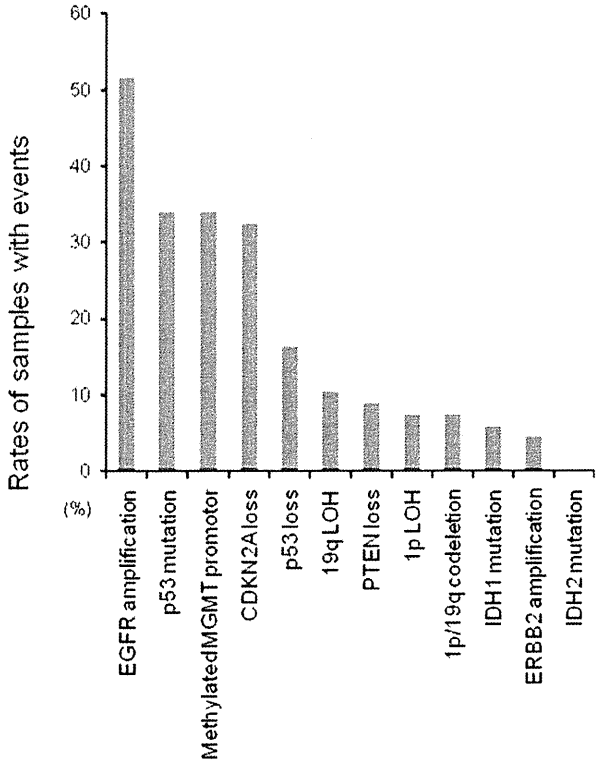


Figure 1. Frequency and pattern of genetic and epigenetic alterations in newly diagnosed primary glioblastoma multiforme (GBM).

arrhythmia and no serious ischemic heart disease. All patients received the standard Stupp regimen,[1] and among these, 39 patients were received combination treatment with IFN-β, as described in the method section.

Frequency of Genetic and Epigenetic Alterations

Of 68 cases, we could obtain sufficient genetic and epigenetic information in all cases. We used direct sequencing for *TP53* and *IDH1/2*. We employed MLPA for the analysis of 1p/19q LOH, loss of *TP53, PTEN* and *CDKN2A*, and amplification of *ERBB2* and *EGFR*. MLPA is a multiplex PCR method that detects abnormal copy numbers of up to 50 different genomic DNA sequences simultaneously. When comparing MLPA to FISH, MLPA not only has the advantage of being a multiplex technique but also one in which very small (50-70 nt) sequences are targeted, enabling MLPA to identify the frequent, single gene aberrations that are very small to be detected by FISH. Furthermore, for the detection of *EGFR* amplification, MLPA can examine exons 1-8, 13, 16, and 22, while previously reported real-time PCR covers only exons 2, 17, and 25. In our preliminary experiments, MLPA was found to be approximately 80% consistent with the real-time PCR method (data not shown). Notably, the methylation status of the MGMT promoter was analyzed by quantitative pyrosequencing technology. Although methylation-specific PCR analysis of MGMT promoter methylation is a widely applicable biomarker for the clinical setting, it is non quantitative and bears a risk of false-positive or false-negative results, especially when the DNA quality and/or quantity is low. Recent attempts to remedy some of these deficiencies have led to the development of an alternative sequence-based approach for methylation analysis, known as pyrosequencing. Pyrosequencing yields continuous methylation values ranging from 0-100%. Based on our comparisons with standard methylation-specific PCR and immunohistochemical study using the anti-MGMT antibody, we determined 14% as the threshold distinguishing unmethylation and methylation of the MGMT promoter in a given tumor.

As indicated in Figure 1 and Table 3, the alterations frequently observed were *EGFR* amplification (51.5%), *TP53* mutation (33.8%), *CDKN2A* loss (32.4%), *TP53* loss (16.2%), methylation of the MGMT promoter (33.8%), and *IDH1* mutation (5.9%). These findings were consistent with those in previous reports.[3,9,20,21]

Clinical, Genetic, and Epigenetic Parameters Associated With Survival in GBM Patients

The median follow-up time was 16.7 months (range, 3.4-46.7 months). The median PFS for all patients was 9.2 months (95% confidence interval [CI], 5.7-12.7). The median OS of all patients was 17.1 months (95% CI, 15.5-18.7) (Figure 2A). The log-rank tests demonstrated that tumor localization ($P = .032$), the MGMT methylation status ($P = .029$), and *TP53* mutation or loss ($P = .035$) were associated with the OS of patients with GBM (Figure 2B-D). These findings were similar to univariate analysis, where deep location ($P = .035$), unmethylated MGMT promoter ($P = .033$) and *TP53* mutation or loss ($P = .038$) were identified as candidate variables for poorer OS (Figure 2). In contrast, well-established prognostic factors such as age, ECOG PS, and the extent of tumor resection did not influence the outcome in this clinical setting. Next, we established multivariate survival models for OS. The model was designed to consider each of these factors without considering the interaction terms. The independent prognostic factors for OS were methylated MGMT promoter ($P = .016$).

Table 3. Relation Between Genetic and Epigenetic Parameters and Overall Survival

Parameter	No.	Months of OS	Log-rank test: *P*
1p LOH			
+	5	16.9	.27
–	63	21.9	
19q LOH			
+	7	17.1	.46
–	61	21.9	
1p/19q codeletion			
+	5	16.9	.27
–	63	21.9	
***PTEN* loss**			
+	6	21.4	.40
–	62	16.9	
***CDKN2A* loss**			
+	22	16.3	.64
–	46	17.4	
***TP53* loss**			
+	11	11.7	**.08**
–	57	17.4	
***ERBB2* amplification**			
+	3	13.9	.77
–	65	17.1	
***EGFR* amplification**			
+	35	17.4	.91
–	33	17.1	
***TP53* mutation**			
+	23	15.7	.128
–	45	17.6	
***TP53* mutation or loss**			
+	29	13.9	**.035**
–	39	17.6	
MGMT promotor			
Unmethylated	45	15.1	**.029**
Methylated	23	21.4	
***IDH1* mutation**			
+	4	19.9	.96
–	64	16.9	
***IDH2* mutation**			
+	0	NA	NA
–	68	NA	

OS indicates overall survival; NA, not available

Combination of IFN-β With TMZ Prolonged Survival

We analyzed whether the use of IFN-β affected the survival of consecutive GBM patients treated with TMZ-based chemotherapy. Of the total 68 patients, 39 (57.4%) received IFN-β in combination of TMZ. Interestingly, the median OS of the combination group was significantly greater with 19.9 months (95% CI, 15.3-24.5) as compared to the TMZ alone group, which was 12.7 months (95% CI, 10.5 to 14.9) (Figure 3A). The 12-month-survival rate was 67.6% for the standard TMZ-treated cohort, whereas it was 83.6% for the combination group. The 24-month survival rates were 22.1% and 34.5%, respectively, for the 2 groups. The difference was statistically significant as determined by the log-rank test and univariate and multivariate analyses.

Benefits of IFN-β for GBM Patients With the Unmethylated MGMT Promoter

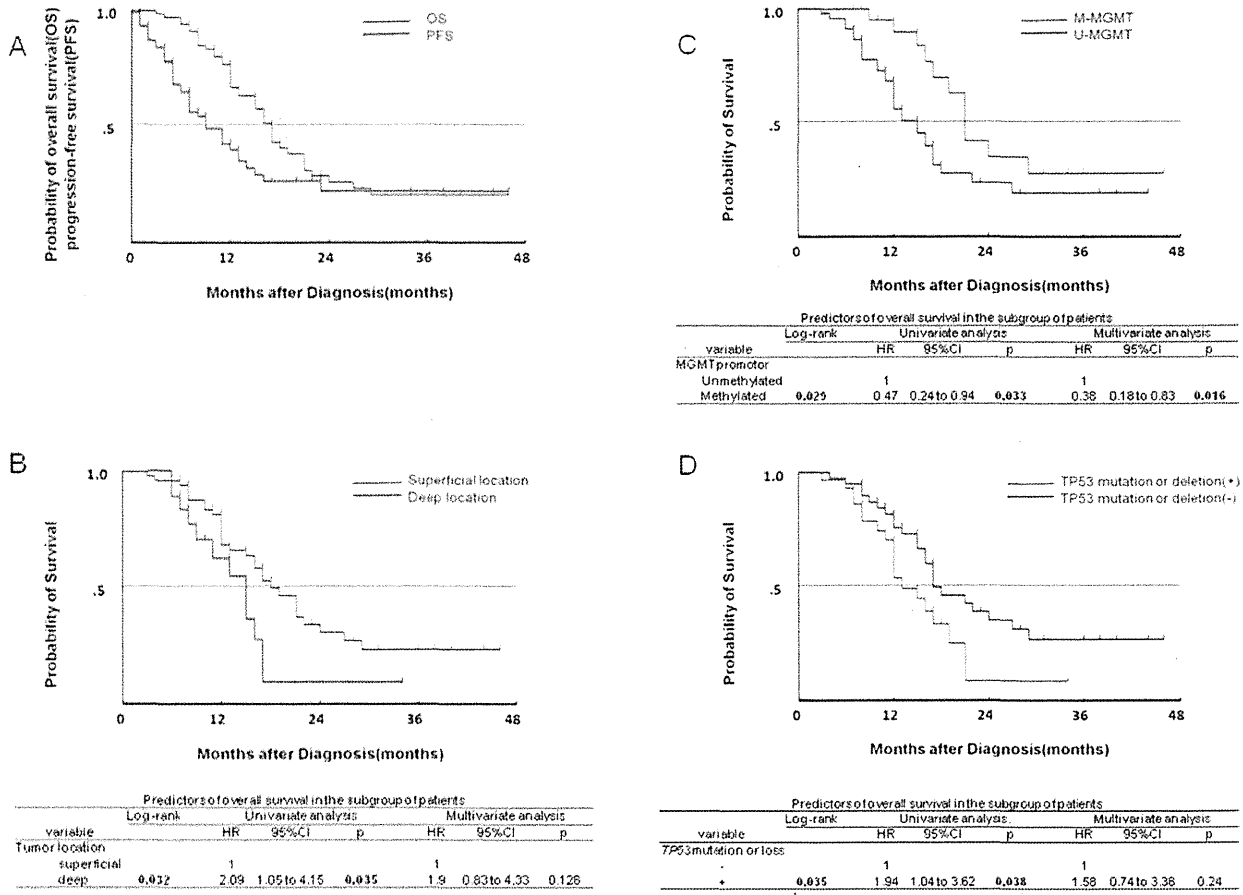
Next, we sought to determine the subpopulation that had benefited from the use of the IFN-β combination treatment. It is well known that patients with GBM containing the methylated MGMT promoter benefit from TMZ, but those with the unmethylated MGMT promoter show no such benefits.[1,2] Consistently, the median OS of 45 patients with the unmethylated MGMT status was significantly lesser than that of the patients with the methylated promoter (median OS = 15.1 months; 95% CI, 11.3-18.9). Notably, even in patients whose tumors had the unmethylated MGMT promoter, the median OS was prolonged to 17.2 months (95% CI, 13.9-20.6) when receiving TMZ with IFN-β as compared to the 12.5 months (95% CI, 11.3-13.7) in those receiving TMZ without IFN-β ($P = .017$) (Figure 3B).

Various associations of these clinical and molecular parameters were evaluated. A complete overview of the pairwise associations between these parameters and chemotherapy with or without IFN-β is provided in Figure 4. The relative hazards of OS between TMZ with or without IFN-β groups according to 6 baseline covariates, calculated by means of multivariate analysis, are shown. There were significant associations among patients under 40 years of age ($P = .025$), with ECOG PS ≤ 1 ($P = .004$), deep tumor location ($P = .028$), non-GTR ($P = .048$), and ummethylated MGMT status ($P = .02$) (Figure 4).

