgery after recurrence. Moreover, 23 patients were initially treated with ACNU but received TMZ at relapse. Among this subgroup, MGMT methylation was still significantly associated with longer overall survival. This result suggests the effectiveness of TMZ might not be affected by preceding chemotherapy with ACNU, as previously reported [38].

Conclusion

The prognostic significance of MGMT protein expression or *MGMT* promoter methylation in patients with glioma remains unclear. The conclusion of this study relies mainly on evaluation of the MGMT predictive value after adjusting for well-recognized clinicopathologic prognostic factors. Further clinical studies are needed to clarify whether the MGMT predictor can discriminate between biologically distinct groups of tumors with different natural histories and treatment responses.

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Conflict of interest statement None.

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CLINICAL ARTICLE

Long-term survivors of glioblastoma: clinical features and molecular analysis

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Abstract
Background Glioblastoma is a highly lethal neoplasm with a median survival of 12–14 months; only 2–5% of patients survive >3 years.
Methods At our institute, patients with glioblastoma are initially treated with maximum tumor resection followed by radiation and the intravenous injection of nimustine hydrochloride (ACNU).
Results Using this strategy, 18 of 123 (14.6%) patients treated at our hospital survived >3 years; 7 manifested no recurrence, and the other 11 had early recurrence and received additional therapies. To identify factors associated with prolonged survival, we compared these patients with 21 short-term (<1.5 years) glioblastoma survivors. In the long-term survivors, the *MGMT* promoter methylation was significantly more frequent. The rate of *p53* mutation was lower, and the rate of *PTEN* mutations and the proliferation index were slightly higher in short-term survivors.
Conclusion By multivariate analysis, we found that a younger age and *MGMT* promoter methylation were significant favorable factors in patients with glioblastoma.

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Abbreviations

STS	short-term survivors
LTS	long-term survivors
ACNU	nimustine hydrochloride
MSP	methylation specific PCR
QRT-PCR	quantitative real-time reverse transcription PCR
TTP	time to progression
OS	overall survival
RS	radiosurgery
RT	radiation therapy
ICE	isfosfamide + cisplatin + etoposide
MTX	methotrexate
GAPDH	glyceraldehydes-3-phosphate dehydrogenase

Introduction

Glioblastoma is the most common primary malignant tumor in adults, and despite therapeutic advances, the median survival continues to be approximately 12 months [11]. Possible factors contributing to the long-term survival of the few reported patients are younger age, female gender, higher preoperative Karnofsky performance score (KPS), more extensive tumor resection, long progression-free survival, radiotherapy, and adjuvant chemotherapies [5, 18, 24–28, 34].

Despite progress in our understanding of the genetic alterations in glioblastoma, clinically useful molecular markers predictive of the therapeutic response and prognosis are still rare.

O^6-methylguanine DNA methyltransferase (*MGMT*) promoter methylation is reportedly strongly associated with survival [10, 13, 18, 19]. In the presence of *MGMT*, a key enzyme for DNA repair, the toxicity of alkylating agents is reduced, the formation of adducts at the O^6 position of guanine is reversed, and the formation of lethal cross-links is inhibited. Thus, *MGMT* activity is a major mechanism underlying the resistance to alkylating drugs such as nitrosourea and temozolomide. Molecular factors such as *p53*, the unaltered copy number of the epidermal growth factor receptor (*EGFR*) gene, and the low rate of tumor proliferation appear to correlate with long-term survival [4, 20]. *PTEN* is a major tumor suppressor that is inactivated in 50% of high-grade gliomas by mutation or epigenetic mechanisms; the result is uncontrolled PI3K signaling in these tumors [17, 21]. In addition, the *PTEN* mutation is an important prognostic factor in patients with anaplastic astrocytoma and in older patients with glioblastoma [30].

In our institute, glioblastoma patients are initially treated by maximum tumor resection followed by radiation and the intravenous injection of nimustine hydrochloride (ACNU). At the first relapse after ACNU maintenance therapy, we apply aggressive therapies, including surgical resection, radiosurgery, and second-line chemotherapy. However, it remains unclear which patients would benefit from additional therapies. To identify molecular markers that may be associated with prolonged survival in adult patients with supratentorial glioblastoma, we compared two cohorts. Tumor samples from patients who survived <1.5 years after the initial diagnosis (short-term survivors, STS, n=21) and patients who survived ≥3 years (long-term survivors, LTS; n=18) were examined for *MGMT* aberrations determined by promoter methylation, immunohistochemical expression, and quantitative real-time reverse transcription PCR (QRT-PCR). We also assessed the proliferation index and *p53* and *PTEN* mutations.

