bevacizumab infusion administered on Day 1 every 2 weeks. Eligible patients received 10 mg/kg bevacizumab as an intravenous infusion administered over 90 (± 15) min on Day 1 of each cycle, which could be reduced to 30 min by Cycle 3 if no infusion reactions occurred. Treatment continued until disease progression (PD) or unacceptable toxicity. Bevacizumab doses were adjusted only for changes of ≥10% in body weight during the study. In the event of unacceptable toxicity, bevacizumab treatment was delayed or discontinued according to pre-specified criteria. Bevacizumab was discontinued if multiple adverse events (AEs) fulfilling the pre-specified delay or discontinuation criteria occurred in the same cycle, if cerebral hemorrhage occurred and if delayed treatment could not be restarted within 6 weeks of the last bevacizumab infusion. Patients who discontinued bevacizumab were followed for survival. Bevacizumab was provided by Chugai Pharmaceutical Co. Ltd (Tokyo, Japan).

Assessment of Efficacy

The primary endpoint was 6-month PFS in patients with recurrent GBM only. Six-month PFS was defined as the percentage of patients who remained alive and progression free at 24 weeks and was chosen based on published evidence demonstrating its extrapolation to the overall survival (OS) (6,7). Secondary efficacy endpoints included the 1-year survival, PFS, objective response rate (ORR), duration of response (DOR), OS and disease control rate (DCR).

Efficacy was assessed every third cycle (i.e. Cycles 3, 6, 9 etc.). Progression and objective response were determined by comprehensive evaluation of the results from MRI scans, corticosteroid dose assessment and neurocognitive function assessment. They were assessed by an independent radiology facility (IRF) by reference to Macdonald's Criteria (34). Response was classified according to the following categories: complete response (CR), partial response (PR), no change (NC) and PD. Confirmation of the response was determined on two consecutive assessments ≥4 weeks apart: patients who were determined as having CR or PR were defined as responders; patients who were determined as having NC or PD were defined as non-responders.

Percentage tumor shrinkage was also assessed and was calculated from the sum of the products of the diameters (SPD) at baseline and the smallest SPD after baseline.

Assessment of Safety

AEs were assessed throughout the study and were graded according to the Common Terminology Criteria for AEs version 3.0 (35). Body weight, vital signs and laboratory tests were assessed prior to the start of each cycle.

Statistical Methods

The efficacy analysis population comprised all patients with recurrent GBM. Patients with Grade III glioma were also evaluated for efficacy, but were not included in the primary analysis. All patients were evaluated for safety.

Statistical analysis to detect a 6-month PFS of 35% was established based on data from previous studies [BRAIN study [24] (42.6% with bevacizumab monotherapy) and the NCI-06-C-0064E study [26] (29% with bevacizumab monotherapy)], in which a 15% threshold for 6-month PFS was defined. Under these conditions, 28 patients with recurrent GBM would provide at least 80% power to detect a 20% increase in 6-month PFS from 15 to 35% at the 5% one-sided significance level. Assuming that other WHO Grade III glioma patients would be enrolled, the overall target sample size was 32 patients.

The 6-month PFS, median PFS, OS and DOR were calculated by the Kaplan–Meier method and confidence intervals (CIs) calculated by Greenwood's formula (36). Exact binomial CIs were used for estimated intervals for response rates.

RESULTS

Patients

Between August 2009 and July 2010, 31 patients were enrolled, 29 of whom were included in the efficacy analysis population. All enrolled patients received a median of 6 bevacizumab doses. Treatment was discontinued in a total of 25 patients: 23 (74.2%) due to PD; 2 (6.5%) due to AEs. Efficacy and safety analyses, except for OS, were performed after an observation period of ≥6 months (data cut-off 7 January 2011); the OS analyses, which included data collected through to 22 August 2011, were performed after all enrolled patients had been observed for ≥1 year.

Demographic Data

The majority of patients (29; 93.5%) had GBM; 2 (6.5%) had Grade III glioma (Table 1). The median age was 54.0 years (range: 23–72); 10 (32.3%) patients were aged ≥65 years. The percentage of males to females was well balanced. Patients were in relatively good health with 61.3% having a KPS of 90–100, and 64.5% of patients not receiving corticosteroids at the start of the study. Similar numbers of patients had experienced 1 [17 (54.8%)] or 2 [14 (45.2%)] relapses.

Efficacy Outcomes

At the time the PFS and OS analyses were performed, 22 PD events and 21 death events had been reported in the 29 patients with recurrent GBM. The 6-month PFS rate in the 29 patients with recurrent GBM (primary endpoint) was 33.9% (90% CI, 19.2–48.5), and this exceeded the 15% threshold ($P = 0.0170$). Kaplan–Meier estimates of PFS showed a steady decline over the initial 6 months with a median PFS of 3.3 months (95% CI 2.8–6.0) (Fig. 1). The 1-year survival rate for these patients was 34.5% (90%

Table 1. Demographic and baseline disease characteristics

Parameter	All patients ($n = 31$)	GBM ($n = 29$)	WHO Grade III ($n = 2$)[a]
Median age, years (range)	54.0 (23–72)	57.0 (23–72)	32.5 (30–35)
Age groups in years, *n* (%)			
≤40	6 (19.4)	4 (13.8)	2 (100)
41–64	15 (48.4)	15 (51.7)	0 (0.0)
≥65	10 (32.3)	10 (34.5)	0 (0.0)
Gender, *n* (%)			
Male	16 (51.6)	14 (48.3)	2 (100)
Female	15 (48.4)	15 (51.7)	0 (0.0)
KPS, *n* (%)			
70–80	12 (38.7)	12 (41.4)	0 (0.0)
90–100	19 (61.3)	17 (58.6)	2 (100)
Relapse/progression status, *n* (%)			
First	17 (54.8)	17 (58.6)	0 (0.0)
Second	14 (45.2)	12 (41.4)	2 (100)
Duration of malignant glioma[b]			
Median, months (range)	15.2 (5.6–213.3)	15.0 (5.6–213.3)	46.8 (27.8–65.8)
Time from RT to bevacizumab[c]			
Median, months (range)	13.2 (3.8–209.6)	13.1 (3.8–209.6)	44.8 (25.5–64.1)
Corticosteroid use at baseline, *n* (%)			
Yes	11 (35.5)	10 (34.5)	1 (50.0)
No	20 (64.5)	19 (65.5)	1 (50.0)

GBM, glioblastoma; WHO, World Health Organization; KPS, Karnofsky performance status; RT, radiotherapy; q2w, every 2 weeks.
[a]One patient had anaplastic astrocytoma and one patient had anaplastic oligoastrocytoma.
[b]Time since the initial diagnosis of malignant glioma.
[c]Time from the last RT to the first dose of bevacizumab.

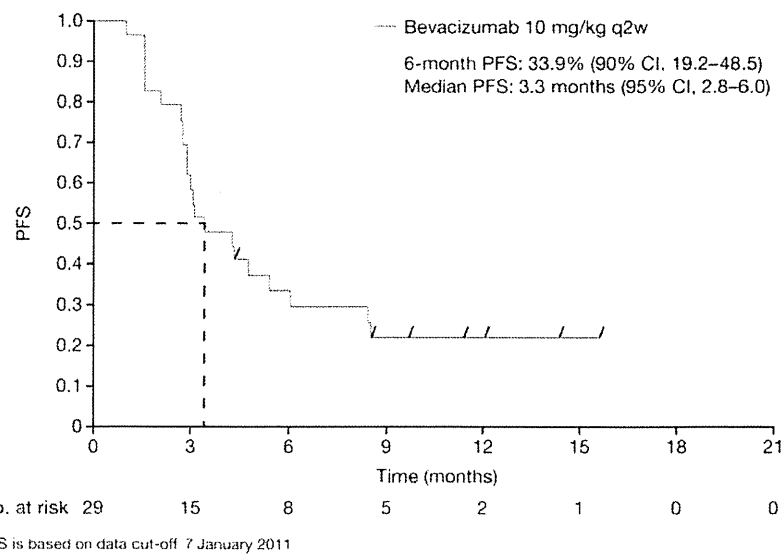

Figure 1. Progression-free survival determined by independent radiology facility in patients with recurrent glioblastoma (GBM).

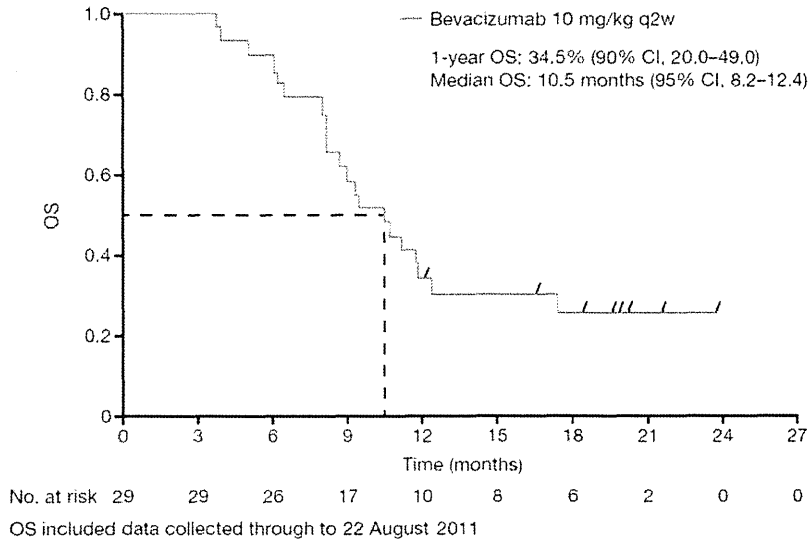

Figure 2. Overall survival in patients with recurrent GBM.