DISCUSSION

Genomic Analysis in Newly Diagnosed GBMs

In this study, we analyzed the genomic abnormalities in 68 consecutive newly diagnosed patients with GBM who were treated with TMZ-based chemotherapy. We observed *TP53* mutation (33.8%), *TP53* loss (16.2%), *EGFR* amplification (51.5%), *CDKN2A* loss (32.4%),



Predictors of overall survival in the subgroup of patients

variable	Log-rank	Univariate analysis HR	95%CI	p	Multivariate analysis HR	95%CI	p
MGMT promotor							
Unmethylated		1			1		
Methylated	0.025	0.47	0.24 to 0.94	0.033	0.38	0.18 to 0.83	0.016

Predictors of overall survival in the subgroup of patients

variable	Log-rank	Univariate analysis HR	95%CI	p	Multivariate analysis HR	95%CI	p
Tumor location							
superficial		1			1		
deep	0.032	2.09	1.05 to 4.15	0.035	1.9	0.83 to 4.33	0.126

Predictors of overall survival in the subgroup of patients

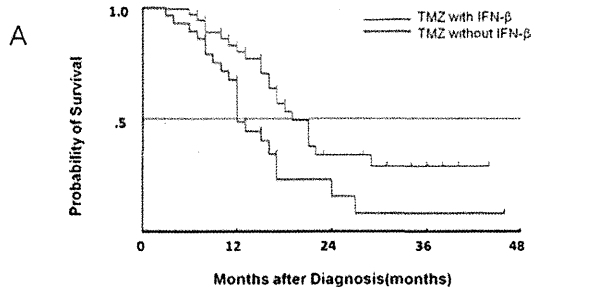
variable	Log-rank	Univariate analysis HR	95%CI	p	Multivariate analysis HR	95%CI	p
TP53 mutation or loss							
-		1			1		
+	0.035	1.94	1.04 to 3.62	0.038	1.58	0.74 to 3.38	0.24

Figure 2. Kaplan-Meier curves showing overall survival (OS) and progression-free survival (PFS) for the entire cohort (A), and OS according to (B) tumor location ($P = .032$), (C) MGMT promoter methylation status ($P = .029$), and (D) *TP53* mutation or loss ($P = .035$) (D). Predictors of overall survival in the subgroups of patients by univariate and multivariate analyses were shown (B-D). The hazards ratio (HR) was adjusted for the factors; age, Eastern Cooperative Oncology Group performance status (ECOG PS), the extent of tumor resection, MGMT promoter methylation status, *TP53* mutation or loss and TMZ with or without interferon-β (IFN-β) in the multivariate analysis.

and methylation of the MGMT promoter (33.8%). Recent large-scale efforts to characterize the GBM genome have identified additional alterations in genes not previously implicated in glioma, such as *ERBB2* and *IDH1/IDH2* mutation in primary and secondary GBM, respectively, and a significant incidence of mutation and genomic loss of *NF1*.[3,4,6] The TCGA study also noted *TP53* mutations and losses in 35% of the cases, which is a surprisingly higher frequency than that reported previously.[3,20,21] Furthermore, this study also revealed *EGFR* amplification (45%), *CDKN2A* loss (52.0%), and methylation of the MGMT promoter (20.9%). These results were consistent with our data. *IDH1* mutations have recently been identified in gliomas, which are a strong predictor of a more favorable prognosis.[6] Our study supported the finding that within the group of primary GBM, *IDH1* mutations are rare and tend to define a prognostically favorable outcome.

Factors for Prognosis and Prediction of Response to Therapy

The current study demonstrated that the methylated MGMT promoter and the combination of IFN-β and TMZ were independent prognostic indicators of GBM patients on multivariate analysis. Epigenetic silencing by the MGMT promoter methylation correlates with improved survival in glioma patients treated with TMZ.[2,22-25] The prognostic significance of MGMT promoter methylation has been shown in several clinical trials. In these studies, MGMT promoter methylation was an independent favorable prognostic factor and patients whose tumor contained a methylated MGMT promoter

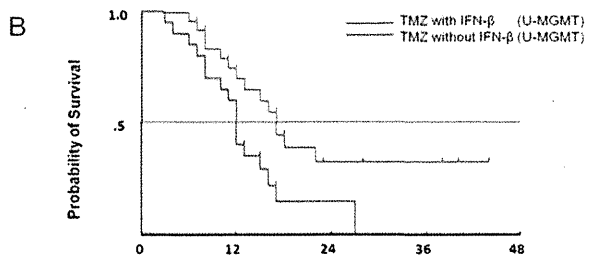


Predictors of overall survival in the subgroup of patients

	Log-rank	Univariate analysis			Multivariate analysis		
Variable		HR	95%CI	p	HR	95%CI	p
Chemotherapy							
TMZ without IFN-β		1			1		
TMZ with IFN-β	**0.007**	0.43	0.23 to 0.81	**0.008**	0.46	0.23 to 0.92	**0.028**

Survival data

	All patients	TMZ with IFN-β	TMZ without IFN-β
Median OS (months)	17.1	19.9	12.7
12M-OS (%)	77	84	68
24M-OS (%)	29	35	22
Median PFS (months)	9.2	11.6	9.2
12M-PFS (%)	41	47	34



Survival data of patients with unmethylated MGMT

	All patients	TMZ with IFN-β	TMZ without IFN-β
Median OS (months)	15.1	17.2	12.5
12M-OS (%)	68	75	60
24M-OS (%)	23	32	14

Figure 3. Kaplan-Meier estimates of overall survival (OS) according to temozolomide (TMZ) with or without interferon-β (IFN-β) for all patients (A) ($P = .007$) and for patients with unmethylated MGMT promoter (U-MGMT) (B) ($P = .017$). The hazards ratio (HR) was adjusted for the factors; age, Eastern Cooperative Oncology Group performance status (ECOG PS), the extent of tumor resection, MGMT promoter methylation status, *TP53* mutation or loss, and TMZ with or without IFN-β in the multivariate analysis.

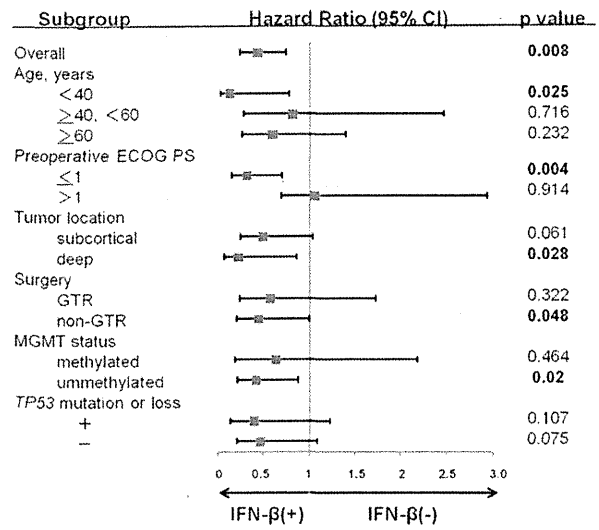


Figure 4. Estimated effect of temozolomide (TMZ) with interferon-β (IFN-β) versus TMZ without IFN-β on the hazard of overall survival (OS), according to baseline characteristics. The hazard ratio was computed using a proportional hazard model by selected factors. There were significant associations under 40 years of age (age, <40), with Eastern Cooperative Oncology Group performance status (ECOG PS) ≤1, deep tumor location, no macroscopic (gross) total resection (non-GTR), and ummethylated MGMT status.

showed overall prolonged survival when treated with TMZ and radiotherapy. Our results demonstrated similarly that MGMT promoter hypermethylation determined by a novel pyrosequencing technology was significantly associated with better OS.

There are several contradicting reports on survival related to the prognostic value of *TP53* mutations in GBM, showing either no association or that the presence of *TP53* mutations was a favorable or an unfavorable prognostic factor.[9,20,21,26] On the other hand, our results demonstrated that *TP53* mutation or loss was significantly associated with poor OS only in univariate analysis, but not in multivariate analysis. These findings were not in conflict with recent evidence, which shows that *TP53* mutations not only disrupt its function but also possess gain-of-function and dominant-negative effects on the wild-type p53 protein, thus making the mutated *TP53* gene an oncogene.[27]

Benefits of IFN-β and TMZ combination treatment for GBM

The current study demonstrated that newly diagnosed primary GBM patients were associated with a favorable outcome on IFN-β and TMZ combination chemotherapy. The IFN-β and TMZ combination group achieved a median OS of 19.9 months (Figure 3A). This excellent result was almost equal to the median OS of only patients with the methylated MGMT promoter in the EORTC/NCIC trial.

IFN-β elicits pleiotropic biological effects such as antiproliferation, immunomodulation, and cell differentiation.[28] Furthermore, it has been widely used either alone or in combination with other antitumor agents in the treatment of malignant brain tumors and melanomas. In our previous studies, we showed that combination therapy with

IFN-β and nitrosourea has been particularly useful in the treatment of malignant gliomas in Japan.[10] IFN-β has multifaceted functions related to antitumor activity, such as cytostatic effects, participating in the differentiation of CTLs and potentiation of their antitumor immunological responses, and behavior as a drug sensitizer to enhance toxicity against various malignant neoplasms when administered in combination with nitrosourea.[10] Previously, in an in vitro study, we corroborated that IFN-β markedly enhanced chemosensitivity to TMZ[29]; this manifestation revealed that one of the major mechanisms by which IFN-β enhances chemosensitivity is the down-regulation of MGMT transcription. This effect was also confirmed in an experimental animal model.[30] A subanalysis in this study showed that patients whose tumor had an unmethylated promoter benefited from the addition of IFN-β, suggesting that the combination of IFN-β and TMZ might provide better clinical outcomes in patients with the unmethylated MGMT promoter (Figures 3B, 4). Although we discovered that the patients under 40 years of age at diagnosis and those who had an initial ECOG PS $\leq$1 seemed to receive the benefit from IFN-β and TMZ combination therapy, our phase I study revealed that the combination regimen of IFN-β and TMZ was safe and well tolerated even in patients with older age and worse PS (Figure 4; manuscript in submission). In addition, the benefit associated with IFN-β was shown in patients whose tumors were deep, who had undergone non-GTR (Figure 4). This finding suggests that IFN-β might be better for use in cases of complicated tumor removal, i.e., when the tumors were deep, all the tumors could not be removed because they were, for example, located in an eloquent area or around essential structures.

In summary, this study supported the hypothesis that in cases of newly diagnosed primary GBM, IFN-β and TMZ combination therapy was significantly associated with a favorable outcome. To our knowledge, this is the first study to associate the survival benefits derived from IFN-β and TMZ combination. These benefits were, in particular, well correlated in patients with an unmethylated MGMT promoter.

Our results are limited as opposed to a prospective clinical trial as retrospective studies might have been influenced by unrecognized biases. However, the subject group we used was a consecutive series of patients, and this study provides novel information on the treatment for GBM. Thus, accumulation of evidence for this treatment will help further improvement of this disease and hopefully become a novel therapy. We are planning a prospective randomized control trial to compare the clinical outcomes between TMZ alone and a combination of TMZ and IFN-β in newly diagnosed GBM patients.

CONFLICT OF INTEREST DISCLOSURES

This work was supported by grants from a Grant-in-Aid (C) for Scientific Research from the Ministry of Health, Labor, and Welfare, Japan (A.N.).