Clinical materials and methods

Patient characteristics

The 123 glioblastoma patients were Japanese individuals treated at the Department of Neurosurgery, Tohoku University Hospital, from 1996 to 2004. Among them, 67 (54.4%) patients underwent total resection. The resection rate was determined by postoperative MR imaging. Long-term survivors (LTS) were defined as patients who survived more than 36 months after the initial diagnosis of glioblastoma [4, 18, 19]. In our institute, all LTS patients (n=18) were treated by total resection followed by radiotherapy and nitrosourea-based chemotherapy. ACNU was administered to all patients in the first-line chemotherapy protocol. These patients were divided into two groups by the time to progression (TTP) of the tumor. LTS-1 patients (n=7) survived ≥36 months without tumor progression. LTS-2 patients (n=11) underwent salvage therapy (surgical resection, radiosurgery, and second-line chemotherapy) for tumor recurrence within 36 months after the initial treatment. Short-term survivors (STS) were defined as patients who survived fewer than 18 months from the initial diagnosis as reported previously [4]. Because all LTS were treated by total resection followed by radiochemotherapy, STS (n=29) were chosen from among patients who had undergone total resection and similar radiochemotherapy. Since eight STS died of other disease or treatment complications, we finally selected 21 STS who clearly died of disease progression in this study. Using WHO criteria, glioblastoma was diagnosed in all patients at the first operation. All cases were re-reviewed by a second pathologist (Y.N.) to confirm the diagnosis. Tumors with significant oligodendroglial components were classified as anaplastic oligodendroglioma or anaplastic oligoastrocytoma and excluded from this study. Clinical details, including the patient's age at the time of diagnosis, gender, preoperative KPS score, extent of resection, adjuvant therapy at recurrence, and the recorded date of disease progression or death were noted.

Tissue specimens and preparation

We obtained 30 brain tumor samples from frozen surgical specimens archived at the Department of Neurosurgery, Tohoku University Hospital. Resected specimens were quick-frozen in liquid nitrogen and kept at −80°C until nucleic acid extraction. The Ethics Committee of Tohoku University Hospital approved this study. Informed consent for use of their tissues was obtained from all study subjects.

MGMT methylation analysis

Genomic DNA was isolated from frozen surgical specimens with the Qiagen kit (Qiagen, Valencia, CA). *MGMT* promoter methylation was analyzed by methylation-specific PCR (MSP) as reported earlier [10]. Tumor DNA (2 ug) was treated with sodium bisulfite using the CpG genome DNA modification kit (Qiagen). The primer sequences for the unmethylated reaction were 5′ TTTGTGTTTTGATGTTTGTAGGTTTTTGT3′ (forward) and 5′AACTCCACACTCTTCCAAAAACAAAACA3′ (reverse). For the methylated reaction, they were 5′ TTTCGACGTTCGTAGGTTTTCGC3′ (forward) and 5′ GCACTCTTCCGAAAACGAAACG3′ (reverse). The annealing temperature was 59°C. The PCR products were separated on 4% agarose gels. The investigators who

selected and analyzed the glioblastoma samples were blinded to all clinical information.

Quantitative real-time reverse transcription PCR (QRT-PCR)

We used 21 glioblastoma samples in this study. mRNA was extracted from frozen tumor tissue using a micro-fast track mRNA isolation kit (Invitrogen, Carlsbad CA). Random hexamer-primed cDNA was synthesized using the first-strand cDNA synthesis kit (GE Healthcare Biosciences, Piscataway, NJ). QRT-PCR was carried out with a Light-Cycler FastStart DNA master hybridization probe (Roche, Basel, Switzerland), 10 uM of each primer, and a 2-uM probe (5′-TCACAACCTTCAGCAGCTTCCATAACAC-3′) in a LightCycler 2.0 Real-Time PCR System (Roche) for 40 cycles. The primers for *MGMT* amplification were 5′-CGAGGCTATCGAAGAGTTCC-3′ (sense) and 5′-GGCTGCTAATTGCTGGTAAG-3′ (antisense). For the quantitative internal control we monitored the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The standard curves for *MGMT* and *GAPDH* mRNA were generated using ten-fold serially diluted standard PCR products of *MGMT* or *GAPDH* as the template; each mRNA expression level was calculated from the standard curve. For precise quantification, the *MGMT* mRNA expression level of each sample was normalized using the expression of *GAPDH*. The mean *MGMT* expression level of three non-neoplastic brain samples was assigned an expression value of 1.0, and the fold increase or decrease in the expression of *MGMT* was determined for the glioblastoma samples.

Immunohistochemistry

We used routine, previously described immunohistochemical procedures. All primary antibodies were mouse monoclonals: anti-MGMT (MT3.1; Chemicon, CA) and anti-Ki67 (MIB-1; DAKO, Tokyo, Japan). Each slide stained for MGMT and Ki-67 was individually reviewed and scored by one neuropathologist (M.W.). Scoring for MGMT was based on nuclear staining, where 0=no staining, 1=<10%, 2=10–50%, and 3=>50% of nuclei were stained. The Ki-67 labeling index was established by determining the percentage of positive nuclei in 1,000 tumor cells. For statistical assessment, all MGMT scores of 0 and 1 were combined and considered stain-negative; scores of 2 and 3 were combined and considered stain-positive.

RT-PCR and DNA sequencing

The results of *p53* and *PTEN* cDNA sequencing of samples from 13 of our patients were reported previously (see Tables 2, 3) [16]. We extracted mRNA from frozen tumor tissues of the other 15 patients and synthesized cDNA as indicated above. For *p53* and *PTEN* cDNA amplification, we performed PCR as described previously [16]. For *PTEN* and *p53* sequencing, each cDNA derived from the tumors was used directly as a template. The PCR products were purified using the high pure PCR product purification kit (Roche). All sequence reactions were performed using the GenomeLab™ DTCS quick-start kit (Beckman Coulter, Inc., Fullerton, CA). The four primers were used as described previously [16]. The reactions were carried out in an automated DNA analyzer (CEQ 8000; Beckman Coulter).