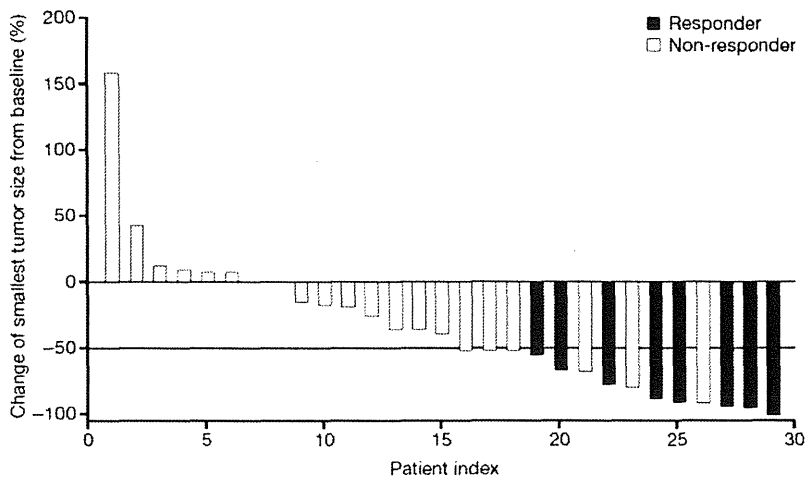

Figure 3. Waterfall plot showing the change in tumor size from baseline.

CI 20.0–49.0) with a median OS of 10.5 months (95% CI 8.2–12.4) (Fig. 2).

There were eight responders (all PR) with an ORR of 27.6% (95% CI 12.7–47.2). The DCR (0 CR + 8 PR + 15 NC) was 79.3% (95% CI 60.3–92.0). The two patients with WHO Grade III glioma completed one and two cycles of treatment, respectively; both experienced PD. Twenty-one patients (72.4%) with recurrent GBM experienced tumor shrinkage during the treatment period (Fig. 3), including 13 patients who were classified as non-responders. Of the 11 patients who were taking corticosteroids at baseline, dose reductions or discontinuation of corticosteroids occurred in 8 patients.

Efficacy endpoints were investigated in different patient subgroups (Table 2). Patients who were aged <50 years or <65 years, male, with a high KPS (90–100), on their first treatment relapse, not receiving corticosteroid therapy at baseline, or having been diagnosed with GBM at the initial diagnosis of malignant glioma, appeared to have a better response to bevacizumab treatment than other patients.

Safety Outcomes

All 31 patients experienced AEs with a total of 220 AEs reported during the study (Table 3). Serious AEs occurred in 11 (35.5%) patients, the most common being convulsion [2 (6.5%) patients]. Two (6.5%) patients discontinued the study due to AEs: one patient experienced a Grade 1 cerebral hemorrhage, and one patient had Grade 2 neutropenia that meant re-treatment within 6 weeks was not possible. A total of 13 (41.9%) patients experienced an AE of Grade ≥3, the most common being hypertension [3 (9.7%) patients]. No

Table 2. Six-month PFS and ORR by subgroup in patients with recurrent GBM

Variable	Bevacizumab 10 mg/kg, q2w ($n = 29$)	
	Six-month PFS, % (95% CI)	ORR, %
Age, years		
<65 ($n = 19$)	42.1 (19.9–64.3)	36.8
≥65 ($n = 10$)	15.0 (0.0–40.2)	10.0
Age, years		
<50 ($n = 11$)	45.5 (16.0–74.9)	45.5
≥50 ($n = 18$)	26.7 (5.7–47.6)	16.7
Gender		
Female ($n = 15$)	24.0 (1.3–46.7)	20.0
Male ($n = 14$)	42.9 (16.9–68.8)	35.7
KPS		
70–80 ($n = 12$)	16.7 (0.0–37.8)	8.3
90–100 ($n = 17$)	47.1 (23.3–70.8)	41.2
Relapse/progression status		
First ($n = 17$)	46.3 (22.3–70.4)	35.3
Second ($n = 12$)	16.7 (0.0–37.8)	16.7
Corticosteroid use at baseline		
Yes ($n = 10$)	20.0 (0.0–44.8)	10.0
No ($n = 19$)	42.1 (19.9–64.3)	36.8
Initial diagnosis of malignant glioma by site		
GBM ($n = 23$)	43.0 (22.6–63.5)	34.8
Other ($n = 6$)	0.0 (0.0–0.0)	0.0

PFS, progression-free survival; ORR, objective response rate; CI, confidence interval.

Table 3. Adverse events ≥Grade 3 and adverse events of special interest to bevacizumab

Patients, *n* (%)	Bevacizumab 10 mg/kg, q2w ($n = 31$)	
	All grade	Grade ≥3
Total patients with at least one AE	31 (100.0)	13 (41.9)
Irregular menstruation	3 (9.7)	2 (6.5)
Pyrexia	7 (22.6)	1 (3.2)
Convulsion	3 (9.7)	1 (3.2)
Depressed level of consciousness	1 (3.2)	1 (3.2)
Hydrocephalus	1 (3.2)	1 (3.2)
Increased intracranial pressure	1 (3.2)	1 (3.2)
Brain edema	1 (3.2)	1 (3.2)
Hemiplegia	1 (3.2)	1 (3.2)
Appendicitis	1 (3.2)	1 (3.2)
Urinary tract infection	1 (3.2)	1 (3.2)
Delirium	1 (3.2)	1 (3.2)
Neutropenia	5 (16.1)	1 (3.2)
Leukopenia	5 (16.1)	1 (3.2)
AEs of special interest to bevacizumab	22 (71.0)	5 (16.1)
Proteinuria	13 (41.9)	—
Hemorrhage[a,b]	10 (32.3)	—
Hypertension	10 (32.3)	3 (9.7)
Congestive heart failure	1 (3.2)	1 (3.2)
Venous thromboembolism	1 (3.2)	1 (3.2)

AE, adverse event.
[a]All events were Grade 1.
[b]Includes: epistaxis, gingival bleeding, conjunctival hemorrhage, infusion site hemorrhage, blood urine present, cerebral hemorrhage, hemorrhage subcutaneous, metrorrhagia.

incidence of Grade 4 or 5 hypertension was observed. One patient died of brain edema (Grade 5 AE), which was considered by the investigator to be related to PD with no causal relationship with bevacizumab treatment.

A total of 22 (71.0%) patients experienced AEs of special interest to bevacizumab, comprising proteinuria, hemorrhage, hypertension, congestive heart failure and venous thromboembolism (Table 3). One Grade 1 cerebral hemorrhage was observed on MRI; this was asymptomatic and resolved without treatment. Five (16.1%) patients had Grade 3 AEs of special interest to bevacizumab, comprising congestive heart failure [1 (3.2%) patient], venous thromboembolism [1 (3.2%) patient] and hypertension [3 (9.7%) patients]. No patients reported the other AEs of special interest to bevacizumab, i.e. reversible posterior leukoencephalopathy syndrome, wound-healing complications, GI perforation or fistulae.

Abnormal laboratory results were reported in 74.2% of patients. The most common abnormal laboratory result was proteinuria, which was reported in 41.9% of patients. Abnormal laboratory results classed as ≥Grade 3 were observed in two patients, reported as neutropenia and leukopenia.

DISCUSSION

This is the first clinical trial to investigate the safety and efficacy of single-agent bevacizumab in Japanese patients with recurrent GBM. Our data demonstrated that single-agent bevacizumab 10 mg/kg was effective in terms of the 6-month PFS, ORR, OS and 1-year survival, and was well tolerated in this Japanese population. In addition, the majority [21 (72.4%)] of patients with recurrent GBM experienced some tumor shrinkage during the treatment period.

The observed 6-month PFS of 33.9% and ORR of 27.6% seen in our study were more favorable than previous published data. These data are numerically higher than those reported for other studies with other chemotherapy and/or RT regimens (6-month PFS 9–21% and ORR 4–9%)

(6,7,10,11,37), and comparable with those reported for single-agent bevacizumab (42.6 and 28.2% for 6-month PFS and ORR, respectively) (24).

The use of Macdonald's Criteria was standard when this study was initiated; however, subsequently the Response Assessment in Neuro-Oncology (RANO) Working Group has recommended assessing MRI T2-weighted or fluid-attenuated inversion recovery (FLAIR) of non-enhancing lesions in addition to enhancing lesions (38). As the Macdonald's Criteria only assess contrast-enhancing lesions, there are risks that pseudoprogression and pseudoresponses may be considered real treatment effects. In our study an IRF assessed the changes in the T2/FLAIR signal, which was not included in the primary response evaluation based on Macdonald's Criteria. No significant increase in the T2/FLAIR signal was confirmed in the eight responders for the DOR, and seven out of eight responders exhibited $\geq$6 months' DOR. Based on these results, we are convinced that the objective response seen in our study is not a pseudoresponse.

Of the 29 GBM patients treated, 21 exhibited tumor shrinkage, including 8 patients who had a PR and 13 'non-responders' who were determined as NC or PD but exhibited some benefit with bevacizumab that was not captured by the response criteria; the maximum percentage of tumor shrinkage in 6 patients was >50%. The apparent discrepancy between the number of responders and the number of patients with tumor shrinkage is likely to be due to the ways in which the endpoints are calculated. The percentage of tumor shrinkage is calculated from the SPD at baseline and the smallest SPD after baseline, whereas for a patient to be classed as a responder, there had to be a decrease in tumor volume by $\geq$50% in the product of two diameters according to confirmation MRI performed $\geq$4 weeks after an observed response, as well as no increase in corticosteroid dosage and no neurologic deterioration. This leads to the difference between the number of patients with tumor shrinkage and the number of responders.