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The Global DNA Methylation Surrogate LINE-1 Methylation Is Correlated with *MGMT* Promoter Methylation and Is a Better Prognostic Factor for Glioma

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Abstract

Gliomas are the most frequently occurring primary brain tumor in the central nervous system of adults. Glioblastoma multiformes (GBMs, WHO grade 4) have a dismal prognosis despite the use of the alkylating agent, temozolomide (TMZ), and even low grade gliomas (LGGs, WHO grade 2) eventually transform to malignant secondary GBMs. Although GBM patients benefit from promoter hypermethylation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) that is the main determinant of resistance to TMZ, recent studies suggested that *MGMT* promoter methylation is of prognostic as well as predictive significance for the efficacy of TMZ. Glioma-CpG island methylator phenotype (G-CIMP) in the global genome was shown to be a significant predictor of improved survival in patients with GBM. Collectively, we hypothesized that *MGMT* promoter methylation might reflect global DNA methylation. Additionally in LGGs, the significance of *MGMT* promoter methylation is still undetermined. In the current study, we aimed to determine the correlation between clinical, genetic, and epigenetic profiles including LINE-1 and different cancer-related genes and the clinical outcome in newly diagnosed 57 LGG and 54 GBM patients. Here, we demonstrated that (1) *IDH1/2* mutation is closely correlated with *MGMT* promoter methylation and *1p/19q* codeletion in LGGs, (2) LINE-1 methylation levels in primary and secondary GBMs are lower than those in LGGs and normal brain tissues, (3) LINE-1 methylation is proportional to *MGMT* promoter methylation in gliomas, and (4) higher LINE-1 methylation is a favorable prognostic factor in primary GBMs, even compared to *MGMT* promoter methylation. As a global DNA methylation marker, LINE-1 may be a promising marker in gliomas.

Citation: Ohka F, Natsume A, Motomura K, Kishida Y, Kondo Y, et al. (2011) The Global DNA Methylation Surrogate LINE-1 Methylation Is Correlated with *MGMT* Promoter Methylation and Is a Better Prognostic Factor for Glioma. PLoS ONE 6(8): e23332. doi:10.1371/journal.pone.0023332

Editor: Maciej S. Lesniak, The University of Chicago, United States of America

Received March 25, 2011; **Accepted** July 14, 2011; **Published** August 4, 2011

Funding: This work was supported by grants from a Grant-in-Aid (B) for Scientific Research from the Ministry of Health, Labor, and Welfare, Japan (AN). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Although Mr. Takashi Fukui is employed by a commercial company (FALCO Biosystems), this does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Glioblastoma multiforme (GBM, WHO grade 4) is one of the most frequently occurring brain tumors in the primary central nervous system of adults and is highly malignant. The median survival time is 14 months from diagnosis, despite the use of aggressive treatment, surgery, postoperative radiotherapy, and adjuvant temozolomide (TMZ)-based chemotherapy [1,2,3]. The efficacy of TMZ for treating GBM is often very limited because of inherent or acquired resistance. The main determinant of resistance to alkylating agents is O^6-methylguanine-DNA methyltransferase (MGMT); this enzyme directly and specifically eliminates the cytotoxic alkyl adducts formed at the O^6 position of guanine and less frequently at the O^4 position of thymine [4,5,6]. A subanalysis in an international randomized trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) compared the results of radiotherapy alone with those of concomitant radiotherapy and TMZ and showed that epigenetic silencing of the *MGMT* gene by promoter methylation increased the survival time of patients with primary GBM [3,7]. MGMT has been used as a therapeutic target because downregulation of MGMT may enhance the chemosensitivity of malignant gliomas to TMZ. Thus, MGMT has been regarded as a predictive factor in the treatment of GBM patients. Although the predictive value of *MGMT* methylation has largely been confirmed in numerous prospective and retrospective clinical investigations, it is unclear if this is directly due to reduced MGMT expression. Indeed, evidence has shown that *MGMT* promoter hypermethylation is better correlated with survival benefit than evaluations of its mRNA and protein levels [8,9]. In addition, Van den Vent et al reported that a methylated *MGMT* promoter was of prognostic significance among patients with anaplastic gliomas treated with radiation alone [10]. These results suggest that a methylated *MGMT*

promoter is prognostic as well as predictive for the outcome of adjuvant therapy in high-grade gliomas [11].

Cancer-specific DNA methylation changes are hallmarks of human cancers, with global DNA hypomethylation often seen concomitantly with hypermethylation of CpG islands [12]. A CpG island methylator phenotype (CIMP) is regarded as cancer-specific CpG island hypermethylation of a subset of genes in some tumors [13]. Colorectal CIMP is associated with microsatellite instability and transcriptional silencing [14]. Recently, The Cancer Genome Atlas (TCGA) project and other groups have attempted to profile GBM genes comprehensively based on genomic and epigenomic aberrations and transcriptomal features [1,15,16]. In GBM, glioma-CIMP status (G-CIMP) was shown to be a significant predictor of improved patient survival [16]. Collectively, these different sets of observations suggest that the level of *MGMT* promoter methylation, serving as a prognostic factor, may reflect an aspect of the global DNA methylation status in GBM.

Recently, long interspersed nuclear element-1 (LINE-1) has attracted attention. LINE-1 is a non-long terminal-repeat class of retroposons that is the most successfully integrated mobile element in the human genome and accounts for approximately 18% of the human genome [17]. The level of LINE–1 methylation is regarded as a surrogate of global DNA methylation. In various cancers such as colon and ovarian cancer, it is thought that hypomethylation of LINE-1 is correlated with poor prognosis [17,18,19]. However, in glioma patients, the level of LINE-1 methylation has not been fully estimated. Recently, many studies have suggested that low-grade gliomas (LGGs, WHO grade 2) including astrocytoma (As), oligodendroglioma (OG) and oligoastrocytoma (OA) display a highly methylated profile, in particular LGGs with mutated *IDH1* [20,21].

In the current study, we aimed to determine the correlation between clinical, genetic, and epigenetic profiles of LINE-1 and of different cancer-related genes and the clinical outcome in newly diagnosed LGG and GBM patients. Here, we demonstrated that (1) LINE-1 methylation levels in primary and secondary GBMs are lower than those in LGGs and normal brain tissues, (2) LINE-1 methylation is directly proportional to *MGMT* promoter methylation in gliomas, and (3) higher LINE-1 methylation is a favorable prognostic factor in primary GBMs. As a global DNA methylation marker, LINE-1 may be a promising marker reflecting the *MGMT* promoter methylation and the G-CIMP status.

Materials and Methods

Ethics Statement

The study was approved by the institutional review board at each participating hospital and complied with all provisions of the Declaration of Helsinki.

Patients and Tumor Samples

We collected 111 freshly frozen tissues from patients with LGGs (WHO grade 2), or GBMs treated at Nagoya University Hospital, Oita University Hospital, Hamamatsu University Hospital, and Shizuoka Cancer Center. Their clinical characteristics are summarized in Table 1. Of 57 LGG patients, 30 patients with residual tumor evaluated by T2-wighted magnetic resonance imaging (MRI) received adjuvant nitrosourea-based or TMZ-based chemotherapy concomitant with radiotherapy (large focal 40 Gy) immediately after initial surgery. All primary GBM patients received TMZ–based chemotherapy and radiotherapy (60 Gy) following initial surgery. Secondary GBM was defined as a prior histological diagnosis of LGG.

Table 1. Clinical characteristics.

Baseline characteristics of all gliomas (n = 111)					
Histological subgroups	**No.**	**Age, years**		**Sex**	
		median	**range**	**male (%)**	**female (%)**
Grade 2 gliomas	57	42.0	21–72	36 (63%)	21 (37%)
As	17	40.0	23–72	13 (76%)	4 (24%)
OG	29	44.0	21–61	17 (59%)	12 (41%)
OA	11	48.0	26–68	6 (55%)	5 (45%)
GBMs	54	59.0	12–84	33 (61%)	21 (39%)
pGBMs	51	59.2	12–84	31 (61%)	20 (39%)
sGBMs	3	42.0	21–50	2 (67%)	1 (33%)

As; Astrocytoma, OG; Oligodendroglioma, OA; Oligo-astrocytoma, pGBMs; primary GBMs, sGBMs; secondary GBMs.
doi:10.1371/journal.pone.0023332.t001

Tumor Samples

DNA was prepared using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of DNA obtained from the tumor was sufficient for the subsequent genomic and epigenomic analyses.

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) was used to determine allelic losses and gains in the tumor samples. The analysis was performed using the SALSA MLPA KIT P088-B1 and P105-C1 in accordance with the manufacturer's protocol (MRC Holland, Amsterdam, Netherland) [22]. Information regarding the probe sequences and ligation sites can be found at www.mlpa.com. Amplification products were separated on an ABI® 3130×I Genetic Analyzer (Applied Biosystems, Foster City, CA) and quantified with Genemapper 4.0 software (Applied Biosystems). Data analysis was performed with an original Excel-based program based on MRC-Holland's procedures. Normalization for sample data was first performed on control probes, and each tumor sample was then normalized using the data on 2 control samples, using peripheral blood DNA. Single regression for control and tumor data slope correction was performed. Abnormal/normal ratio limits were set at 0.65 and 1.3. Statistical analysis was performed using the same Coffalyser software.

Pyrosequencing

Tumor DNA was modified with bisulfate using the EpiTect bisulfite kit (Qiagen). Pyrosequencing technology was used to determine the methylation status of the CpG island region of the *MGMT* promoter and LINE-1, as described previously [18,23]. We used the touchdown PCR method for the *MGMT* promoter and the conventional PCR method for LINE-1. The primer sequences used were the *MGMT* forward primer (5′-TTGGTAAATTAAGGTATAGAGTTTT-3′), the *MGMT* biotinylated reverse primer (5′-AAACAATCTACGCATCCT-3′), the LINE-1 forward primer, (5′-TTTTGAGTTAGGTGTGGGATATA-3′), and the biotinylated reverse primer (5′-AAAATCAAAAAATTCCCTTTC-3′). PCR for the *MGMT* promoter included a denaturation step at 95°C for 30 s, followed by annealing at various temperatures for 45 s, and extension at 72°C for 45 s. PCR for LINE-1 included a denaturation step at 95°C for

30 s, annealing at 50°C for 60 s, and extension at 72°C for 45 s. After PCR, the biotinylated PCR product was purified as recommended by the manufacturer. In brief, the PCR product was bound to streptavidin sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the sepharose beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 N NaOH solution, and then washed again. Next, 0.3 mM pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Westborough, MA). The pyrosequencing primer for the *MGMT* promoter was 5′–GGAAGTTGGGAAGG-3′ and for LINE-1 was 5′–AGTTAGGTGTGGGATATAGT-3′. Methylation was quantified using the provided software.

TP53 and IDH1/IDH2 Sequencing

Direct sequencing of *TP53* exons 5 to 8 and *IDH1/2* was performed as previously described [24,25]. The primer sequences are listed in Table 2. For *IDH* sequencing, 2 fragments were amplified: (1) a 129-bp fragment spanning the sequence encoding the catalytic domain of *IDH1*, including codon 132 and (2) a 150-bp fragment spanning the sequence encoding the catalytic domain of *IDH2*, including codon 172. For sequencing *TP53*, we applied touchdown PCR using the standard buffer conditions; the reaction mixture included 5 ng of DNA and AmpliTaq Gold DNA Polymerase (Applied Biosystems). The reaction was run for 16 cycles with denaturation at 95°C for 30 s, annealing at 65–57°C (decreasing by 0.5°C per cycle) for 30 s, and extension at 72°C for 60 s, in a total volume of 12.5 ml. Then, an additional 30 cycles were performed with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, ending at 72°C for 7 min to complete extension. Direct sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems). For *IDH1/2* mutations, we applied conventional PCR at 35 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 50 s, ending at 72°C for 7 min to complete extension.