Statistical analysis

All statistical analyses were carried out with SPSS for Windows (SPSS, Chicago IL). The Mann-Whitney test was used to compare data acquired in each group for the patient age, KPS, fold increase in the *MGMT* mRNA expression level, and the Ki-67 index. For category variables, cross-tabulations were generated, and the Fisher test was used to compare their distribution. Overall survival was defined as the time between the first surgery and death. Survival distributions were estimated by Kaplan-Meier analysis and compared among patient subsets using log-rank tests. Stepwise multivariate logistic regression was used to determine the factors that were best distinguished between the LTS and STS groups.

Results

Patient characteristics and histological features

Among 123 glioblastoma multiforme patients treated at Tohoku University Hospital between 1996 and 2004, 18

Table 1 Comparison between long- and short-term glioblastoma survivors

	LTS (n=18)	STS (n=21)	p value
Median age at diagnosis (years)	48	62	0.002*
KPS at diagnosis	85	60	0.026*
Gender (% males)	67	67	NS**
Median survival (months)	48	14	<0.001***

*Mann-Whitney test, **Fisher's exact test, ***log-rank test

LTS: long-term survivors (>36 months); STS: short-term survivors (<18 months)

Table 2 Long-term (>36 months) glioblastoma survivors (n=18)

Case	Subgroup	Age/ gender	KPS	TTP	OS	Treatment at recurrence	MGMT promoter hypermethylation	MGMT mRNA expression	MGMT protein expression	Ki-67	p53 gene	PTEN gene
1	LTS-1	54/M	90	36	55	RS	M	0.17	ND	16	WT	WT
2	LTS-1	25/M	70	41	48	None	ND	ND	−	20	ND	ND
3	LTS-1	64/F	90	47	53	None	M	0.87	ND	35	WT[a]	WT[a]
4	LTS-1	39/M	80	50	66	S+ICE	M	0.2	+	24	WT	WT
5	LTS-1	51/M	50	90*	90*	NA	M	0.16	−	20	WT	WT
6	LTS-1	59/F	90	87*	87*	NA	ND	ND	−	60	ND	ND
7	LTS-1	41/M	80	94*	94*	NA	M	0.79	−	10	R175H(CGC→CAC)	WT
8	LTS-2	64/M	90	3	44	RT+MTX+S	M	0.18	−	20	WT	L182V (GTT→GGT)
9	LTS-2	28/M	90	4	82*	S+ICE	U	1.43	+	45	R273C(CGT→TGT)	WT
10	LTS-2	22/M	90	8	64*	RT+ICE+ MTX+S	M	1.32	+	19	R273C(CGT→TGT)	WT
11	LTS-2	58/F	70	11	63	RS	U	ND	−	25	WT	WT
12	LTS-2	52/F	90	11	46	S+RS	U	0.74	ND	27	WT	WT
13	LTS-2	27/M	90	13	44	S+RS	M	0.59	−	52	WT[a]	WT[a]
14	LTS-2	54/M	90	17	57	RS	M	0.62	ND	9	WT[a]	WT[a]
15	LTS-2	45/F	80	27	43	RT+ICE+ S+RS	M	0.04	−	50	V31I(GTT→ATT)	S226T (TTC→TAC)
16	LTS-2	44/F	70	27	46	None	ND	ND	+	60	ND	ND
17	LTS-2	63/M	60	31	39	S	M	0.48	ND	48	WT[a]	WT[a]
18	LTS-2	29/M	40	34	44	None	ND	ND	−	30	ND	ND

M, male; F, female; ND, not done; M, methylated; U, unmethylated; WT, wild type; TTP, time to progression; OS, overall survival

*censored; S, surgery; RS, radiosurgery; RT, conventional radiotherapy; ICE, ifosfamide + cisplatin + etposide; MTX, methotrexate; [a] results were reported previously [18]

Table 3 Short-term (<18 months) glioblastoma survivors (n=21)

Case	Age/ gender	KPS	TTP	OS	Treatment at recurrence	MGMT promoter hypermethylation	MGMT mRNA expression	MGMT protein expression	Ki-67	p53 gene	PTEN gene
1	49/F	60	4	9	RS	U	ND	ND	32	Y234C (TAC→TGC)[a]	WT[a]
2	61/M	90	8	12	RS	M	ND	ND	41	WT[a]	WT[a]
3	45/M	90	9	13	S	U	ND	ND	31	WT[a]	nt.742 ins C (CCT→CCCT)[a]
4	65/M	50	6	14	None	U	ND	ND	15	WT[a]	WT[a]
5	65/M	60	1	8	None	ND	ND	+	44	ND	ND
6	53/F	90	4	9	RS	ND	ND	−	37	ND	ND
7	61/M	60	6	14	RS	M	0.31	−	44	WT	N320L(TTA→CTA)
8	66/M	50	8	14	RS	ND	ND	+	46	ND	ND
9	67/M	80	7	15	RT+S	ND	ND	+	32	ND	ND
10	70/M	50	14	16	None	U	ND	+	45	ND	ND
11	55/F	50	12	18	RS	ND	ND	ND	50	ND	ND
12	60/M	90	1	5	MTX+S	M	0.07	−	35	ND	ND
13	55/F	80	5	13	None	M	0.67	−	20	WT	nt.261-262 del (TATGAC→TGAC)
14	58/M	80	5	15	None	U	0.48	+	23	WT	WT
15	58/M	50	5	17	None	U	0.07	+	31	WT	WT
16	62/M	60	4	16	RT	U	0.56	+	10	WT[a]	WT[a]
17	49/M	70	4	17	RS+S	U	1.21	+	52	WT[a]	WT[a]
18	58/F	70	3	8	RS+S	U	ND	ND	35	R248W (CGG→TGG)[a]	C71Y(TGT→TAT)[a]
19	66/F	40	3	9	RS	U	ND	ND	38	WT[a]	Y155C(TAT→TGT)[a]
20	63/M	90	5	10	None	U	ND	ND	34	WT[a]	nt.741 ins A (ACC→AACC)[a]
21	67/F	60	5	7	None	U	0.77	+	40	WT	R233X(CGA→TGA)[a]