The 6-month PFS and ORR results were better for patients who had experienced one relapse than for those who had experienced two relapses, which is the same as a previously published observation (24). In addition, in our study bevacizumab improved the 6-month PFS and the ORR in the subgroups of patients who were aged <50 or <60 years compared with older patients. Although neither our study nor the previously published study (24) was powered to detect a statistical difference in these subgroups, the results could suggest that earlier administration of bevacizumab, or treatment with bevacizumab in younger patients, may lead to better tumor response and is something that requires investigation in further clinical trials.

Regarding the survival endpoints, our study showed results that were better than previously published data. The median OS of 10.5 months in GBM patients and 9.4 months in all patients was longer than that reported in other GBM trials (5.0–7.3 months) (6–8,10,11) and comparable with data with single-agent bevacizumab (9.3 months) (24,25). In addition, the 1-year survival rate for GBM patients (34.5%) was as good as the published data (14–32%) (6–8,10,11).

In addition to the favorable efficacy measures, a trend was also observed where 8 of the 11 patients who were taking corticosteroids at baseline were able to reduce their dose or discontinue corticosteroids altogether during the course of the study. This is consistent with other findings that suggest that bevacizumab may have corticosteroid-sparing effects in patients with recurrent GBM (39). Corticosteroid reduction may reduce infection rates and other related toxicities and therefore is expected to improve the health-related quality of life for patients.

Bevacizumab was well tolerated in our study and the incidence of AEs of special interest to bevacizumab was similar to that seen in other published studies with single-agent bevacizumab (24–26,40). No new bevacizumab safety signals were seen in this Japanese population.

In our study, and in the other single-agent bevacizumab studies (24–26,40), bevacizumab was administered after prior treatment with TMZ and RT. We observed an apparently greater benefit with bevacizumab in those patients with one relapse compared with those who have had two relapses following treatment with TMZ and RT. It is expected that bevacizumab may also provide benefit when administered concurrently with TMZ and RT rather than after TMZ/RT therapy. Currently, two randomized, double blind, Phase III studies are ongoing (AVAglio (41) and RTOG 0825 (42)) in which the addition of bevacizumab to standard of care (concurrent RT plus TMZ followed by adjuvant TMZ) is being evaluated in patients with newly diagnosed GBM.

There are many novel targeted agents under investigation for the treatment of gliomas (43); however, results with these new agents have been disappointing to date. Single-target agents alone may not be able to prevent tumor growth given the multiple pathways involved in many intracellular processes of tumor development. A key to future improvements in the treatment of gliomas will be the combination of other chemotherapeutic agents or molecular targeted therapies with bevacizumab to block these multiple pathways. This potential approach needs to be explored in future clinical trials.

In conclusion, the results of this study show that single-agent bevacizumab could provide significant clinical benefit for Japanese patients with recurrent GBM.

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Conflict of interest statement

Dr Masao Matsutani is a coordinating investigator of this study, a member of the advisory committee on MSD KK and a member of the independent safety review board for Nobelpharma Co. Ltd; consulting fees as a coordinating investigator of this study have been received by him from Chugai Pharmaceutical Co. Ltd. Dr Ryo Nishikawa is a member of the Avaglio study steering committee (funded by F. Hoffmann-La Roche, Ltd) and has received research funding and speaking fees from MSD KK, and honoraria from Nobelpharma Co. Ltd. No other conflicts of interest were declared.

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ORIGINAL ARTICLE

Predictive significance of mean apparent diffusion coefficient value for responsiveness of temozolomide-refractory malignant glioma to bevacizumab: preliminary report

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Abstract
Background Recurrent glioblastoma after initial radiotherapy plus concomitant and adjuvant temozolomide is problematic. Here, patients with temozolomide-refractory high-grade gliomas were treated with bevacizumab (BV) and evaluated using apparent diffusion coefficient (ADC) for response.
Methods Nine post-temozolomide recurrent or progressive high-grade glioma patients (seven with glioblastoma and two with anaplastic astrocytoma) were treated with BV monotherapy. Average age was 57 years (range, 22–78), median Karnofsky Performance Scale (KPS) was 70 (30–80) and median BV line number was 2 (2–5). Two had additional stereotactic radiotherapy within 6 months prior to BV. Magnetic resonance (MR) imaging after BV therapy was performed within 2 weeks with calculation of mean ADC (mADC) values of enhancing tumor contours.
Results Post-BV treatment MR imaging showed decreased tumor volumes in eight of nine cases (88.9 %). Partial response was obtained in four cases (44.4 %), four cases had stable disease, and one had progressive disease. Of 15 evaluable enhancing lesions, 11 shrank and four did not. Pretreatment mADC values were above 1100 (10^{-6} mm^2/s) in all responding tumors, while all non-responding lesions scored below 1100 ($p = 0.001$). mADC decreased after the first BV treatment in all lesions except one. KPS improved in four cases (44.4 %). Median progression-free survival and overall survival for those having all lesions with high mADC (>1100) were significantly longer than those with a low mADC (<1100) lesion ($p = 0.018$ and 0.046, respectively).
Conclusions Bevacizumab monotherapy is effective in patients with temozolomide-refractory recurrent gliomas and tumor mean ADC value can be a useful marker for prediction of BV response and survival.

Keywords Glioblastoma · Bevacizumab · Apparent diffusion coefficient · Prediction of response and survival · Recurrent high-grade glioma

Introduction

Standard care for glioblastoma (GBM) is radiation therapy (RT) plus concomitant and adjuvant temozolomide (TMZ) [1]. The median overall survival (OS) of patients with GBM remains at 15 months from initial diagnosis [1], and there are no standard therapies established for recurrent GBMs.

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Glioblastoma is highly vascularized and vascular endothelial growth factor (VEGF) has been identified as the major promoting factor for glioma angiogenesis [2]. VEGF expression correlates with aggressiveness and histopathological grade of glioma [3]. VEGF induces an increase in vascular permeability and disruption of the blood–brain barrier (BBB) in tumors. High-grade gliomas with abundant VEGF expression exhibit an increase in interstitial fluids, causing peri- and intra-tumoral edema [4], and further neurological deterioration. It would, therefore, be reasonable to target VEGF as a potential therapeutic strategy against intractable GBM [5].

Bevacizumab (BV) is a humanized monoclonal antibody that binds to and inhibits the activity of VEGF. The efficacy of BV for recurrent GBM was demonstrated in initial phase II clinical trials in combination with irinotecan [6]. BV monotherapy for patients with TMZ-pretreated, recurrent GBM achieved progression-free survival (PFS) at 6 months (PFS-6 m) of 43 % and median OS of 9.3 months [7]. This and another similar phase II study [8] led to accelerated approval for use of BV as a single-agent in adults with recurrent GBM in the United States. However, a subset of GBM lesions do not respond to BV, and this necessitates a way to differentiate tumors that will respond from those that will not, given the adverse effects of BV such as intracerebral hemorrhage and deep venous thrombosis, and also the high cost of the agent.

Bevacizumab decreases interstitial fluid load in the brain and tumor tissue by normalizing the BBB, leading to rapid tumor shrinkage with reduction of perifocal edema [5, 7]. An imaging technique that specifically detects such pathological conditions would be useful for prediction of BV response. One physiological imaging biomarker that might be associated with degradation of cellular integrity, such as necrosis, is apparent diffusion coefficient (ADC) obtained on diffusion-weighted magnetic resonance (MR) imaging. The ADC value represents movement of water molecules and tends to be low in tissues with high cellular density (packed tumor) where extracellular space is restricted [9]. Conversely, tissue edema and necrotic components induced by tumor burden and cytotoxic therapies may well increase the ADC value [10, 11]. The ADC value has been shown to correlate with response to radiation therapy (RT) and prognosis in patients with glioma [12, 13], to predict progression-free survival (PFS) after BV treatment in patients with recurrent GBM, and the minimum ADC values were reportedly prognostic of outcomes in glioma [12, 14]. This prompted us to investigate whether the ADC value in recurrent high-grade glioma may predict rapid shrinking response or survival after BV monotherapy, thereby facilitating selection of patients who are likely responders.

Patients and methods

Patient eligibility

Patients (≥20 years old) had histologically proven high-grade glioma (HGG) for which they had received RT and TMZ. All had experienced tumor progression determined by the Macdonald criteria [15], had measurable enhancing disease(s) on MR imaging, and had recovered from their prior treatment. The minimum 4 weeks from surgical therapy and 8 weeks after RT must have elapsed before the start of BV treatment. The patients had to have adequate organ functions and were excluded if they had experienced cerebral hemorrhage or stroke. Patients were required to have provided written informed consent. The treatment protocol including off-label use of BV at patients' own cost was reviewed and approved by the institutional review board.

Treatment

All patients received BV 10 mg/kg intravenously every other week, until disease progression or discontinuation by their withdrawal, grade 2 or more cerebral hemorrhage, grade 4 non-hematological toxicities, or any other condition that would make the treatment unsafe.