Table 2. List of Primer Sequences for Direct DNA Sequencing.

Gene name	Exon	Sequence
TP53	Exon 5	F 5′-TTATCTGTTCACTTGTGCCC-3′
		R 5′-ACCCTGGGCAACCAGCCCTG-3′
	Exon 6	F 5′-ACGACAGGGCTGGTTGCCCA-3′
		R 5′-CTCCCAGAGACCCCAGTTGC-3′
	Exon 7	F 5′-GGCCTCATCTTGGGCCTGTG-3′
		R 5′-CAGTGTGCAGGGTGGCAAGT-3′
	Exon 8	F 5′-CTGCCTCTTGCTTCTCTTTT-3′
		R 5′-TCTCCTCCACCGCTTCTTGT-3′
IDH1		F 5′-CGGTCTTCAGAGAAGCCATT-3′
		R 5′-GCAAAATCACATTATTGCCAAC-3′
IDH2		F 5′-AGCCCATCATCTGCAAAAAC-3′
		R 5′-CTAGGCGAGGAGCTCCAGT-3′

F indicates forward primer, R,reverse primer.
doi:10.1371/journal.pone.0023332.t002

Statistical Analysis

Statistical analysis was performed using the statistical software SPSS for Windows, version 19.0 (SPSS Inc, Chicago,I ll). The Mann-Whitney U test, the Student's t-test, the χ^2 test, and the Fisher exact test were used to test for the association of clinical variables and molecular markers. Correlation of methylation level between *MGMT* promoter and LINE-1 was analyzed by using Spearman rank correlation coefficient, and analyzed by using Pearson product - moment correlation coefficient in LGGs. Survival was estimated by using the Kaplan-Meier method, and survival curves were compared by using the log-rank test. Overall survival (OS) was calculated from the day of initial surgery until death or the end of follow-up, and progression-free survival (PFS) was until tumor progression or re-treatment. Among LGGs, univariate and multivariate analyses were performed to test the potential influence of baseline characteristics on OS and PFS. The effect of each single factor on OS and PFS was investigated using the Cox proportional hazards model, adjusting for the major clinical prognostic factors, including age at diagnosis (<40 vs. ≥40), Sex (male vs. female), Eastern Cooperative Oncology Group (ECOG) performance status score (ECOG PS; ≤1 vs. >1), extent of resection (macroscopic [gross] total resection [GTR] or subtotal resection [STR] vs. partial resection or biopsy), *MGMT* promoter methylation status, chromosome *1p* loss of heterozygosity (LOH), *19q* LOH, *PTEN* loss, *CDKN2A* loss, *TP53* loss and mutation, *ERBB2* amplification, *EGFR* amplification, *IDH1* and *IDH2* mutation, and adjuvant therapy immediately following the surgery (with radiotherapy or chemotherapy vs. none). The factors in the multivariate proportional hazard model ($p<0.05$) were considered independent factors correlated with prolongation of OS and PFS.

Results

Frequency of Genetic and Epigenetic Alterations in LGGs, and Primary and Secondary GBMs

We used direct sequencing for *TP53* and *IDH1/2* and employed MLPA for the analysis of *1p/19q* loss, *PTEN* and *CDKN2A* loss, and amplification of *ERBB2* and *EGFR*. Moreover, we used pyrosequencing technology for quantitative estimation of the methylation status of the *MGMT* promoter and LINE-1. Based on comparisons using standard methylation-specific PCR and immunohistochemical studies using the anti-MGMT antibody, we determined 14% as the threshold distinguishing unmethylation from methylation of the *MGMT* promoter in a given tumor, as reported previously [26]. The data are summarized in Table 3 and Figure 1. In LGGs, *IDH1/2* mutation and methylation of the *MGMT* promoter were frequently observed (~80%). Of the 46 tumors with *IDH1* mutations, 44 exhibited R132H, one R132G, and one R132S. The *1p/19q* codeletion was detected more often in OG (72%) than in As (6%) and OA (18%). In contrast, *TP53* mutation was more frequently observed in As (41%) and OA (45%) than in OG (10%). We did not detect amplification of *EGFR and ERBB2* in LGGs. In comparison with primary GBM, secondary GBM had more *IDH1/2* and *TP53* mutations and *CDKN2A* loss, a higher frequency of methylated *MGMT* promoter, and less *EGFR* amplification, although the number of secondary GBM (n = 3) was limited (Table 3, Figure 1B).

Recently, emerging evidence revealed correlations between the methylation status of the *MGMT* promoter, *IDH1* mutations, and *1p/19q* codeletions [27,28,29,30]. Using the χ^2 test in LGGs, *IDH1/2* mutation was correlated significantly with a methylated *MGMT* promoter ($p = 0.038$) and *1p/19q* codeletion ($p = 0.024$). Further, the presence of a methylated *MGMT* promoter was correlated significantly with *1p/19q* codeletion ($p = 0.026$). Addi-

Table 3. Genetic, Epigenetic Alterations in all gliomas.

	Grade 2 gliomas			GBMs		Total
	As	OG	OA	pGBMs*	sGBMs	
	n = 17	n = 29	n = 11	n = 51	n = 3	n = 111
IDH1/2 mutation	14 (82%)	24 (83%)	9 (82%)	3 (6%)	2 (67%)	52 (47%)
TP53 mutation	7 (41%)	3 (10%)	5 (45%)	19 (37%)	3 (100%)	37 (33%)
1p LOH	1 (6%)	21 (72%)	2 (18%)	4 (8%)	1 (33%)	29 (26%)
19q LOH	7 (41%)	22 (76%)	4 (36%)	5 (10%)	1 (33%)	39 (35%)
1p/19q codeletion	1 (6%)	21 (72%)	2 (18%)	4 (8%)	0	28 (25%)
PTEN loss	2 (12%)	0	0	4 (8%)	0	6 (5%)
CDKN2A loss	1 (6%)	1 (3%)	1 (9%)	20 (39%)	3 (100%)	26 (23%)
ERBB2 amplification	0	0	0	1 (2%)	0	1 (1%)
EGFR amplification	0	0	0	25 (49%)	0	25 (23%)
Methylated *MGMT*	12 (71%)	24 (83%)	8 (73%)	16 (31%)	2 (67%)	62 (56%)
LINE-1 methylation**	67.6±3.0	69.0±2.6	70.0±2.3	66.6±4.1	60.7±1.8	67.6±3.6

As; Astrocytoma, OG; Oligodendroglioma, OA; Oligo-astrocytoma, pGBMs; primary GBMs, sGBMs; secondary GBMs.
*Motomura K et al reported these alterations of primary GBMs previously [26].
**LINE-1 methylation indicates mean methylation level ± S.D. (%).
doi:10.1371/journal.pone.0023332.t003

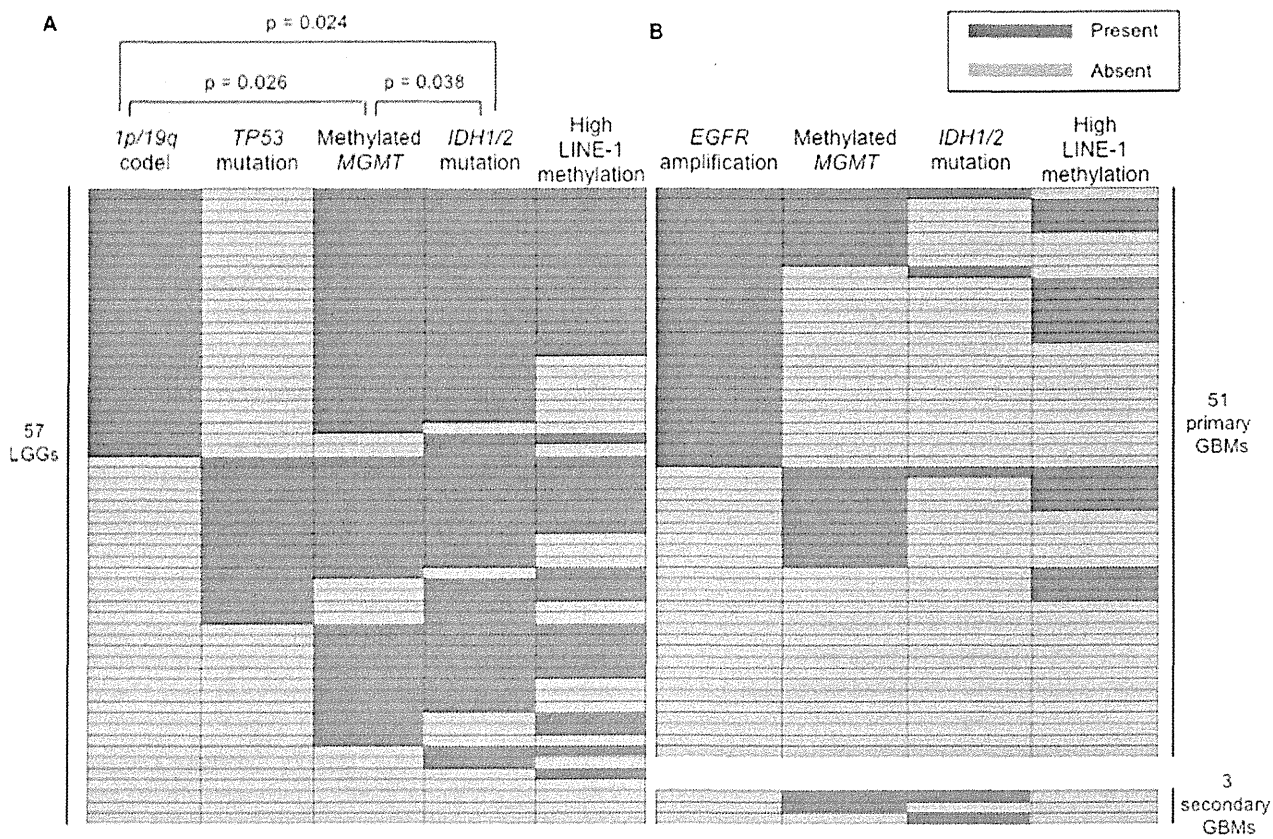

Figure 1. Correlations between the methylation status of the *MGMT* promoter, *IDH1/2* mutations, and *1p/19q* deletions, higher LINE-1 methylation in low-grade gliomas (LGGs), *EGFR* amplification, *MGMT* promoter, *IDH1/2* mutations, high LINE-1 methylation in primary and secondary GBMs. Using the χ^2 test in grade 2 gliomas, *IDH1/2* mutation was correlated significantly with a methylated *MGMT* promoter (p = 0.038) and *1p/19q* codeletion (p = 0.024). Further, the presence of a methylated *MGMT* promoter was correlated significantly with *1p/19q* codeletion (p = 0.026). Additionally, of the 24 cases with *1p/19q* codeletion, 23 and 22 cases exhibited *IDH1/2* mutations and methylated *MGMT* promoters, respectively, but none showed *TP53* mutations. Of the 44 cases with methylated MGMT promoters, 39 cases exhibited *IDH1/2* mutations (A). In primary and secondary GBMs, *EGFR* amplification, which is the most frequent, and methylated *MGMT* promoter, *IDH1/2* mutation and high LINE-1 methylation was shown (B).
doi:10.1371/journal.pone.0023332.g001