M, male; F, female; ND, not done; M, methylated; U, unmethylated; WT, wild type; TTP, time to progression; OS, overall survival

S, surgery; RS, radiosurgery; RT, conventional radiotherapy; MTX, methotrexate; [a] results were reported previously [18]

patients fulfilled the criteria for LTS. The LTS group consisted of 12 men and 6 women; their median age at diagnosis was 48 years (range 22–64 years), and their median survival was 53 months. Five LTS patients were alive at a median follow-up time of 75 months (range 52–82). The median KPS of the 18 LTS patients at initial diagnosis was 85 (range 40–90). The STS group consisted of 14 men and 7 women. Their median age at diagnosis was 62 years (range 49–70), their median survival was 14 months (range 5–18 months), and their median KPS at the time of diagnosis was 60 (range 40–100). As shown in Table 1, there was a significant difference in the median age (48 vs 62, p=0.002, Mann-Whitney test) and the median KPS (85 vs 60, p=0.026; Mann-Whitney test) between LTS and STS patients.

The 18 LTS patients were divided into two groups according to the TTP (LTS-1: TTP≥36 months, LTS-2: TTP<36 months). The LTS-1 group consisted of seven patients, five men and two women whose median age at diagnosis was 51 years (range 25–64). Their median survival was 66 months. The LTS-2 group contained 11 patients, 7 men and 4 women with a median age of 45 years (range 22–64) at diagnosis. Their median survival was 46 months. There was no significant difference in the median age between the two groups.

Treatment at first recurrence

Tables 2 and 3 show the results of treatment administered at the first recurrence. Of the 18 LTS patients, 15 (83.3%) suffered recurrence during the follow-up period (range 3–50 months; median 27 months). A second resection was performed in 8 of 15 (53.3%) LTS and 4 of 21 (19%) STS patients (p=0.071; Fisher's exact test); 8 of 15 LTS (53.3%) and 10 of 21 STS patients (47.6%) underwent radiosurgery and/or radiotherapy (p=1.00; Fisher's exact test). At the first relapse, five LTS (33%) and one STS patient (5%) received second-line chemotherapy (p=0.063; Fisher's exact test). There was no significant difference with respect to additional therapies between the two groups. An ifosfamide + cisplatin + etoposide (ICE) regimen was the second-line chemotherapy in four LTS patients; 2 LTS and one STS patient with cerebrospinal fluid dissemination received an intrathecal injection of methotrexate. In four LTS and nine STS patients, we administered only conservative therapy. The median survival after recurrence in LTS patients who received at least one additional therapy was significantly longer than in STS patients (33 vs. 6 months, p<0.001; log rank). On the other hand, there was no difference in post-recurrence median survival between LTS and STS patients treated with conservative therapy alone (8.5 vs. 8 months).

Immunohistochemistry and molecular analysis

Tables 2 and 3 show the results of the *MGMT* aberration, *p53* and *PTEN* mutation status for LTSs and STSs, respectively. As shown in Tables 2 and 3, *MGMT* promoter methylation was more common in LTS (11/14, 79%) than in STS patients (4/16, 25%); this difference was statistically significant (p=0.006; Fisher's exact test). Two samples from LTS patients (cases 4 and 8) showed signals from methylated DNA alone; in the other methylated samples, signals from unmethylated DNA were also present (Fig. 1). We subjected 21 glioblastoma and 3 normal brain samples to QRT-PCR analysis. The mean expression level of the three normal samples was assigned an arbitrary value of 1.0, and the fold increase in expression relative to this value was determined for each glioblastoma sample. There was no significant difference in the fold increase in MGMT expression between STS (0.52) and LTS patients (0.59) (p=0.726; Mann-Whitney test). Of 13 STS patients, 9 (69.2%) were positive for MGMT protein expression; this was true for 4 of 9 (44.4%) LTS patients; the difference between STS and LTS patients was not statistically significant (p=0.115; Fisher's exact test).

In all tumor samples we observed a heterogeneous MGMT immunostaining pattern in different regions of the same tumor; immunostaining ranged from absent to strong. Although there was a correlation between aberrant promoter methylation and loss of expression, we noted no correlation between mRNA expression and MGMT promoter methylation and MGMT protein expression.

There was no significant difference in the Ki-67 labeling index between LTS and STS patients (p=0.368, Mann-Whitney test).