Patient evaluation

The response to therapy was assessed using MR imaging and neurological examination. The Macdonald criteria were employed to evaluate the MR imaging [15]. The criteria use the largest cross-sectional area of the post-contrast T1-weighted images and also take into account the steroid dose and clinical findings. We also evaluated non-contrast T1-weighted images, T2-weighted images, FLAIR images, and diffusion-weighted images. All MR examinations were carried out at 1.5 T. MR imaging was performed after the first and the third cycles of BV treatment, and images were reviewed by a neuroradiologist (K.T.).

Measurement of mean apparent diffusion coefficient ADC value of tumors

A mean ADC (mADC) value of the tumor was calculated on a terminal of the Picture Archiving and Communication System (PACS). Gadolinium-enhancing tumor contours were manually segmented on sequential post-contrast T1-weighted images, eliminating non-enhancing regions within the tumor, and the same segmented areas were selected on the corresponding ADC maps, thereby obtaining a mADC value (10^{-6}mm^2/s) and an area (mm^2) of the tumor in each image (Fig. 1). The mADC value of the image slice was multiplied by the area to produce a total ADC value of

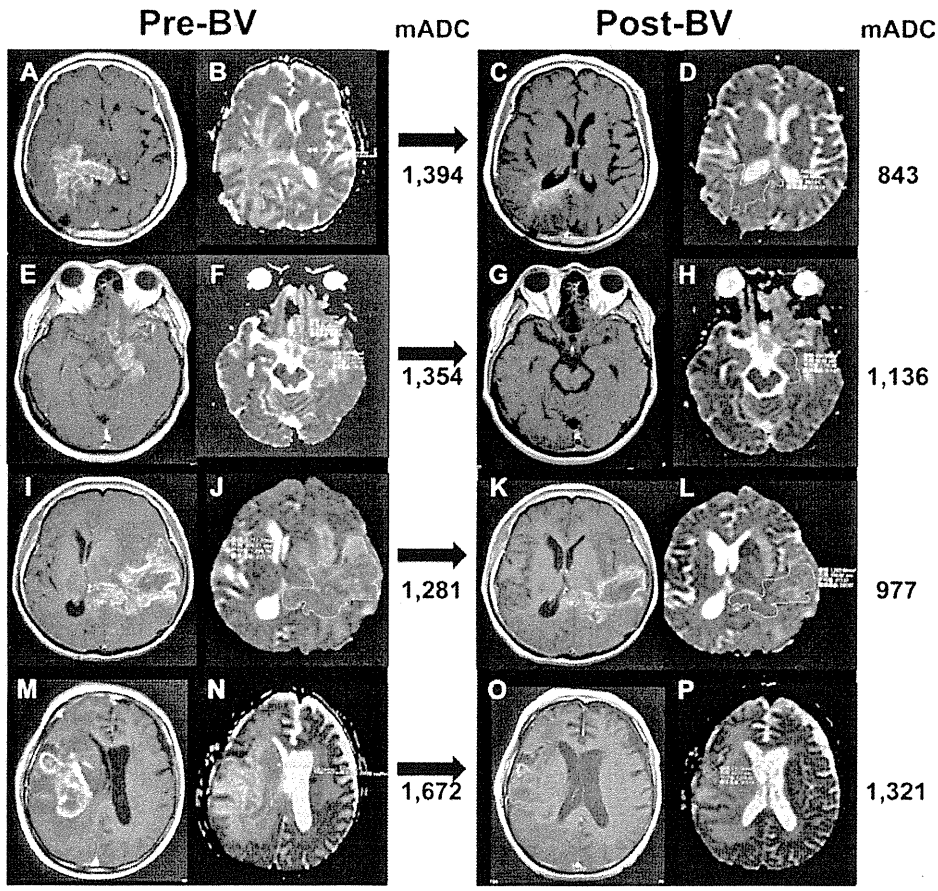

Fig. 1 Postcontrast T1-weighted images aligned side-by-side with the corresponding ADC maps of both pre- (*left-hand column*) and post-treatment (*right-hand column*) with BV in representative patients who achieved immediate response after a single BV administration. The mADC values of the lesions of interest are indicated at the *right margin* of the ADC maps. Each mADC value is calculated as described in "Patients and methods." Note that all pretreatment mADC values are above 1100 (10^{-6} mm^2/s), which subsequently decrease after BV treatment. **a, b, c, d**: case 1; **e, f, g, h**: case 2; **i, j, k, l**: case 8; **m, n, o, p**: case 9

the slice. The sum of the total ADC value of all slices for a lesion was divided by the sum of areas of all slices to achieve the net mADC value of the single enhancing lesion.

Immunohistochemistry

Immunohistochemistry analysis of surgical specimens of the original tumors entailed: (1) fixation with 10 % buffered formalin, and embedding in paraffin; (2) deparaffinizing 5-μm-thick sections of the tissues in xylene, and rehydration in 90, 70, and 50 % ethanol; (3) antigen retrieval, by autoclaving in buffered citrate (pH 7.0) at 120 °C for 10 min; (4) incubation with the primary antibody, anti-VEGF antibody (code SC-152, Santa Cruz, CA, USA) at room temperature for approximately 12 h; and (5) detection of immunoreactivity using the EnVision system (Dako, Carpinteria, CA, USA), followed by hematoxylin counterstaining.

O^6-methylguanine-DNA methyltransferase (*MGMT*) status

Methylation status of the *MGMT* gene promoter region in tumors was determined by the methylation-specific polymerase chain reaction as described elsewhere [16].

Statistical analysis

The correlation of the mADC value with response to BV was evaluated by Fisher's exact test and the Mann–Whitney *U* test. The change of mADC values before and after the first BV treatment was assessed by a paired *t* test. PFS and OS were calculated according to the Kaplan–Meier method, and differences in progression and survival according to mADC values were evaluated with the log-rank test. All the probability values were two-sided, and all statistical analyses were done at a significance level of $p = 0.05$, using the statistical package SPSS 17.0J (SPSS, Inc., Chicago, IL, USA).

Results

Bevacizumab treatment of patients with recurrent HGG after TMZ failure

From August 2009 to December 2010, nine eligible patients with recurrent or progressive HGGs (seven with GBM and two with anaplastic astrocytoma) were treated with BV monotherapy. The average patient age was 57

years (range, 22–78), and median Karnofsky Performance Scale (KPS) was 70 (30–80) (Table 1). Five patients underwent RT plus concomitant TMZ at the initial therapy, and others received TMZ monotherapy on disease progression. BV treatment was primarily (67 %) given as the second line therapy (range 2–5). The cycles of BV therapy ranged from 1 to 21 (median 7). There were no serious adverse events in any of the patients.

Response to BV

After the first BV cycle, early post-treatment MR imaging (taken between days 3 and 21, median 13) demonstrated rapid shrinkage of both enhancing and T2-elongated (hyperintense on T2-weighted and FLAIR images) areas in most tumors (Fig. 1). By a patient-based analysis, single BV treatment resulted in a decrease in evaluable enhancing tumor volume in eight of nine cases (88.9 %) (Table 1). Four patients (44.4 %) had a partial response (PR), four had a stable disease (SD), and one had a progressive disease (PD) by the Macdonald criteria; the overall response rate was 44.4 %. In cases 1, 3, and 8, both hemiparesis and disturbance of consciousness recovered soon after the first BV administration with a marked reduction of extent of hyperintensity on T2-weighted or FLAIR images along with the enhancing tumor shrinkage. KPS improved immediately in four cases (44.4 %).

Association of tumor mADC values with the response to BV

Of 15 individual evaluable enhancing lesions in the nine patients, 11 tumors shrank (Fig. 1), while four did not respond upon initial BV treatment (Fig. 2). We then evaluated parameters obtained in the MR images to determine if there were any predictors for tumor response to BV. The pretreatment MR images were obtained on days −1 to −25 (median −10). The average pretreatment mADC value for all lesions was 1249 (10^{-6} mm^2/s) (range 964–1672). Tumor mADC values were above 1100 in all of the responding tumors, in contrast to those in all non-responding lesions that scored below 1100 (Fisher's exact test, $p = 0.001$) (Fig. 3), suggesting that a pretreatment mADC value higher than 1100 may be predictive for a rapid shrinkage of enhancing tumors. Interestingly, the tumor mADC values decreased significantly after the first BV treatment in all lesions except for one that did not respond (paired t test, $p < 0.001$) (Fig. 4). The average mADC value after the first cycle of BV was 1051 (range 828–1320).