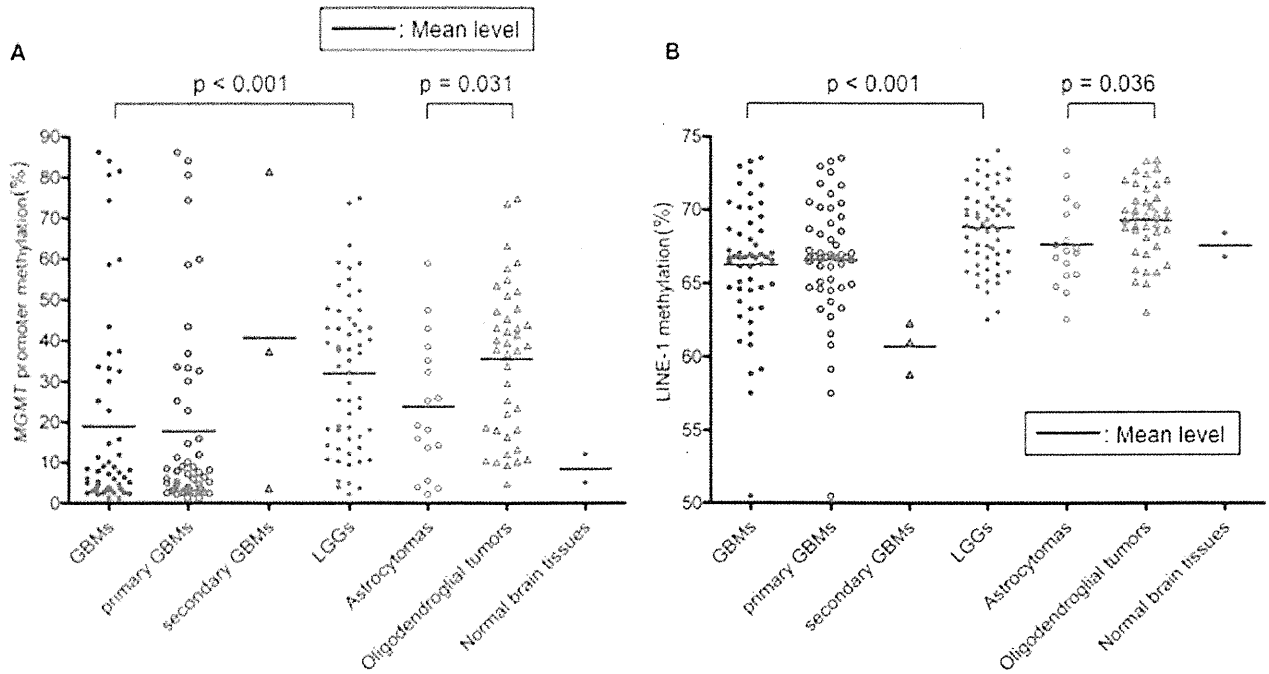

Figure 2. Differences in the methylation levels of *MGMT* promoter and LINE-1 between low-grade gliomas (LGGs) and glioblastoma multiforme (GBM), and between grade 2 astrocytomas and oligodendroglial tumors. A higher proportion of LGGs including astrocytoma, oligodendroglioma, and oligoastrocytoma, exhibited a methylated *MGMT* promoter (A) and LINE-1 (B) compared to GBMs, although the level of LINE-1 in GBMs varied (see also Table 3). Compared among histological subgroups, the level of LINE–1 methylation in astrocytomas was significantly lower than that in oligodendroglial tumors (B), which was similar to the *MGMT* promoter methylation (A). Horizontal line in the graph indicated the mean level.
doi:10.1371/journal.pone.0023332.g002

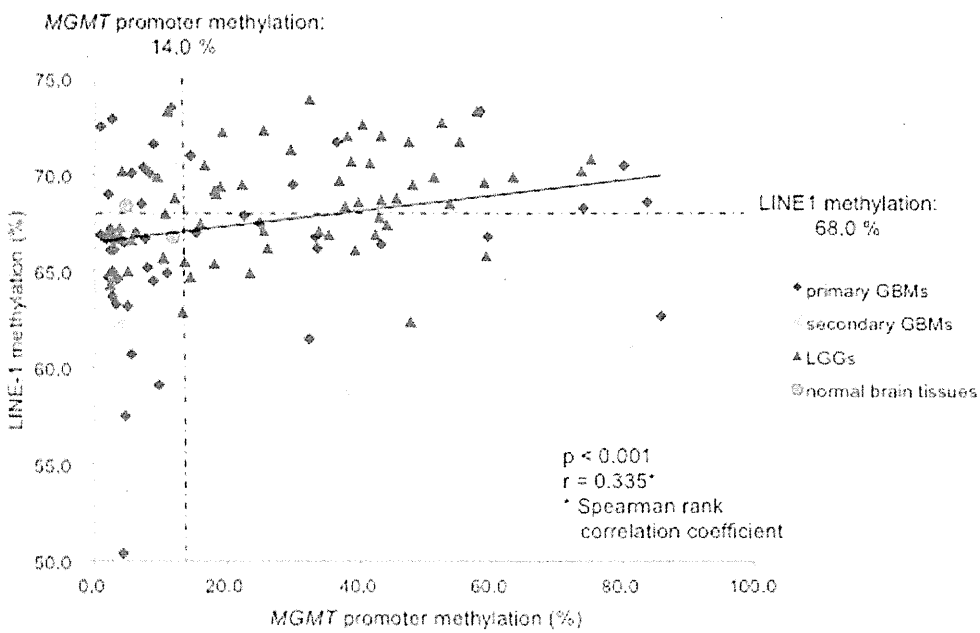

Figure 3. LINE-1 methylation is directly proportional to *MGMT* promoter methylation in gliomas. *MGMT* promoter methylation level was directly proportional to LINE–1 methylation in a statistically significant manner ($r = 0.335$, $p<0.001$) for all samples quantified, including LGGs, primary and secondary GBMs, and normal brain tissue. Cut-off line of LINE-1 methylation, *MGMT* promoter methylation was indicated.
doi:10.1371/journal.pone.0023332.g003

tionally, of the 24 cases with *1p/19q* codeletion, 23 and 22 cases exhibited *IDH1/2* mutations and methylated *MGMT* promoters, respectively, but none showed *TP53* mutations. Of the 44 cases with methylated *MGMT* promoters, 39 cases exhibited *IDH1/2* mutations. These results suggest that almost all patients having tumors with *1p/19q* codeletions exhibited methylated *MGMT* promoters and that almost all tumors with methylated *MGMT* promoters exhibited *IDH1/2* mutations (Figure 1A).

LINE-1 Methylation Is Proportional to MGMT Promoter Methylation in gliomas

The level of LINE–1 methylation is regarded as a surrogate of global DNA methylation. Recently, many studies have suggested that low-grade gliomas (LGGs, WHO grade 2) including astrocytoma (As), oligodendroglioma (OG) and oligoastrocytoma (OA) display a highly methylated profile [20,21]. We examined the level of LINE-1 methylation in comparison with that of *MGMT* promoter methylation in glioma patients. To date, studies have revealed that the level of methylated *MGMT* promoters among LGGs was higher than that among GBMs [20,30,31]. Similar to the previous reports, a higher proportion of LGGs exhibited a methylated *MGMT* promoter and LINE-1 compared to GBMs, although the level of LINE-1 in GBMs varied [*MGMT*, mean 18.9% vs. 31.9% ($p<0.001$); LINE-1, 66.2% vs. 68.8% ($p<0.001$); Table 3 and Figure 2AB]. Compared among histological subgroups, the level of LINE–1 methylation in As was significantly lower than that in oligodendroglial tumors, including OG and OA, which was similar to the *MGMT* promoter methylation (mean LINE-1 methylation level, 67.6% vs. 69.3%; $p = 0.036$, Figure 2B).

The results described above prompted us to analyze the correlation between the quantitative methylation values of LINE-1 and the *MGMT* promoter. We found that the *MGMT* promoter methylation level was directly proportional to LINE–1 methylation in a statistically significant manner ($r = 0.335$, $p<0.001$) for all glioma samples and normal brain tissue (Figure 3). However, while LINE-1 methylation is significantly proportional to *MGMT* promoter in LGGs ($r = 0.336$, $p = 0.011$), statistical significance was not found when primary GBMs only were analyzed, probably due to non-parametric distribution of the *MGMT* promoter methylation level (Figure S1AB).

Previously, it was reported that G-CIMP tumors are more prevalent among LGGs, and are tightly associated with *IDH1* mutation [16]. Thus, it may be interesting to know whether LINE-1 methylation is correlated with *IDH1* mutation in our sample sets. Although we did not observe the significant correlation between *IDH1/2* mutation and higher LINE-1 methylation both among LGGs and GBMs (Figure S2), we showed that LGGs exhibited higher LINE-1 methylation than GBMs did, and oligodendroglial tumors showed higher LINE-1 methylation than astrocytomas (Table 3, Figure 2B), which was consistent with the previous report demonstrating that LGGs, in

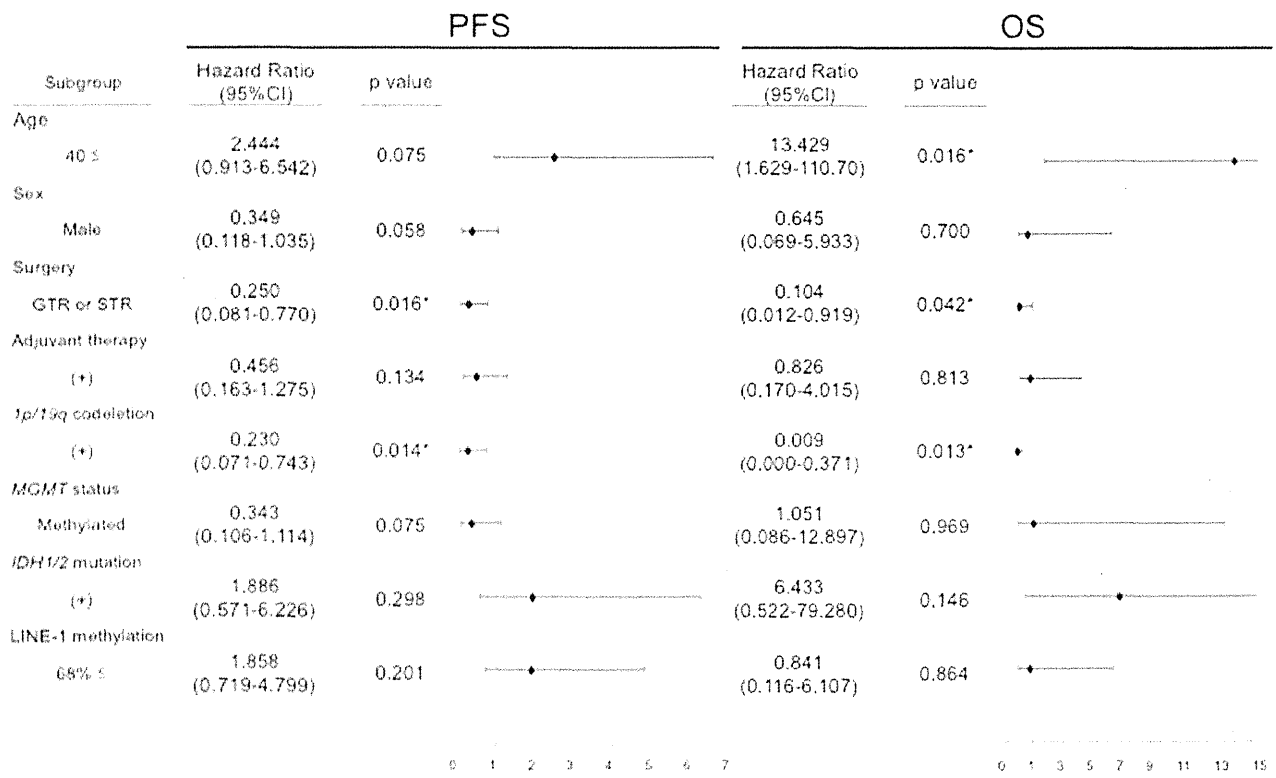

Figure 4. Clinical, genetic, and epigenetic parameters in correlation with progression-free survival (PFS) and overall survival (OS) in low-grade glioma patients. The presence of *1p/19q* codeletion and the extent of resection were independently correlated with prolonged PFS, as shown with multivariate analysis ($p = 0.014$ and $p = 0.016$, respectively). The presence of *1p/19q* codeletion, the extent of resection and the age were correlated with prolonged OS ($p = 0.013$, 0.042, 0.016, respectively).
doi:10.1371/journal.pone.0023332.g004

particular oligodendroglial tumors are characteristics of G-CIMP positive group.