In all studied samples from 14 LTS and 14 STS patients, we successfully amplified the 1.2-kb *p53* cDNA and 1.2-kb *PTEN* cDNA by RT-PCR. With respect to *p53*, 4 of 14 (29%) LTS and 2 of 11 STS samples (14%) manifested mutations (p=0.622; Fisher's exact test). On the other hand, 2 of 14 LTS (14%) and 7 of 14 STS samples (50%) manifested *PTEN* mutations (p=0.103; Fisher's exact test). There was no significant difference between LTS and STS

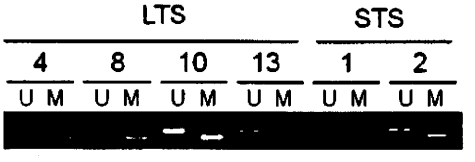

Fig. 1 Methylation analysis of the MGMT gene in LTS and STS glioblastomas by methylation-specific PCR. The presence of a PCR band under lanes M or U indicated methylated or unmethylated genes, resprectively. Numbers indicate case number. Case 4, 8, 10 and 13 in the LTS group and case 2 in the STS group are methylated, whereas case 1 in the STS group is unmethylated

patients with respect to *p53* and *PTEN* mutations. In Table 4 we present a comparison of these results for the LTS and STS groups.

As shown in Table 5, we divided our 18 LTS patients into two subgroups based on the length of progression-free survival. The rate of *PTEN* mutations and the proliferation index was lower in the LTS-1 group, but there was no significant difference. There was a significant difference among the three groups with respect to *MGMT* promoter hypermethylation (p=0.006; Fisher's exact test).

Multivariate analyses

Factors that were significantly associated with survival on univariate analysis were included in logistic regression models. After stepwise elimination of variables with p> 0.05, age and *MGMT* promoter methylation remained significant in multivariate analysis (Table 6).

Discussion

Although more than 95% of glioblastoma patients die within 36 months of their initial diagnosis, those who survive for 36 months have a relatively good chance for extended survival. Among our 18 LTS patients, 6 (33.3%) lived for 5 years or longer after their initial diagnosis. It remains unclear which of the numerous changes in gliomas are most closely associated with a poor outcome.

Among 123 glioblastoma patients we encountered between 1996 and 2002, 18 (14.6%) survived more than 36 months. This number of long-term survivors is higher than in previously reported series [18, 28]. To avoid the inclusion of glioblastoma patients with high-grade tumors of oligodendroglial lineage, a second pathologist (Y.N.) reviewed and confirmed our diagnoses. In our series, only 3 of 18 included LTS harbored a minor oligodendroglial component in an otherwise typical glioblastoma. There was no evidence of post-treatment recurrence in only 7 of 123 patients (5.7%) who were treated at our institute and survived for more than 3 years. This finding coincides with earlier reports [18, 28]. Of our 11 operated LTS-2 patients, 9 received at least one additional therapy due to glioblastoma recurrence within 36 months of the first surgery. Although we have no direct evidence that the resection rate and additional therapy are associated with survival prolongation, intensive therapy may increase the chance for prolonged survival in glioblastoma patients [7, 12, 14, 31].

On the other hand, the median survival after glioblastoma recurrence in our operated STS patients who received at least one additional treatment was only 6.0 months (range 2–12 months) and did not differ from STS patients who received only conservative therapy (8 months; range 2–10 months). Our findings suggest that STS patients did not benefit from additional therapies.

As did others [3, 8], we found that a younger age and a higher preoperative KPS were favorable factors associated with longer survival. Although it has been reported that among LTS patients there is a slight preponderance of females [18, 28], the male:female ratio in our series of LTS and STS patients was 2.0 and 2.0, respectively. Gender is not a well-established prognostic factor in glioblastoma.

All of our LTS patients were initially treated by total tumor resection. According to Stummer et al. [31], the extent of resection is associated with prolonged time to progression and survival. To exclude the effect of the resection rate at the initial treatment on patient survival, we chose our STS patients from among patients who had received total resection. Stupp et al. [32] demonstrated that concomitant and adjuvant temozolomide chemotherapy had a positive effect on the survival of glioblastoma patients [32]. In Japan, temozolomide as a drug to treat malignant gliomas was approved in 2006; as all patients in our series were treated between 1996 and 2002, none received temozolomide treatment.

Translational studies revealed that hypermethylation of the *MGMT* promoter is associated with prolonged progression-free and overall survival in glioblastoma patients treated with alkylating agents [10, 13], and most glioblastoma patients experiencing long-term survival exhibited *MGMT* promoter methylation [18, 19]. A previous report showed that *MGMT* promoter methylation is associated with a longer survival time in patients with anaplastic astrocytoma, but not in patients with glioblastoma [15]. However, we found that significantly more LTS than STS patients subjected to an ACNU-based regimen exhibited *MGMT* promoter methylation. This finding was

Table 4 Comparison of markers in long- and short-time glioblastoma survivors

Marker	LTS	STS	*P*
MGMT promoter hypermethylation	79% (11/14)	25% (4/16)	0.01
MGMT mRNA	0.59 (0.04–1.43)	0.52 (0.07–1.21)	NS
MGMT immunopositivity	31% (4/13)	69% (9/13)	NS
p53 gene	29% (4/14)	14% (2/14)	NS
PTEN gene	14% (2/14)	50% (7/14)	NS
Ki-67	25% (9–60)	35% (10–52)	NS

p53 and PTEN genes are expressed as a percentage of mutations

With the exception of Ki-67, all p values were obtained with Fisher's exact test

MGMT mRNA and Ki-67 values are the mean; for comparisons we used the Mann-Whitney test