To determine whether the mADC value after the first BV treatment (post-BV mADC) could also predict a further response of the lesion to additional cycles of BV, the ratio

Table 1 Summary of cases treated with bevacizumab monotherapy on temozolomide failure

No	Age (years)	Gender	KPS (pre-BV)	Pathological Dx	RT (Gy)	TMZ cycles	TMZ OR	*MGMT* status	BV line[a]	BV cycles	Months from last RT	mADC (pre-BV) (10^{-6} mm^2/s)	Total TV (ml) (pre-BV)	BV OR	Relative TV[b]	KPS (post 1st BV)	Relapse	PFS (months)	Out-come	OS (months)
1	74	Female	40	AA	60	46	CR	nd	3	7	77	1394	61.7	SD	0.63	50	+	2.2	D	5.7
2	51	Male	70	AA	60 + SRT	14	SD	nd	2	21	15.2	1354	45.0	PR	0.03	70	+	7.7	D	16.0
3	60	Female	70	GBM	60 + SRT	11	SD	M	2	4	5.6	964–1490[c]	25.7	SD	0.88	90	+	1.5	D	3.3
4	32	Female	70	GBM-s	50	10	SD	M	3	8	35.8	1190	23.7	SD	0.73	70	+	2.3	D	5.5
5	22	Male	80	GBM-s	60	1	PD	U	2	12	2.1	1273	16.8	PR	0.13	80	+	5.5	D	10.3
6	73	Female	60	GBM	60	5	SD	U	2	1	6.7	1009–1281[c]	7.9	PD	1.78	50	+	0.4	D	3.9
7	58	Female	80	GBM	60	8	SD	U	2	8	7.6	1323	8.4	PR	0.67	80	+	3.9	D	11.1
8	51	Female	30	GBM	60 + SRT	40	CR	M	5[a]	5	4.3	1281	152.8	SD	0.54	50	+	1.4	D	2.9
9	78	Male	30	GBM	40	1	PD	U	2	3	1.9	1257–1672[c]	72.9	PR	0.24	40	+	2.1	D	8.3

KPS Karnofsky Performance Scale, *Dx* diagnosis, *RT* radiotherapy, *TMZ* temozolomide, *OR* objective response, *MGMT* O^6-methylguanine-DNA methyltransferase, *BV* bevacizumab, *mADC* mean apparent diffusion coefficient, *pre-BV* before the first BV treatment, *TV* tumor volume, *PFS* progression-free survival, *OS* overall survival, *AA* anaplastic astrocytoma, *GBM* glioblastoma, *GBM-s*, secondary GBM, *SRT* stereotactic radiotherapy, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *nd* not determined, *M* methylated promoter, *U* unmethylated promoter, *D*, dead

[a] Previous chemotherapy included bevacizumab

[b] The ratio of tumor volume at the first MR imaging post-bevacizumab treatment compared with that of the baseline

[c] The lowest and highest values of multiple lesions

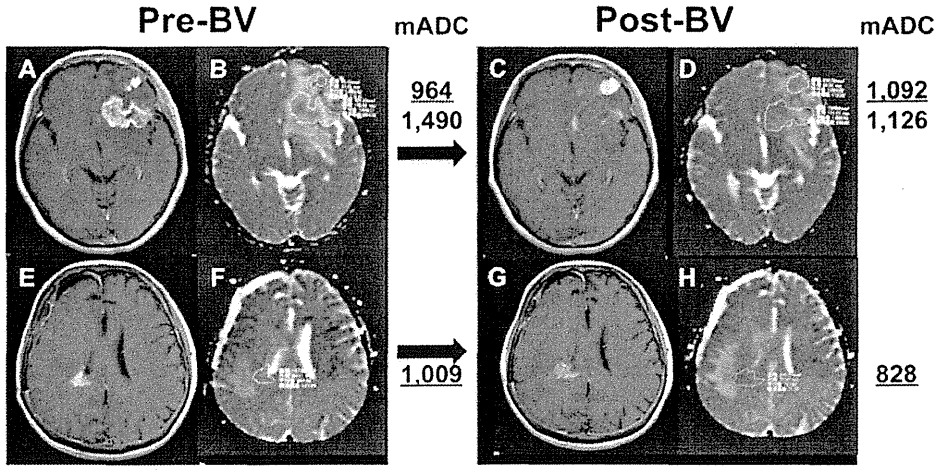

Fig. 2 Post-contrast T1-weighted images aligned side-by-side with the corresponding ADC maps in patients with tumors which did not respond to BV (**a, b, c, d**: case 3; **e, f, g, h**: case 6). Note that the non-responding lesions have mADC values below 1100 (10^{-6} mm^2/s). In case 3, the anterior frontal lesion with an initial mADC of 964 continues to grow, whereas the posterior frontal lesion that has undergone stereotactic radiotherapy before further progression shows an initial mADC of 1490 and its enhancement reduces after BV treatment

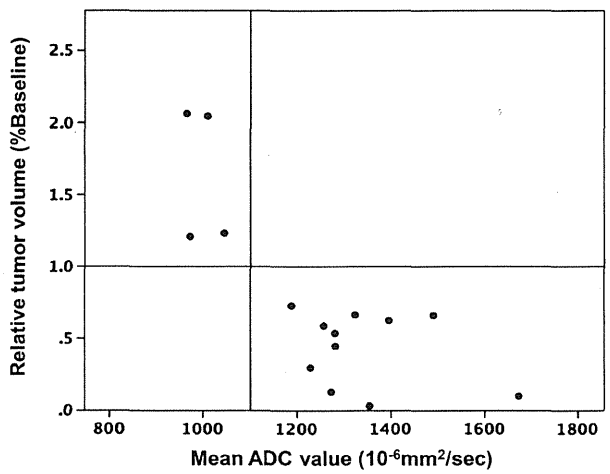

Fig. 3 Clear correlation between mean ADC values and changes in tumor size after a single BV treatment in recurrent high-grade glioma lesions. All lesions with mADC above 1100 (10^{-6} mm^2/s) shrink, while those with lower mADC continue growth (Fisher's exact test, $p = 0.001$)

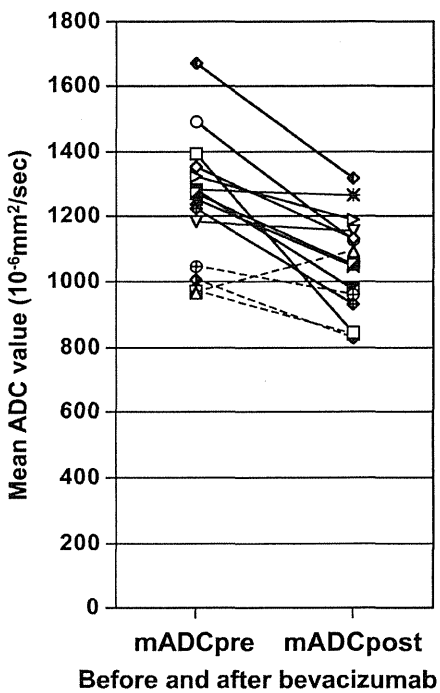

Fig. 4 Changes in mADC value between pre- and post-treatment with BV. Corresponding mADC value points of each lesion are connected with *lines*; the responding lesions are in *solid lines* and the non-responding are in *dashed lines*. mADC values of all lesions decrease after BV except for one non-responding

of tumor volumes at the first MR imaging to those at the second MR imaging taken after 2 more BV cycles (median interval, 39 days; range, 35–56 days) was measured (Table 2). Post-BV mADC significantly correlated with response to additional BV (Mann–Whitney *U* test, $p = 0.045$) and all five high post-BV mADC (>1100) lesions shrank after the additional BV treatments. Although three of eight (37.5 %) low post-BV mADC (<1100) lesions also decreased in size, the majority (5/8, 62.5 %) did not respond further, which approached statistical significance (Fisher's exact test; $p = 0.075$). These observations suggest that even the mADC immediately after the first BV treatment may be of predictive value for further response to BV.

Table 2 mADC values before and after the first bevacizumab treatment and the change in tumor volume

Case	Lesion	mADC (10^{-6} mm^2/s)		TV (%baseline) (1st F/U MRI)	TV (%baseline) (2nd F/U MRI)	TV (%1st MRI) (2nd F/U MRI)
		Before	After			
1	P/T	1394.2	842.5	62.7	21.8	34.9
2	T	1353.6	1135.8	3.3	0.0	0.0
3	F	1490.1	1125.6	66.1	63.6	96.3
	F-AL	964.3	1092.0	206.4	445.5	215.9
	F-AM	971.1	841.1	120.9	135.5	112.1
	F-MF	1227.6	930.0	29.8	30.4	103.1
	F-PM	1044.5	961.8	123.2	225.6	183.1
4	F-BG	1187.5	1155.4	72.8	15.2	20.9
5	T	1272.6	1053.0	12.9	0.0	0.0
6	T	1008.9	827.6	204.6	na	na
	F	1281.1	1267.7	44.8	na	na
7	CC	1323.4	1192.9	66.5	18.3	27.9
8	PT	1280.9	977.4	53.8	106.7	198.4
9	F	1672.4	1320.5	10.0	7.3	73.7
	F	1256.7	1046.5	58.9	50.1	85.1

TV, tumor volume, *F/U* follow-up, *P* parietal, *T* temporal, *F* frontal, *AL* antero-lateral, *AM* antero-medial, *MF* mid-frontal, *PM* premotor, *BG* basal ganglia, *CC* corpus callosum

Survival

After a median follow-up of 5.7 months (range, 2.9–16.0), all patients had progressed and died despite a high rate of early response. PFS from the initiation of BV therapy was 2.2 months [95 % confident interval (CI) 1.8–2.6 months]. PFS for two patients (cases 3 and 6) having a lesion with mADC value <1100 (low mADC) was short (0.4 months), whereas PFS for others having all lesions with mADC >1100 (high mADC) was significantly longer (2.3 months, 95 % CI 2.0–2.6) (log-rank test, $p = 0.018$). Median OS after the start of BV treatment was 5.7 months (95 % CI 5.1–6.2). Patients having all lesions with a high mADC (>1100) survived significantly longer (median 8.3 months, 95 % CI 1.4–15.2) than those with a low mADC tumor(s) (median 3.3 months, $p = 0.046$), despite the small sample number (9 cases). The tumor volume of enhancing lesions prior to initiation of BV was not associated with either PFS or OS (Table 1, data not shown).

VEGF expression in the original tumor specimens

Immunohistochemistry staining of VEGF-A was performed in the primary tumors and all but one were found to express VEGF to a variable extent (data not shown). Expression at the beginning of BV treatment was not determined due to lack of re-resection prior to BV therapy. The tumor with negative VEGF staining (case 6) did not respond to BV.