Clinical, Genetic, and Epigenetic Parameters in Correlation with PFS and OS in Low-grade Glioma Patients

We investigated the correlations of the genetic and epigenetic alterations with OS and PFS among LGGs. Among all LGGs, the median PFS was 45.7 months (95% confidence interval [CI]: 17.1–74.3 months), the median OS was 172.8 months (95%CI; 8.9–336.8 months). Patients with As, OG, and OA had a PFS of 45.1, 74.9, and 37.3 months, respectively. As shown in Figure 4, the presence of *1p/19q* codeletion, the extent of resection were independently correlated with PFS, as shown with multivariate analysis (p = 0.014, 0.016), and the presence of *1p/19q* codeletion, the extent of resection and the age were correlated with prolonged OS (p = 0.013, 0.042, 0.016, respectively). Using a log-rank test, a univariate analysis revealed that prolonged PFS and OS was significantly correlated only with the presence of *1p/19q* codeletion (p = 0.013, p = 0.013, supplementary Figure S3AB). Univariate analysis showed that a methylated *MGMT* promoter was not significantly correlated with prolonged PFS (p = 0.128); however, if patients undergoing partial removal or biopsy at initial surgery were selected, it became significantly correlated with PFS (p = 0.017, supplementary Figure S4). Of particular note, high LINE-1 methylation (68% ≤) was significantly correlated with prolonged OS of patients aged over 40 (p = 0.039), whereas statistical significant association was not obtained between high LINE-1 methylation and PFS (Figure 5).

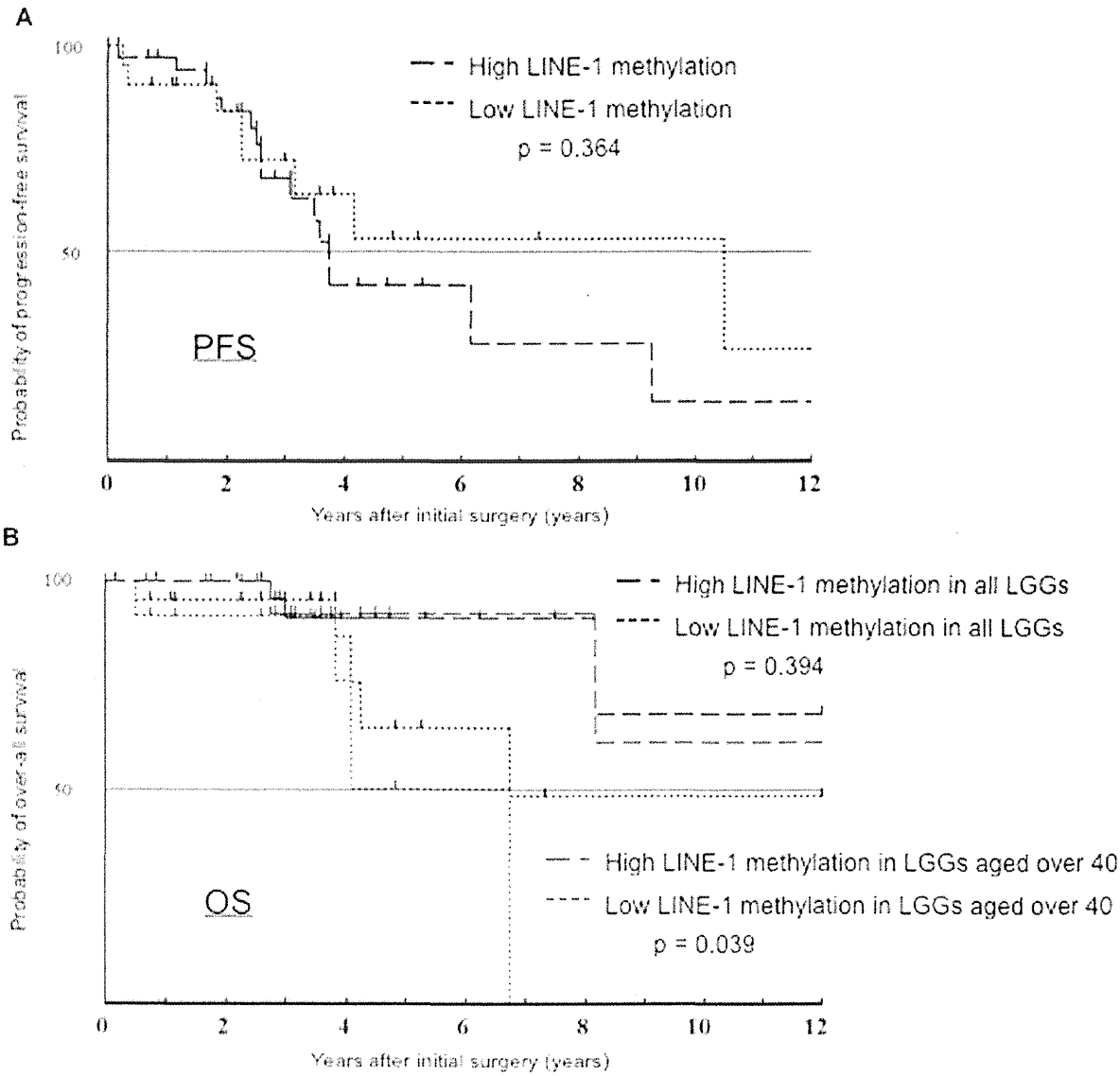

Figure 5. High LINE-1 methylation status in correlation with progression-free survival (PFS) and overall survival (OS) in low-grade glioma patients. In the Kaplan-Meier survival curve of patients with LGGs, High LINE-1 methylation status was not correlated with PFS in LGGs, using log-rank test (p = 0.364); (A). However in correlation with OS, in LGGs aged over 40, High LINE-1 methylation prolonged OS (p = 0.039), black line indicated the Kaplan-Meier survival curve of all LGGs (high LINE-1 methylation and low), red line LGGs aged over 40 (B).
doi:10.1371/journal.pone.0023332.g005

LINE-1 Methylation is a Prognostic Factor Among primary GBMs

Next, we examined whether LINE-1 could be a prognostic factor in primary GBMs. To our surprise, in the Kaplan-Meier survival curve of patients with primary GBM, univariate analysis indicated a lower p value in the comparison of <68% and ≥68% of LINE-1 methylation than in the comparison of <14% and ≥14% of *MGMT* promoter methylation (p = 0.010 and 0.015, Figure 6AB). Furthermore, in multivariate analysis, the hazard ratio was computed using a proportional hazard model by selected factors. Prolonged overall survival time was significantly correlated with a high LINE-1 methylation status but not with a methylated *MGMT* promoter (p = 0.031, Figure 6C).

Genetic and Epigenetic Changes From Low-grade Glioma to Secondary GBM

We experienced 3 secondary GBM cases and obtained serial tumor samples of 2 cases at the time of grade 2 glioma (As and OA) and at the time of progression to GBM. The secondary GBM tumors already had *TP53* mutation and *IDH1* mutation at the time of the low-grade tumors but displayed a 2-fold increase in methylation of the *MGMT* promoter and an 8% decrease in methylation of LINE-1 during malignant transformation.

Discussion

Previously, we demonstrated clinical, genetic, and epigenetic profiles in newly diagnosed primary GBMs [26]. In this study, we extended those analyses to LGGs, in comparison with GBMs. We also included secondary GBMs in order to provide a possible clue into the profile changes that occur during malignant transformation. Of great interest, the principal and novel finding of the current study is that a global DNA methylation surrogate, LINE-1 methylation, is positively proportional to the *MGMT* promoter methylation in gliomas.

In this study, 57 LGG samples exhibited *IDH1/2* mutations most frequently (82%), followed by methylated *MGMT* promoters (77%), *1p/19q* codeletion (42%), and *TP53* mutations (26%). Our results were consistent with data reported previously [20,32,33,34,35]. We demonstrated that higher methylation levels of LINE-1 and the *MGMT* promoter and *1p/19q* codeletion were associated with oligodendroglial tumors. Additionally, the presence of *1p/19q* codeletion was significantly correlated with higher *MGMT* promoter methylation.

Of these alterations, *1p/19q* codeletion was most strongly correlated with prolonged OS and PFS in both univariate and multivariate analysis of LGGs. In our study, *IDH1/2* mutation was not correlated with prolonged PFS and OS in LGG patients. The