Table 5 Comparison of markers in long- and short-time glioblastoma survivors

Marker	LTS-1	LTS-2	STS	*P*
MGMT promoter hypermethylation	100% (5/5)	67% (6/9)	25% (4/16)	p=0.006
MGMT mRNA	0.44 (0.17–0.79)	0.68 (0.04–1.43)	0.52 (0.07–1.21)	NS
MGMT immunopositivity	20% (1/5)	38% (3/8)	69% (9/13)	NS
p53 gene	20% (1/5)	33% (3/9)	14% (2/14)	NS
PTEN gene	0% (0/5)	22% (2/9)	50% (7/14)	NS
Ki-67	26% (10–60)	35% (9–60)	35% (10–52)	NS

p53 and PTEN genes are expressed as a percentage of mutations

With the exception of Ki-67, all p values were obtained with Fisher's exact test

MGMT mRNA and Ki-67 values are the mean; for comparisons we used the Mann-Whitney test

confirmed by immunohistochemical analysis: MGMT protein expression was found in 4 of 13 (30.8%) LTS and 9 of 13 (69.2%) STS patients. However, due to the small size of our study population, we were unable to examine the statistical significance of this difference. Investigations on the relationship between *MGMT* promoter hypermethylation and protein expression in human gliomas returned contradictory results [2, 9, 22]. Since MSP is a highly sensitive method, a methylated band might be detected in a small portion of tumor cells with *MGMT* promoter methylation. In our study, the MGMT immunostaining patterns were heterogeneous in different regions of the same tumor. Although we cannot rule out the presence of contaminating normal cells, explanations for the observed variability include monoallelic promoter methylation, methylation of a small portion of malignant cells, and loss of heterozygosity in 10q26 [1, 9].

While the quantitation of *MGMT* mRNA by QRT-PCR is reportedly useful for predicting the effect of nitrosourea [33], we found no significant difference between samples from LTS and STS patients. Although QRT-PCR is appropriate for estimating the MGMT status of the whole sample, contamination by normal cells and heterogeneous expression by tumor cells strongly affect the results.

The prognostic significance of *p53* mutations in glioblastoma remains unclear [6, 23, 29]. Burton et al. [4] noted higher p53 protein expression in LTS patients, although there was no significant difference in the mutation rate of the *p53* gene between LTS and STS glioblastoma patients. Our cDNA-based direct sequencing method covered the full length of the *p53* coding sequence, and although the incidence of the *p53* mutation was higher in LTS than STS patients (29% vs. 11%), the difference was not statistically significant.

Table 6 Multivariate analysis (logistic regression showing) factors independently associated with survival

	P	OR	95%CI
Age	0.02	1.15	1.030–1.292
MGMT promoter hypermethylation	0.02	0.08	0.009–0.643

p values, odds ratio (OR), and 95% confidence interval (95% CI) are indicated

The prognostic significance of *PTEN* mutations in glioblastoma is also unclear. Although *PTEN* mutations have been associated with a poor prognosis in elderly glioblastoma patients [30], other studies [23] failed to substantiate their prognostic significance. Our findings suggest that *PTEN* mutation is not a prognostic factor in glioblastoma patients.

The proliferation rate has not been a consistent predictor of outcome in glioblastoma. Burton et al. [4] reported that a lower proliferation rate was associated with long-term survival. The median proliferation index was lower in our LTS than STS patients; however, the small size of our study population did not permit statistical assessment of this difference.

We divided our 18 LTS patients into two subgroups based on the length of progression-free survival. *MGMT* promoter hypermethylation was higher, the *PTEN* gene mutations and the median Ki-67 labeling index were lower in LTS-1 than LTS-2 and STS patients. Glioblastomas in LTS-1 might be less aggressive and chemosensitive tumors. On the other hand, the median Ki-67 labeling index was the same in LTS-2 as in STS. Although more aggressive tumors recurred earlier in LTS-2 than LTS-1 patients, LTS-2 with frequent *MGMT* promoter methylation could survive more than 36 months by extensive therapies.

There was a difference between LTS and STS patients with respect to the age, preoperative KPS, and the rate of *MGMT* promoter methylation. Stepwise multivariate logistic regression was used to determine the factors that best distinguished between the LTS and STS groups. Age and *MGMT* promoter methylation remained significant in

multivariate analysis. Furthermore, as there are currently no markers to analyze the significance of differences between our LTS-1 and LTS-2 patients, these factors may be useful to identify patients with recurrent glioblastoma eligible for additional therapies.

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Comment

The most reliable marker in gliomas for biological behavior has been the 1p19q abnormality described in oligodendroglial tumors. Concerning the pure astrocytic tumors, no other marker has been described that has predictive value. Early indications from a few years ago indicated that those patients who received nitrosoureas were more likely to do better if the promoter region of the MGMT gene was methylated. This study seems to corroborate this finding and indeed moves the MGMT promoter methylation status into the forefront in terms of its being predictive for outcome in patients with high-grade gliomas. More retrospective studies are obviously required to decide whether or not this is a robust marker for long- versus short-term survival. This manuscript serves as an excellent substrate on which to base those studies.