Relationship of mADC with *MGMT* promoter methylation status

While *MGMT* status is prognostic/predictive for survival in GBM patients [17, 18], tumoral mADC values were not associated with *MGMT* methylation status (Fisher's exact test; $p = 0.445$) in the seven GBM cases (Table 1). Taken together, while we observed that response of recurrent lesions after a single dose of BV was significantly correlated with a mean ADC value of the tumors above 1100 (10^{-6}mm^2/s), it was not with VEGF expression or *MGMT* status.

Discussion

Although BV has shown efficacy against TMZ-refractory GBMs, some tumors may also be resistant to BV, progressing to fatality [19], and thus determining their sensitivity to BV prior to initiation of the treatment could have significant clinical value. Here, we demonstrated that the pretreatment mADC value of enhancing tumors was predictive for initial response to BV monotherapy in recurrent HGGs. All lesions with the mADC above 1100 (10^{-6} mm^2/s) responded, in clear contrast to those with the mADC below 1100, which did not respond ($p = 0.001$) (Fig. 3). The mADC values decreased after the first BV treatment in all lesions, except for one that did not respond. The second mADC value obtained after the first BV treatment was also a good indicator for further response to additional BV when it remained above 1100 ($p = 0.045$). Furthermore, the high mADC value also significantly correlated with elongation of both PFS and OS after BV treatment in patients with TMZ-refractory recurrent HGGs.

Anti-VEGF therapy is expected to normalize vascular structure, capillary permeability and interstitial pressure more effectively in areas with strong edema and necrotic changes than in those with "packed" tumor cells. The ADC value represents movement of protons of water molecules and may be increased in areas where tissue edema and necrotic components have been induced by tissue damage from tumor burden and cytotoxic therapies [9–11]. This may account for our findings that glioma lesions with a high pretreatment ADC value shrank upon BV challenge whereas those with a low ADC value did not. Such non-responding lesions also tended to be strongly enhanced (e.g., a subcortical lesion in the left frontal lobe in Fig. 2a), consistent with the observation that GBMs that relapsed after BV treatment exhibited low ADC values and hyperintensity on diffusion-weighted images [20].

Pope et al. reported that PFS correlated with crude average ADC values within areas showing contrast enhancement in 41 recurrent GBM cases who underwent BV treatment. Of note, when the average ADC value of the lower peak of biphasic peaks in ADC histograms ($mADC_L$) was lower than 1201, PFS was significantly extended [21]. Our use of whole ADC values within an enhancing tumor to calculate a mean value on a PACS terminal in clinics without specific software successfully segregated responding from non-responding lesions. This simple method might incorporate regions of extremely high ADC values containing necrotic tissues, resulting in a shift of the mean value toward a higher value, compared with the histogram-based analysis. However, regions exhibiting apparent necrosis or cyst could be readily excluded when defining a tumor on each ADC map, in order to avoid such data contamination. We also eliminated T2-elongated areas surrounding contrast-enhancing lesions because they may contain both edematous white matter and non-enhancing tumor to a variable extent. As a result, our data show clear segregation of lesions for response by ADC value at 1100 (10^{-6} mm^2/s), smaller than the cut-off value of 1201 in the Pope study [21]. It would be beneficial in daily practice to use this simple measurement, as it does not require specific histogram analysis, though it needs further accumulation of cases for validation.

Conflicting findings that BV induces rapid shrinkage of the enhancing lesions while they subsequently regrow in a relatively short term might represent, at least partly, the recently recognized "pseudo-response," a decrease in enhancing tumor on MR imaging without a decrease in tumor activity [22]. This has been reported in a clinical trial where radiation necrosis was successfully treated with BV [23]. To date, there are no validated imaging methods to determine whether the observed shrinkage of areas of contrast enhancement were due to real tumor reduction or pseudo-response, as well as progression without an emergence or increase of enhancing lesion [24]. Indeed, the potential value of changes in T2 relaxation time is recently suggested by an observation that an elevated residual, post-treatment, median T2 may be predictive of both PFS and OS [25]. Efforts to clarify these issues include application of the newly-developed response criteria, Response Assessment in Neuro-Oncology (RANO), utilizing elongation of T2 relaxation time as a surrogate for non-enhancing tumor [26], and MR imaging techniques such as MR perfusion studies that are under investigation in large trials for validity. In our study, there were two patients (cases 3 and 8) who underwent stereotactic RT on recurrence prior to BV initiation, and one patient (case 5) who received BV within 3 months after completion of induction-concomitant RT plus TMZ. Two of these three patients had a PR by single BV treatment with further progression in 2 and 5.5 months, indicating no clear relationship between response and potential radiation injury.

A recent study reported that the lower ADC_L value rather correlated with longer PFS for patients with newly-diagnosed GBM [27], in contrast to recurrent GBM. It also showed an association between ADC values and *MGMT* methylation status [27], which was not observed in our series. Whether this association might be influenced by the difference of tumor microstructure in the setting of newly-diagnosed or treatment-modified recurrent tumors needs to be clarified.

Limitations of our study include the small number of patients and lesions analyzed, heterogeneous prior treatment regimens applied in some patients, and specimens from primary newly-diagnosed tumors used for determination of VEGF and *MGMT* status. This might hamper drawing definite conclusions about the relationship of the mADC value with survival gain or VEGF/*MGMT* status, as well as analyzing whether pathological findings of treated tumors may also correlate with either mADC values or responsiveness to BV, or both. However, even with the small sample size, a clear segregation of responders from non-responders and survival prediction were seen using our simplified mADC measurement and this warrants further investigation in a larger series.

Conclusions

Bevacizumab monotherapy is an active regimen for patients with TMZ-refractory recurrent gliomas leading to rapid lesion shrinkage, and the tumor mADC value can be a useful marker for prediction of BV response and survival, and thus for patient selection. It would be also intriguing to investigate methods of delineating tumors in which BV would provide long-term response in the future.

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Conflict of interest The authors declare that they have no conflict of interest.

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CLINICAL STUDY – PATIENT STUDY

O^6-methylguanine-DNA methyltransferase promoter methylation in 45 primary central nervous system lymphomas: quantitative assessment of methylation and response to temozolomide treatment

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Abstract Favorable responses to temozolomide chemotherapy have recently been reported in primary central nervous system lymphoma (PCNSL) patients who are refractory to high-dose methotrexate therapy. The gene encoding the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) is transcriptionally silenced by promoter methylation in several human tumors, including gliomas and systemic lymphomas. *MGMT* promoter methylation is also a prognostic marker in glioblastoma patients treated with temozolomide. To validate temozolomide treatment in PCNSL, we applied methylation-sensitive high resolution melting (MS-HRM) analysis to quantitate *MGMT* methylation in PCNSL. *MGMT* promoter methylation was detected in tumors from 23 (51%) of 45 PCNSL patients, 11 of which were considered to have high (more than 70.0%) methylation status. Of the five recurrent PCNSLs treated with temozolomide, four cases responded, with three achieving complete response and one, a partial response. All four responsive PCNSLs had methylated *MGMT* promoters, whereas the non-responsive recurrent PCNSL did not. Thus, the use of quantitative MS-HRM analysis for the detection of *MGMT* promoter methylation has been suggested in PCNSL for the first time. The assay allows rapid and high-throughput evaluation of the *MGMT* methylation status, and seems to be promising in clinical settings. *MGMT* promoter methylation may become a useful marker for predicting the response of PCNSLs to temozolomide.

Keywords Primary central nervous system lymphoma · MGMT · Temozolomide · Chemotherapy

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Introduction

Primary central nervous system lymphoma (PCNSL) is characterized by rapid progression, frequent cerebrospinal fluid involvement, and early recurrence [1]. The introduction of combined pre-irradiation high-dose methotrexate (HD-MTX) with radiation therapy has resulted in a median survival time of 33 months [2, 3]. HD-MTX, however, is associated with considerable systemic toxicity affecting renal, cardiac, and hematological function [4]. There are also important limitations of radiation therapy, including an increased risk of late neurotoxicity, especially in elderly patients [5]. Thus, there is a need for an alternative chemotherapy that is both efficient and well-tolerated. Cyclophosphamide–adriamycin–vincristine–prednisolone (CHOP) chemotherapy is the standard chemotherapy for systemic malignant lymphoma, but the drugs do not penetrate the brain effectively and do not improve survival of patients with PCNSL [6, 7]. Temozolomide is an alkylating agent with good cerebrospinal fluid penetration [8], and there have been some reports of temozolomide efficacy with acceptable toxicity in cases of PCNSL [9–12].

Previous studies on glioma and systemic lymphoma associated the presence of the methylated form of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene

with a more favorable response to alkylating agents [13–15]. MGMT is a DNA repairing enzyme that removes alkyl adducts from the O^6 position of guanine and prevents the cytotoxic, mutagenic, and carcinogenic effects of alkylating agents such as temozolomide [16–19]. It has been demonstrated that silencing of *MGMT* is involved in carcinogenesis in humans [20, 21]. The loss of *MGMT* function in human cancer cells is mainly due to the hypermethylation of CpG islands in the gene's promoter sequence [22, 23]. Therefore, evaluation of the methylation status of the *MGMT* promoter is important to validate temozolomide treatment in PCNSL.

In this study, we applied, for the first time, methylation-sensitive high resolution melting (MS-HRM) analysis to quantify the *MGMT* methylation status in 45 cases of PCNSL. In addition, the *MGMT* methylation level was compared with the clinical response to temozolomide treatment.