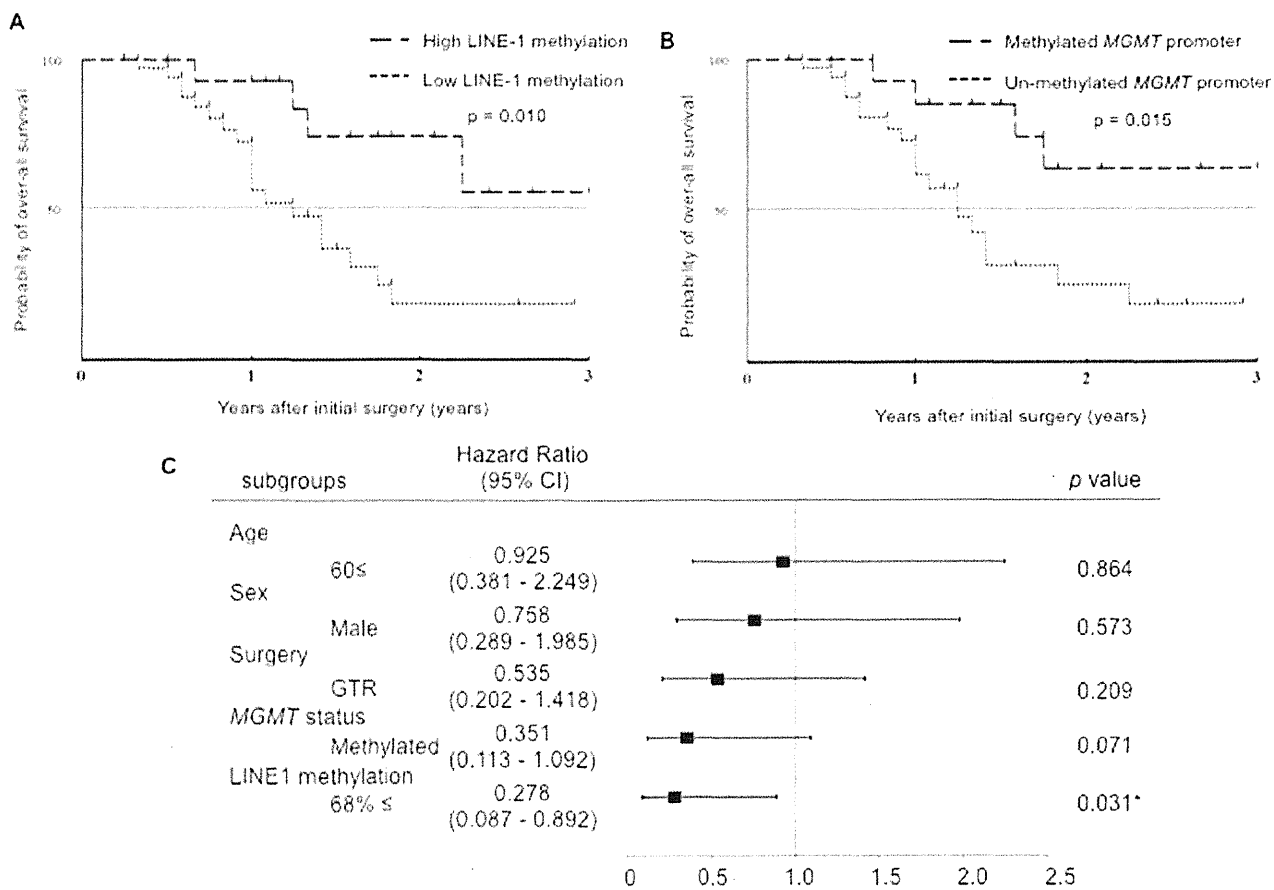

Figure 6. LINE-1 methylation is a better prognostic indicator in primary GBMs. In the Kaplan-Meier survival curve of patients with primary GBM, univariate analysis indicated a lower p value in the comparison of <68% and ≥68% of LINE-1 methylation (A) than in the comparison of <14% and ≥14% of *MGMT* promoter methylation (B). In multivariate analysis, the hazard ratio was computed using a proportional hazard model by selected factors. Prolonged overall survival time was significantly correlated with a high LINE-1 methylation status but not with a methylated *MGMT* promoter (C).

doi:10.1371/journal.pone.0023332.g006

finding was consistent with previous reports demonstrating that *IDH1/2* mutations are not a prognostic factor for LGGs [27,36], but there was opposed evidence showing significant and independent associations between *IDH* mutation and improved survival in LGGs [21,32]. The prognostic significance of *IDH1/2* mutation in LGGs remains controversial.

To date, *MGMT* promoter methylation has been regarded as a prognostic as well as predictive for the outcome to adjuvant chemotherapy [10]. In various cancers, such as colorectal cancer, global DNA hypomethylation was correlated with poor prognosis [17,18]. We hypothesized that *MGMT* promoter hypermethylation reflects global DNA hypermethylation in gliomas. To demonstrate our hypothesis, we quantified the level of LINE-1 methylation in gliomas. Higher methylation levels of LINE-1 and the *MGMT* promoter were observed in LGGs than in GBMs (LINE-1: mean 68.8% vs. 66.2%, $p<0.001$; *MGMT* promoters: 31.9% vs. 18.9%, $p<0.001$). Additionally, we investigated the correlations between LINE-1 and *MGMT* promoter methylation levels. Among gliomas, in particular LGGs, LINE-1 methylation levels were significantly proportional to *MGMT* promoter methylation. Notably, only low LINE-1 methylation indicated poor prognosis in primary GBM patients, as analyzed by both univariate and multivariate analyses. Prolonged overall survival time was significantly correlated with high LINE-1 methylation status but not with a methylated *MGMT* promoter. Additionally, higher LINE-1 methylation was correlated with prolonged OS in LGG patients aged over 40. This is consistent with other cancers such as colorectal cancer and ovarian cancer, in which hypomethylation of LINE-1 is correlated with shortened survival [17,18,37].

LINE-1 methylation and *MGMT* promoter methylation were also correlated with tumor grading; LGGs displayed a higher methylation level of LINE-1 and the *MGMT* promoter than GBMs (WHO grade 4). Thus, in order to determine whether DNA methylation relies on malignant transformation, we investigated changes in genetic and DNA methylation patterns from LGGs to secondary GBMs in identical cases. However, secondary GBMs paradoxically displayed an increase in *MGMT* promoter methylation and a decrease in LINE-1 methylation. The limited number of samples studied warrant further investigations.

Previously, it was reported that G-CIMP tumors are tightly associated with *IDH1* mutation [16]. More recently, *IDH* mutations and resultant 2-hydroxyglutarate (2HG) production in leukemia cells were reported to induce global DNA hypermethylation through impaired TET2 catalytic function [38]. In this study, LGGs with *IDH1/2* mutation tended to exhibit higher LINE-1 methylation although there was no statistical significance. Our study demonstrated the correlation of LINE-1 methylation with good prognosis among GBMs for the first time, however, the mechanism was not interpreted and the number of samples in our study was limited. The higher levels of LINE-1 methylation in low grade gliomas may be attributable to the differential prevalence of *IDH* mutation in low versus high-grade glioma, and the methylator phenotype associated with *IDH* mutation. Thus, interpreting LINE-1 methylation values for prognosis may be more difficult than interpreting *IDH1/2* mutation. We need further investigation to validate our findings.

In summary, we demonstrated that LINE-1 methylation levels in primary and secondary GBMs are lower than those in LGGs and normal brain tissues, that LINE-1 methylation is directly proportional to *MGMT* promoter methylation in gliomas, and that higher LINE-1 methylation is a favorable prognostic factor in primary GBMs. LINE-1 is a global DNA methylation marker, which may be a promising marker reflecting the *MGMT* promoter or the G-CIMP status.

Supporting Information

Figure S1 Correlation between the methylation levels of LINE-1 and *MGMT* promoter. Among LGGs, LINE-1 is derectly proportional to *MGMT* promoter, $p = 0.011$, $r = 0.336$ (A). However among primary GBMs, the correlation between the methylation levels of LINE-1 and *MGMT* promoter are statistically insignificant, $p = 0.187$, $r = 0.188$ (B).
(TIFF)

Figure S2 Differences of methylation levels of LINE-1 between mutated *IDH1/2* and wild-type. Among LGGs, *IDH1/2* mutation exhibited higher methylation level of LINE-1, although insignificant, than wild-type *IDH1/2*, mean; $69.0\pm2.5\%$, $67.6\pm3.4\%$, $p = 0.144$ (A). Among primary and secondary GBMs, mutated *IDH1/2* did not exhibited the differences of methylation level of LINE-1, compared with wild-type *IDH1/2* although we analyzed only 5 mutated *IDH1/2*, mean; $65.5\pm4.8\%$, $66.3\pm4.2\%$, $p = 0.449$ (B).
(TIFF)

Figure S3 *1p/19q* codeletions in correlation with overall survival, progression-free survival in low-grade glioma patients. Using a log-rank test, a univariate analysis revealed that prolonged PFS (A) and OS (B) was significantly correlated only with the presence of *1p/19q* codeletion.
(TIFF)

Figure S4 *MGMT* promoter methylation in correlation with progression-free survival (PFS) in low-grade glioma patients. Methylated *MGMT* promoter was not significantly correlated with prolonged PFS (A); however, if patients undergoing partial removal or biopsy at initial surgery were selected, it became significantly correlated with PFS (B).
(TIFF)

Author Contributions

Conceived and designed the experiments: AN YK. Performed the experiments: FO KM YK TF HM KI SK MI. Analyzed the data: KW TW. Contributed reagents/materials/analysis tools: TA YN HN MF. Wrote the paper: AN FO.

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J Cancer Res Clin Oncol (2012) 138:1239–1247
DOI 10.1007/s00432-012-1198-y

ORIGINAL PAPER

Effect of chemotherapy on survival after whole brain radiation therapy for brain metastases: a single-center retrospective analysis

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Received: 10 January 2012 / Accepted: 5 March 2012 / Published online: 23 March 2012

Abstract
Background and purpose Whether chemotherapy for systemic disease affects survival of patients with brain metastases or not has not been elucidated before. We performed comprehensive analysis of patients with newly-diagnosed brain metastases primarily treated with whole brain radiation therapy (WBRT) alone.
Materials and methods Data from 134 patients with newly-diagnosed brain metastases primarily treated with WBRT from 2007 to 2008 was retrospectively reviewed. Univariate and multivariate analyses were performed to identify significant prognostic factors.
Results Median survival time (MST) of this cohort from the start of WBRT was 5.7 months. MST of patients with RPA Class 1, 2 and 3 were 10.3, 7.8 and 2.2 months, respectively. Multivariate analysis revealed that karnofsky performance status ($\geq$70, $p < 0.0001$), gender (female, $p < 0.0001$), activity of extracranial disease (stable, $p = 0.015$), time to develop brain metastasis (<3 months, $p = 0.042$) and use of chemotherapy after WBRT (multiple regimens, $p < 0.0001$) were independent prognostic factors for better survival.
Conclusions Systemic chemotherapy for chemo-responsive cancer prolongs survival despite the presence of treated brain metastases. Irradiated brain metastases will lose their prognostic significance in a large number of patients. Systemic chemotherapy will be a treatment of choice for patients who have systemic disease after WBRT for brain metastases. These results should be validated in the future prospective clinical trials.

Keywords Brain metastasis · Brain metastases · Radiation therapy · Whole brain radiation therapy · Chemotherapy · Prognostic factors

Introduction

Brain metastasis affects 20–40 % of cancer patients (Soffietti et al. 2002). Brain metastasis is one of the major causes of morbidity in cancer patients. The prognosis of patients with brain metastasis is generally poor with a median survival time (MST) of 1–2 months with corticosteroids only (Weissman 1988; Lagerwaard et al. 1999).

The route of metastatic dissemination to the brain is often hematogeneous, therefore, the entire brain can be seeded with micrometastatic focus. Traditionally, whole brain radiation therapy (WBRT) has been regarded as the standard treatment for patients with brain metastasis. Overall survival of the patients after WBRT ranges 3–6 months (Lagerwaard et al. 1999; Gaspar et al. 2010; Tsao et al. 2005). Various dose/fractionation schedules of WBRT were tested in clinical studies, which resulted in no significant difference in median survival time after WBRT (Tsao et al. 2005; Gaspar et al. 2010).

Recently, significant progress has been made for a subset of patients with single or few brain metastases and well controlled systemic disease. Surgical resection or stereotactic radiosurgery (SRS) combined with WBRT significantly prolonged survival (Patchell et al. 1990; Vecht et al. 1993; Andrews et al. 2004). Median survival of

This study was presented in part at the 53rd Annual Meeting of the American Society for Radiation Oncology in Miami, October 2-6, 2011.

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patients who received these aggressive therapies ranges 7–10 months. Unfortunately, patients who entered into these clinical trials represent only a small minority of the patients with brain metastases. For the majority of patients with multiple brain metastases and uncontrolled systemic disease, only WBRT is the standard treatment of choice.

The role of chemotherapy in brain metastasis has been limited because of the concern about the activity of chemotherapeutic agent to cross the blood–brain barrier (BBB). Recently, the activity of chemotherapy in brain metastasis is highlighted (Robinet et al. 2001; Walbert and Gilbert 2009; Mehta et al. 2010). Concurrent chemoradiation therapies with BBB permeable agents, such as Temozolamide or topotecan are currently under investigation in prospective clinical trials. Some investigators suggested that the permeability of BBB can alter after fractionated radiotherapy for brain metastasis (Yuan et al. 2006; Wilson et al. 2009). However, whether the use of chemotherapy affects survival of the patients with brain metastasis or not has not been elucidated before.