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Decreased expression of germinal center–associated nuclear protein is involved in chromosomal instability in malignant gliomas

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Malignant glioma (MG) is highly proliferative and invasive, with the malignant characteristics associated with aneuploidy and chromosomal instability (CIN). Here, we found that the level of germinal center–associated nuclear protein (GANP), a mammalian homologue of yeast Sac3, was markedly decreased in MGs with a poor prognosis; and thus we explored the effect of its decrease on cell-cycle progression of MG cell lines. Glioblastomas showed a significantly lower level of *ganp* mRNA than anaplastic astrocytomas, as measured by real-time reverse transcription-PCR, in 101 cases of adult MG. MGs of *ganp*^{Low} expression displayed more malignant characteristics, with loss of heterozygosity on chromosome 10, epidermal growth factor receptor gene amplification, and significantly poorer prognosis than the *ganp*^{High} group. Human diploid fibroblasts depleted of *ganp* mRNA by the RNA interference (RNAi) method showed a decreased percentage of S-phase cells and a cellular-senescence phenotype. MG cell lines harboring abnormalities of various cell-cycle checkpoint molecules displayed slippage of mitotic checkpoints and an increased proportion of hyperploid cells after *ganp* RNAi-treatment. These results suggest that GANP protects cells from cellular senescence caused by DNA damage and that a significant decrease in GANP expression leads to malignancy by generating hyperploidy and CIN. (*Cancer Sci* 2009; 100: 2069–2076)

Human malignant gliomas (MGs), diagnosed as anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM), are composed of different cell types displaying a wide spectrum of heterogeneity regarding morphology, biological aggressiveness, invasive potentiality, and treatment sensitivity.[1] Extensive genetic studies have shown that MGs reveal distinctive features of complex chromosome aberrations, resulting in the loss of heterozygosity of the chromosomes carrying tumor suppressors of PTEN and p16/Cdkn2/Ink4, and in the amplification of epidermal growth factor receptor (EGFR) and human double minute-2 oncoprotein (HDM2).[2–5] Chromosomal instability (CIN), appearing as chromosome gains or losses, occurs frequently during the cell-cycle processes of DNA synthesis, chromosomal duplication and segregation, and cytokinesis[6] as a result of various kinds of DNA damage. A sensor mechanism in cells initially recognizes DNA damage as single-stranded DNA breaks or double-stranded DNA breaks (DSBs) and then induces a DNA damage response (DDR) acting through either a cellular-senescence mechanism that keeps the damaged cells proliferation silent at cell-cycle checkpoints or an apoptotic mechanism that eliminates the DNA-damaged cells.[7]

Accumulated evidence has demonstrated that DNA damage occurs to genes during active gene transcription as transcription-coupled DNA damage during the G1-phase[8] in a yeast model that lacks mRNA export molecules.[9] The molecular mechanisms of mRNA export have been intensively studied by using yeast cells.[10,11] The yeast Sac3 protein is associated with Thp1 protein as a Sac3/Thp1 complex that is necessary for the transport of ribonucleoprotein complexes bound to SAGA complexes to the nuclear pore and toward the cytoplasm.[12] The lack of either one of these components causes homology-mediated DNA hyper-recombination at a high frequency that is measured by an artificial reporter construct of tandem-repeat *leu2* gene.

A mammalian homologue of Sac3 was identified as germinal center–associated nuclear protein (GANP), which is a protein required for affinity maturation of antigen-stimulated B-cells.[13–15] GANP contains two functional domains potentially involved in DNA replication: the NH_2-terminal RNA-primase domain[16] and the COOH-terminal MCM3-binding/acetylating domain.[17] Overexpression of *ganp* cDNA in Daudi B-cells causes DNA synthesis to exceed that in the mock-transfectants.[16] Transgenic mice (*ganp*-Tg) that express the *ganp* transgene in B lineage cells show a high incidence of lymphomagenesis (29.5%) after they have aged.[18] The middle region of GANP is homologous to Sac3, implying that GANP might be involved in the mRNA export complex. The introduction of *ganp* into NIH-3T3 cells suppressed homology-mediated DNA hyper-recombination caused by DSBs after restriction enzyme digestion with *I-sce*I, and this activity was restricted to the Sac3-homology region of GANP.[19] In mammals, however, there are few studies providing evidence for the involvement of GANP in mRNA export and none indicating its association with transcription-coupled DNA damage and cancer cells in clinical cases. Here, we examined whether an abnormality in GANP related to the export of mRNA complexes exists in clinical cancer cases by focusing on various malignant neoplasm originating in the central nervous system (CNS). GANP expression was significantly decreased in MGs with poor prognosis. Remarkably, a decrease in GANP expression caused the accumulation of senescence-phenotype cells among human diploid fibroblast cells, presumably indicating that GANP expression is normally essential to suppress DNA damage during cell culture. GANP insufficiency was associated with the induction of CIN in the cells harboring various genetic abnormalities in their cell cycle and checkpoints, indicating that a decrease in GANP expression is a critical factor for the progression of malignancy in gliomas.

Materials and Methods

Patients and samples. Samples were obtained from the Department of Neurosurgery at Kumamoto University Hospital. The 101 patients were from a consecutive series, and no

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Table 1. Primer sequences

Primer name	Forward (5′–3′)	Reverse (5′–3′)
siRNA		
ganp siRNA	CCAGCGUCUUCUGGAGUAAGUCAUU	AAUGACUUACUCCAGAAGACGCUGG
ganp siRNA2	CCCAAACCUCAAGUGUUGGACCCUU	AAGGGUCCAACACUUGAGGUUUGGG
scrambled control siRNA	CCCACCUCAAGUGUUGGACCAACUU	AAGUUGGUCCAACACUUGAGGUGGG
Real-time RT-PCR		
ganp	CGTGGAGCTGATGGAACG	GCAGAAGCACTGAAGCTCCT
ganp donor probe	AGTGGGCACAGACATCCTCACAGCAACG	—
ganp acceptor probe	GCCACACGGACCCTCTGGTCTGTCTCTA	—
gapdh	CAGCCTCAAGATCATCAGC	GGCCATCCACAGTCTTCT
gapdh donor probe	GGTCATCCATGACAACTTTGGTATCATGGAA	—
gapdh acceptor probe	GACTCATGACCACAGTCCATGCCATCACTG	—