Patients and methods

Patients and treatment

We studied 45 patients with newly diagnosed PCNSL between 2002 and 2011. Tumor samples were obtained by surgical resection or biopsy prior to therapy. On the basis of pathological examination, a diagnosis of diffuse large B cell lymphoma was made in all cases. Written informed consent for use of their tissues was obtained from study subjects.

The primary treatment was high-dose methotrexate (HD-MTX)-based chemotherapy in 43 patients, and exclusive radiation therapy (RT) in one patient, and one patient received no adjuvant treatment after surgery. The MTX dose was 1–3.5 g/m^2. MTX was used as a single agent in 15 patients, and was combined with 375 mg/m^2 rituximab in 28 patients. After primary chemotherapy, RT was performed in 32 of 43 cases. The remaining 11 cases were patients older than 65 years, and were treated without RT to avoid cognitive dysfunction due to radiation-induced leukoencephalopathy. We continued HD-MTX-based chemotherapy for these 11 patients until the tumor progressed.

Fourteen patients received oral temozolomide at 150–200 mg/m^2 per day for 5 days every 4 weeks (Table 1). Five (cases 1–5) of 14 cases were treated with temozolomide as salvage chemotherapy upon relapse after standard HD-MTX/radiation therapy. For the remaining nine patients (cases 6–14), temozolomide was used as adjuvant chemotherapy after first-line HD-MTX therapy.

DNA extraction and *MGMT* quantitative methylation assay

Genomic DNA was extracted from fresh frozen (43 of 45 cases) or paraffin-embedded formalin-fixed (2 of 45 cases)

Table 1 Summary of 14 PCNSL patients treated with temozolomide

Case No.	Age at temozolomide treatment (years)/ gender	No. of temozolomide cycles[a]	Maximum response to temozolomide	Duration (months) keeping CR or PR	Therapy prior to temozolomide treatment	Degree of *MGMT* promoter methylation[b]
1	75/M	19	CR	38	M, R, RT	61.7 ± 4.04
2	60/M	3	CR	12	M, R, RT	99.0 ± 1.00
3	47/M	5	PR	7	M, R, RT	72.3 ± 2.52
4	72/M	2	PD		M, R, RT	1.3 ± 1.26
5	50/M	8	CR	7	M, R, ICE, RT	94.3 ± 2.50
6	64/F	2	CR	11	M	7.7 ± 0.58
7	73/F	6	CR	11	M, RT	0.8 ± 0.96
8	70/M	5	CR	21	M, RT	1.5 ± 1.28
9	55/M	4	CR	27	M, R, RT	26.7 ± 0.58
10	57/M	3	CR	24	M, R, RT	1.8 ± 0.96
11	54/M	3	PD		M, R, RT	32.0 ± 1.83
12	62/F	2	CR	24	M, R, RT	0.3 ± 0.50
13	72/M	10	PR	9	M, R, RT	1.0 ± 0.82
14	85/F	9	CR	7	M, R	74.7 ± 0.58

Cases 1–5 were treated with temozolomide as salvage chemotheapy at relapse after standard HD-MTX/radiation therapy. Cases 6–14 were treated with adjuvant temozolomide therapy after first-line HD-MTX therapy

CR complete response; *PR* partial response; *PD* progressive disease

M HD-MTX; *R* rituximab; *ICE* Ifosfamide + Cisplatin + Etoposide; *RT* radiation therapy

[a] Patients received oral temozolomide for 5 days every 4 weeks

[b] Percentage of *MGMT* methylation level in our assay. The mean ± standard deviation is indicated

Table 2 The primers designed to target the promoter sequences of the *MGMT* gene

	Primer sequences 5′–3′
MGMT MS-HRM[a]	F-GCGTTTCGGATATGTTGGGATAGT
	R-CCTACAAAACCACTCGAAACTACCA

[a] Methylation-sensitive high-resolution melting

brain biopsy specimens using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

We quantified the methylation status of the *MGMT* promoter's CpG-rich region using MS-HRM analysis [24]. Briefly, 500 ng of genomic DNA was treated with sodium bisulfite using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification and MS-HRM analysis were carried out sequentially on a LightCycler480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). The primer sets to amplify 18 CpG-rich sites in the *MGMT* promoter were designed according to previous reports (Table 2) [24, 25]. Each PCR run contained 2 μl of bisulfite-converted DNA in a total volume of 20 μl: 2× master mix containing high-resolution melting dye (Roche Diagnostics, Mannheim, Germany), 4 mM Mg^{2+}, and 500 nM of each primer. Cycling conditions were 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 60°C for 20 s. The melting step was 95°C for 1 min, 50°C for 1 min, 72°C for 5 s, and continuous acquisition to 95°C at 30 acquisitions per 1°C. As positive (100% methylated) and negative (0% methylated) controls, we used CpGenome™ Universal Methylated and Unmethylated DNA (Chemicon, Millipore, Billerica, MA), respectively. The duration of the run from the onset of PCR was approximately 80 min. All reactions were performed at least in triplicate.

Methylation-specific polymerase chain reaction (MSP)

Bisulfite-treated DNA was also subjected to MSP assay of *MGMT* promoter methylation in 14 PCNSL patients treated with temozolomide. We developed nested PCR using primers described in Esteller et al. and Palmisano et al. [23, 27]. The first round of PCR was performed to amplify a 289 bp fragment of the *MGMT* gene including a portion of their CpG-rich promoter region. Primer sequences used in the first round amplification are as follows: 5′-GGA TATGTTGGGATAGTT-3′ (sense) and 5′-CCAAAAACC CCAAACCC-3′ (antisence). 5 μl of the first round PCR products was subjected to the second round PCR in which primers specific to methylated or unmethylated template. A 93 bp unmethylated product was amplified using the primers: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTG T-3′ (sense) and 5′-AACTCC AACACTCTTCCAAA AACAAAACA-3′ (antisense). An 81 bp methylated product was amplified using the following primers: 5′-TTT CGACGTTCGTAGGTTTTCGC-3′ (sense) and 5′-GCAC TCTTCCGAAAACGAAACG-3′ (antisense). The PCR mixture contained 5 μl DNA, 10× PCR Buffer (TaKaRa), 5 nmol each deoxynucleotide, 2.5 pmol of each primer and 0.8 U *Taq* HS (TaKaRa), in a final volume of 50 μl. The first round PCR cycling conditions were as follows: 95°C for 10 min, then denature at 95°C for 30 s, anneal at 52°C for 30 s, extension at 72°C for 30 s for 40 cycles followed by a 10 min final extension. In the second round PCR, annealing temperature was increased to 62°C and all of the cycling times were reduced to 15 s for a total of 34 cycles. An aliquot of 10 μl of the second round PCR was loaded onto a 12.5% polyacrylamide gel, stained with ethidium bromide and visualized under UV illumination.

Results

Melting data collected using the LightCycler480 Instrument can be analyzed by the "Tm (Melting Temperature) calling" algorithm that converts the melting profiles into derivative plots, which allows methylated and unmethylated samples to be distinguished. Products amplified from methylated DNA have a higher Tm due to the presence of CpGs in the amplicon. In contrast, products amplified from unmethylated DNA have a low Tm due to the conversion of unmethylated cytosine to uracil in the bisulfite-modified DNA sample, which results in thymine in the amplicon. If the sample contains a mixture of methylated and unmethylated DNA, two peaks are displayed, as shown in Fig. 1. This assay was able to detect 1.0% methylated DNA over a background of unmethylated DNA. We used the LightCycler480 Gene Scanning Software ver.1.5 to generate standard curves for methylated *MGMT* using serial samples having known ratios of methylated to unmethylated template. The *MGMT* methylation level of an unknown sample could then be estimated from these standard curves. The samples were analyzed at least in triplicates, and we determined the methylation levels from the average value of the experiments. A cut-off value of 4.0% methylated *MGMT* was previously validated by strong correlation of protein expression loss with MGMT promoter methylation [26]. Therefore, samples with an average *MGMT* methylation level of less than 4.0% were defined as unmethylated.

Of the 45 PCNSLs examined by MS-HRM assay, all samples were amplifiable and gave interpretable results. The *MGMT* promoter was methylated in 23 PCNSLs (51%), and was unmethylated in the remaining 22 cases

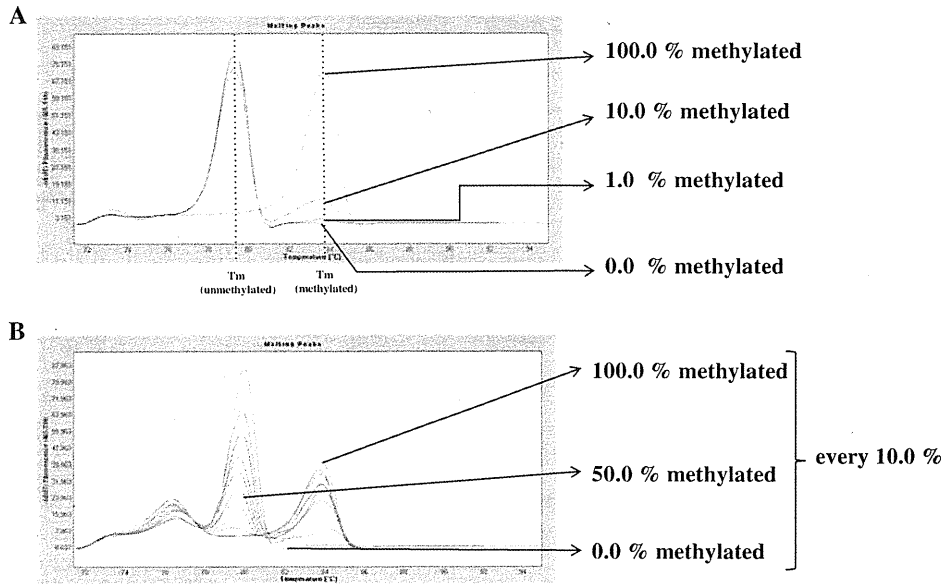

Fig. 1 **A** *MGMT* methylation-sensitive high-resolution melting (MS-HRM) analysis with 100.0% methylated, 0.0% methylated controls, and methylation standards at 10.0 and 1.0% in 0.0% methylated background. Data were analyzed using the Tm calling software module. Two different peaks are obtained for the PCR product derived from the methylated and unmethylated templates. The samples containing a mixture of methylated and unmethylated DNA (methylation standards at 10.0 and 1.0% in 0.0% methylated background) display two peaks. Tm (unmethylated), melting temperature derived from unmethylated DNA; Tm (methylated), melting temperature derived from methylated DNA. **B** *MGMT* MS-HRM analysis with 100.0% methylated, 0.0% methylated, and serially diluted standards at every 10.0%. Data were analyzed using the Tm calling software module

(Table 3). The average methylation level in the 23 methylated samples ranged from 4.1 to 100%. Tumors with a high methylation status (more than 70.0% methylated) were detected in 11 (48%) of the 23 *MGMT*-methylated cases.

Four of five patients with recurrent PCNSLs responded to temozolomide treatment; two cases had a complete response (CR) over 12 months after 1 or 2 cycles of temozolomide, two patients had a partial response (PR), and the remaining patient had progressive disease (Fig. 2). Tumors of all four responders showed relatively high methylation (61.7–99.0%) of the *MGMT* gene (Table 1), and significant *MGMT* methylation was not detected in the patient with progressive disease. In MSP, both unmethylated and methylated *MGMT* were detected in all patients including three cases demonstrated in Fig. 3. It was quite difficult to evaluate the degree of *MGMT* methylation from the result of MSP.

Of the nine cases treated with adjuvant temozolomide after first-line HD-MTX therapy, eight achieved a CR or PR of 7 to 27 months with temozolomide. In addition to HD-MTX and temozolomide, these nine patients received differing treatments (rituximab plus RT in five patients, RT in two patients, rituximab in one patient, and none in one patient). Therefore, we did not investigate the relationship between survival time and methylation status in the patients receiving adjuvant temozolomide.

Table 3 Methylation status of the *MGMT* promoter in 45 PCNSL patients

Degree of *MGMT* promoter methylation (%)[a]	No.
<4.0 (=unmethylated cases)	22
4.1–9.9	3
10.0–19.9	1
20.0–29.9	2
30.0–39.9	2
40.0–49.9	2
50.0–59.9	0
60.0–69.9	2
70.0–79.9	4
80.0–89.9	2
90.0–100.0	5

[a] Percentage of *MGMT* methylation level in our assay

Discussion

The *MGMT* gene is known to be methylated in some systemic B-cell lymphomas [15, 28, 29], but to our knowledge, there has been only one report of *MGMT* methylation in PCNSL[30]. In that study, 6 (60%) of 10 assessable PCNSL patients had methylated MGMT promoters, as measured by gel-based MSP, which is a highly sensitive method to determine epigenetic silencing of genes. As shown in our MSP assay, MSP can detect very low levels

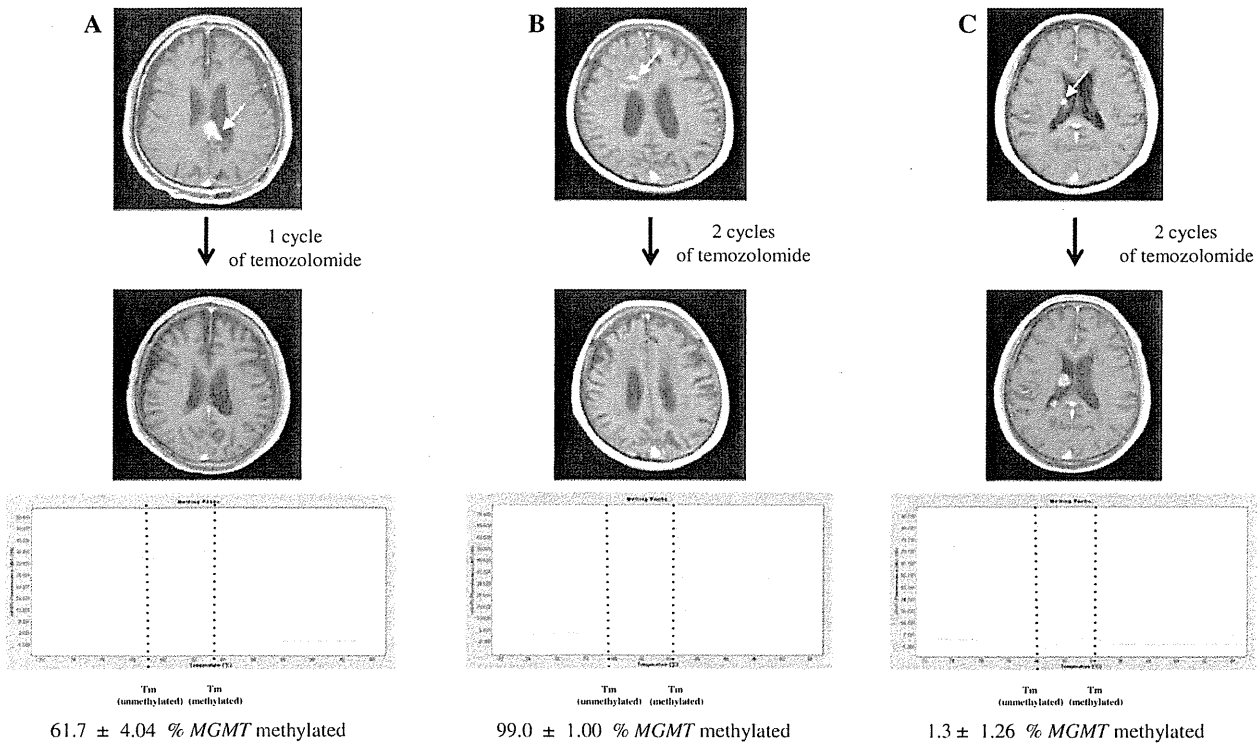

Fig. 2 Relapsed PCNSL cases treated with temozolomide as salvage chemotherapy. **A–C** are cases 1, 2, and 4 in Table 1, respectively. Upper panels show gadolinium-enhanced T1-weighted magnetic resonance images before and after temozolomide treatment. The *white arrows* indicate relapsed tumors. After chemotherapy, tumors completely disappeared in cases 1 and 2, whereas the tumor progressed in case 4. Lower panels show the representative profile of *MGMT* methylation in each case. The mean percentage ± standard deviation of *MGMT* methylation is indicated at the *bottom*

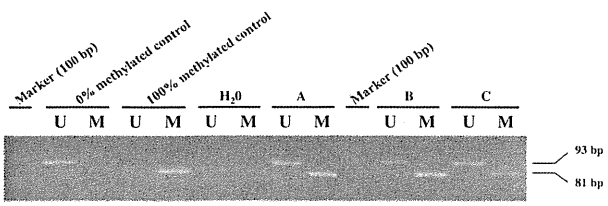

Fig. 3 Methylation-specific PCR results of *MGMT* promoter in cases shown in Fig. 2. PCR products in *lanes* marked as (*U*) indicate the presence of unmethylated *MGMT* alleles. PCR products in *lanes* marked as (*M*) indicate the presence of methylated *MGMT* alleles. 0% methylated control was used as a negative control for methylation, 100% methylated control was used as a positive control for methylation, and water (H_2O) was used as negative PCR control. Marker is a 100 bp DNA marker. The sizes of PCR products are indicated on the right scale. **A–C** are cases 1, 2, and 4 in Table 1, respectively

of DNA methylation or unmethylation. However, several recent studies have raised serious concerns about the application of MSP in a clinical setting. Because of the qualitative nature of the assay, MSP cannot distinguish between high levels of methylation and low levels that have little or no biological significance. Ogino et al. [26] showed that MGMT protein expression was not silenced in tumors with low levels of methylation (less than 4%) in the *MGMT* promoter . Uccella et al. [29] demonstrated that the *MGMT* methylation status correlated with survival in systemic B-cell lymphoma patients treated with alkylating agents; the outcome of survival analysis was unfavorable both in patients with less than 4.0% *MGMT* methylation and in patients with fully unmethylated tumors. Our assay identified some cases with negligible methylation (less than 4.0%), which should have been considered as unmethylated but were defined as methylated by the MSP method. Therefore, it seems reasonable that the *MGMT* methylation frequencies in our series were slightly lower than that found in a previous study using MSP [30]. In addition, a recent study suggested that the MSP assay for *MGMT* methylation is not sufficiently reproducible to make it suitable for clinical use [31]. Thus, a quantitative assay should be used to evaluate *MGMT* promoter methylation in clinical settings. Our study is the first to quantify *MGMT* methylation levels in 45 PCNSLs, which is the largest number of samples reported so far.

Salvage temozolomide treatment has been effective for PCNSL patients who experienced a relapse after HD-MTX chemotherapy. Although cases were small in number, our