exclusion criteria were applied. RNA extraction from samples and reverse transcription (RT) reaction were performed.[20] The patients and/or their legal guardians gave written informed consent for use of their specimens. Median age of the patients was 51 years (range, 17–78 years). All patients underwent surgical resection (including biopsy) with or without postoperative radiotherapy and/or nitrosourea-based chemotherapy. GBM patients younger than 70 years of age received both radiotherapy and chemotherapy; older patients usually received radiotherapy only. Clinical details, date of recurrence (or regrowth) on magnetic resonance imaging, and date of death were recorded. The survival time was measured as the time from the date of the initial surgery to the date of death or to the date of analysis (1 June 2007). Progression-free survival time was measured from the date of initial surgery to the onset of clinical deterioration or the tumor recurrence confirmed radiologically. MIB-1 labeling index (LI), loss of heterozygosity on the chromosome 10 (LOH10), and the epidermal growth factor receptor gene amplification (*EGFR* amplification) were analyzed.[3,4,21] An immunohistochemical analysis (IHC) was carried out on CNS

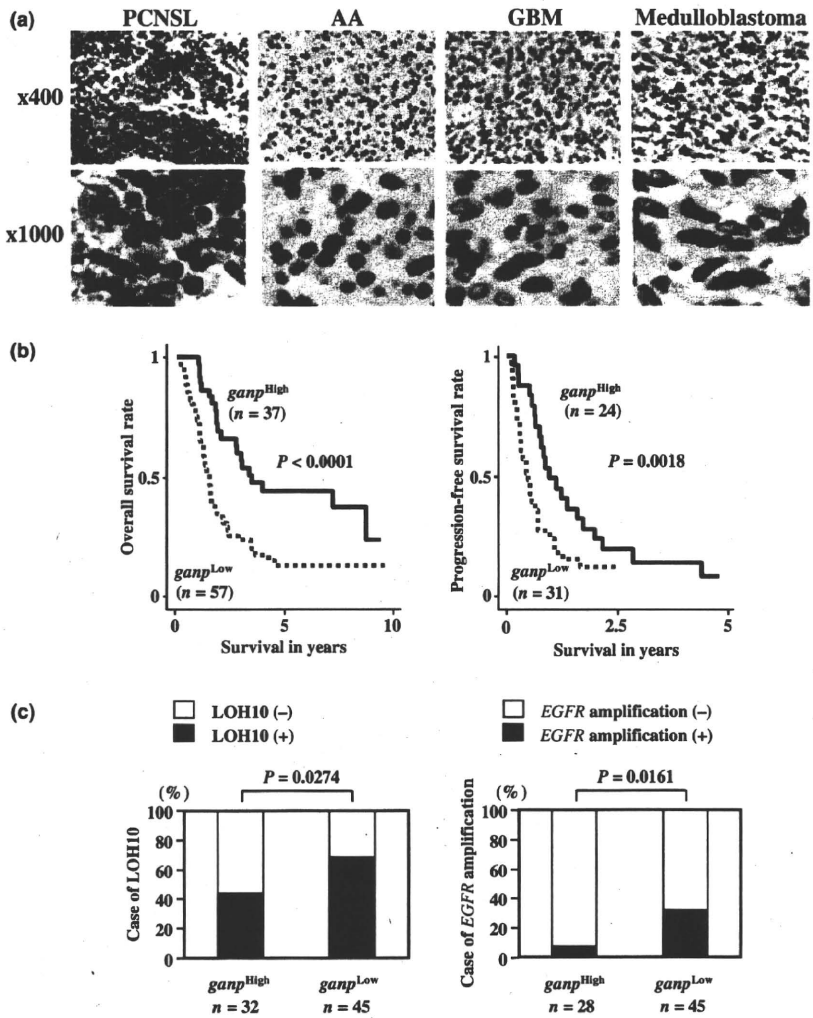

Fig. 1. Germinal center–associated nuclear protein (GANP) expression in central nervous system (CNS) tumors. (a, upper panel) GANP expression in various CNS tumors by immunohistochemistry (IHC). Original magnification ×400 for each panel. (lower panel) The larger magnification of images is shown (×1000). (b, left panel) Comparison of overall survival of malignant gliomas (MGs) between the cases of either high or low *ganp* mRNA expression using the Kaplan–Meier method. The patient group of low *ganp* mRNA expression (5 ± 6) (*ganp*^{Low}) showed a worse prognosis than the group of high expression (20 ± 11) (*ganp*^{High}) (log-rank [Mantel–Cox] test, $P < 0.0001$). (right panel) Statistical analysis of the different progression-free survivals between *ganp*^{Low} and *ganp*^{High} patients using the Kaplan–Meier method. The patients of *ganp*^{Low} (5 ± 2) showed a worse prognosis than those with *ganp*^{High} (19 ± 9) (log-rank [Mantel–Cox] test, $P = 0.0018$). (c) Comparison of other genetic abnormalities between *ganp*^{Low} and *ganp*^{High} patients. Loss of heterozygosity on the chromosome 10 (LOH10) and epidermal growth factor receptor (*EGFR*) amplification were reported[3,4] and the percentage of their positive cases is shown.

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