The primary aim of this study was to perform comprehensive analysis of 134 consecutive patients with newly-diagnosed brain metastases primarily treated by WBRT alone in a single institution. The secondary aim was to define independent prognostic factors associated with longer survival after WBRT. The final aim was to investigate the prognostic value of chemotherapy on survival after WBRT in patients with brain metastases.

Materials and methods

Patient characteristics

The database of patients who underwent radiotherapy for brain metastases at our institution was reviewed. A total of 264 patients were treated with WBRT between 2007 and 2008. Of these, 23 patients received WBRT as a salvage therapy after SRS. Another 39 patients received WBRT as an adjuvant therapy after resection of metastatic brain tumor. Forty-seven patients were metastases from radiosensitive primary tumor such as leukemia, lymphoma or small cell carcinoma. Excluding these patients, we reviewed the medical records of 155 patients with newly diagnosed brain metastases treated with WBRT as a primary therapy. Of these, 19 patients presented with symptoms or radiographic findings of leptomeningeal metastasis. We excluded these patients with leptomeningeal metastasis because they are known to have extremely limited survival. Two patients were ineligible for evaluation because of allergy to contrast media. Finally, a group of 134 patients were subjected to extensive analysis. The clinical and image interpretation data from these patients were entered into database in December 2010. Distribution of baseline patient and tumor characteristics is shown in Table 1.

Table 1 Distribution of baseline patient and tumor characteristics

Parameters	*n*	%	Parameters	*n*	%
Median age (years)	60		Extracranial distant metastases		
Gender			Absent	11	8
Male	69	51	Stable	16	12
Female	65	49	Progressive	107	80
Karnofsky performance status (KPS)			Activity of extracranical tumor		
100–90	46	34	Absent/stable	20	15
80–70	49	37	Progressive	114	85
60–50	29	22	Time to diagnosis of brain metastasis		
40–0	10	7	<3 months	21	16
Neurologic status			3–12 months	33	25
0	45	34	1–2 years	22	16
1	27	20	≥2 years	58	43
2	34	25	Type of the diagnostic brain image		
3	21	16	MRI	106	79
4	7	5	CT	28	21
RPA criteria			Number of brain metastases		
Class 1	5	4	1–4	40	30
Class 2	91	68	5–10	39	29
Class 3	38	28	11–24	29	22
Site of primary tumor			≥25	26	19
Lung	75	56	Size of the largest lesion		
Breast	27	20	≤10	31	23
Upper gastrointestinal tract	11	8	11–20	46	34
Colorectum	10	8	21–30	34	25
Genitourinary tract	5	4	>30	23	17
Others	6	5	Chemotherapeutic regimens before WBRT		
Histological type			None	22	16
Adenocarcinoma	114	85	Single	28	21
Squamous cell carcinoma	9	7	Multiple	84	63
Others	11	8	Chemotherapeutic regimens after WBRT		
Primary tumor status			None	70	52
Absent	57	42	Single	31	23
Stable	25	19	Multiple	33	25
Progressive	52	39	Molecular targeted therapy after WBRT (>1 month)		
			No	100	74
			Yes	34	26

RPA recursive partitioning analysis, *MRI* magnetic resonance imaging, *CT* computed tomography, *WBRT* whole brain radiation therapy

Imaging studies

Diagnosis of brain metastases was performed mainly with magnetic resonance images (MRI). In our institute, all patients with lung cancer routinely undergo brain imaging for initial staging or scheduled follow-up. Patients with other solid tumors underwent brain imaging when brain metastasis is clinically suspected. In this study, initial diagnostic brain images included MRI in 106 patients (79 %) and CT in 28 patients (21 %). Radiological features assessed included number, maximum tumor diameter and location. For follow-up brain images, change in size of the tumors and presence of new metastases were recorded. At least 20 % increase in diameter of the each preexisted tumor before WBRT, taking as reference on the smallest diameter after WBRT, was defined as local progression.

Treatment strategy

Treatment strategy for brain metastasis at our institution was previously described elsewhere (Narita and Shibui 2009; Hashimoto et al. 2011). Patients who received WBRT alone as a primary treatment for brain metastases were subjected for this study. Patients with brain metastases generally have extracranial systemic disease. After WBRT, patients with known systemic disease were indicated to start or continue chemotherapy if they still had active chemotherapeutic regimen with sufficient organ function and with Karnofsky performance status (KPS) of 70 or more. Salvage SRS was considered for recurrent brain metastases after WBRT. Some patients with known chemo-sensitive tumor continued palliative chemotherapy for recurrent brain metastases.

Consent for the treatment was obtained from each patient after the sufficient explanation of potential risks of treatment. All the patients provided written informed consent. Our institutional review board has approved this study.

Whole brain radiation therapy

One hundred and thirty-four patients were intended to receive WBRT. Of these, 128 patients were delivered to a dose of 30 Gy in 10 fractions. Another 3 patients were delivered to 37.5 Gy in 15 fractions, whereas one patient was delivered to 20 Gy in 5 fractions. Two patients discontinued irradiation course because of the deterioration of general condition at a dose of 12 and 24 Gy, respectively.

Retrospective analysis

All the medical charts of the eligible patients were reviewed. Information on potential prognostic factors (age, gender, KPS, neurologic status, site of primary tumor, primary tumor status, activity of extracranial distant metastases, time to develop brain metastasis, number of brain metastases, size of the largest lesion, use of chemotherapy before or after WBRT) was collected.

Initial neurological function was classified into 4 categories (No symptoms: grade 0, Minor symptoms; fully active without assistance: grade 1, Moderate symptoms; fully active but requires assistance: grade 2, Moderate symptoms; less than fully active: grade 3, Severe symptoms; totally inactive: grade 4). Radiation Therapy Oncology Group's (RTOG) recursive partitioning analysis (RPA) classes were coded into 3 categories as follows: Class 1: Patients with KPS $\geq$ 70, <65 years of age with controlled primary and no extracranial metastases; Class 3: KPS < 70; Class 2: all the others (Gaspar et al. 1997).

For the evaluation of extracranial disease status, if there were no evidence of residual tumor after therapy, the activity was coded as "absent". If any tumor existed and there is no increase in size of the tumor for more than 6 months, the activity was coded as "stable". A continuous use of same chemotherapeutic regimen didn't impair the coding of "stable". If any tumor existed with any situation other than "stable", the activity was coded as "progressive".

Patients whose brain metastases were detected at the same time or soon after the diagnosis of primary tumor (so-called "synchronous" brain metastasis) may have different prognosis. We defined "synchronous" brain metastasis as those detected at the same time or detected within 3 months of the initial diagnosis of primary tumor.

For the analysis of prognostic effect of chemotherapy before or after WBRT, three different cohorts were defined: none, single regimen and multiple regimens. If a patient received two or more different types of chemotherapeutic regimens, the status was coded as multiple regimens. Any type of hormonal therapy was regarded as a single regimen. The status of the use of molecular targeted therapy was defined as "yes", if a patient continued to receive a specific regimen for more than 1 month.

Statistical analysis

Overall survival from the start of WBRT was calculated with the Kaplan–Meier method. For univariate and multivariate analysis, all the variables were dichotomized according to the clinical relevance from previous literature. Univariate analyses were performed by using log-rank test. Possible confounded variables were excluded from multivariate analysis. A Cox's proportional hazards model was developed to identify significant factors influencing survival after WBRT. All the tests of hypotheses were

conducted at the alpha level of 0.05 with a 95 % confidence interval. All the statistical analyses were performed by using SPSS Statistics version 17.0 (SAS Institute, Tokyo, Japan).

Results

Outcomes for the entire group

Median survival time (MST) for the entire patients from the start of WBRT was 5.7 months. The 6 months, 1- and 2-year survival rate were 43, 28 and 12 %, respectively. MST of the patients with RTOG's RPA Class 1 ($n = 5$), 2 ($n = 91$) and 3 ($n = 38$) were 10.3, 7.8 and 2.2 months, respectively (Fig. 1). Median intracranial progression-free survival (PFS) were 4.7 months, with 6 months, 1- and 2-year PFS of 35, 14 and 4 %, respectively. A total of 49 patients developed intracranial recurrence after WBRT. The sites of first recurrence after WBRT were as follows: local only (regrowth of preexisted tumors): 25 (51 %); new metastasis only: 10 (20 %); both of local and new metastasis: 12 (24 %); and leptomeningeal dissemination: 2 (4 %). Median local progression-free duration and median intracranial new metastasis-free duration for the entire patients were 9.7 and 18.0 months, respectively. At the time of analysis, 5 patients were alive with disease. The causes of death were identified in 118 patients. Of these, 38 patients (32 %) were due to intracranial tumor progression, whereas 76 patients (64 %) were due to systemic disease. Four patients (3 %) died from intercurrent disease. None had died directly from toxicity of WBRT.

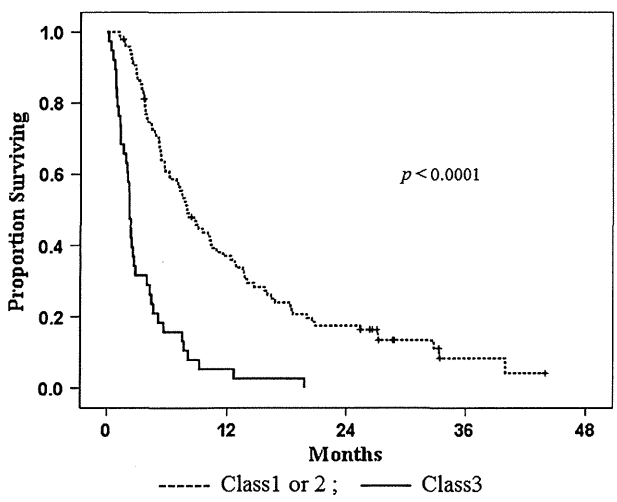

Fig. 1 Kaplan–Meier survival curve for overall survival by RPA criteria

Factors influencing survival after WBRT: univariate and multivariate analyses

Univariate analysis was performed on 12 different variables to evaluate their potential value on survival after WBRT. Univariate analyses identified 9 variables which significantly associated with good prognosis (Table 2).

Multivariate analysis was performed on 9 independent variables. Table 3 summarizes the result of the multivariate analysis for survival after WBRT. Multivariate analysis revealed that KPS (≥70 vs. 70, hazard rate (HR): 2.540, $p < 0.0001$), gender (female vs. male, HR: 2.293, $p < 0.0001$), activity of extracranial disease (absent/stable vs. progressive, HR: 2.134, $p = 0.015$), time to develop brain metastasis (<3 vs. ≥3 months, HR: 1.926, $p = 0.042$), and use of chemotherapy after WBRT (multiple vs. none/single regimens, HR: 3.406, $p < 0.0001$) were independent prognostic factors for overall survival.

Survivals depending on chemotherapy after WBRT

After WBRT, only two patients had no evidence of extracranial tumor. The two patients didn't receive further chemotherapy until disease progression. Another 132 patient had known extracranial tumor including primary, nodal or distant sites. They were indicated to start or continue chemotherapy when it was clinically applicable. A total of 64 patients with extracranial systemic disease underwent chemotherapy after WBRT. Thirty-one patients (23 %) received only a single chemotherapeutic regime, and 33 patients (25 %) received multiple regimens. Figure 2 shows the survival curve by the use of chemotherapy after WBRT. The MST of the patients who received none, single and multiple regimens after WBRT were 3.3, 7.5 and 16.4 months, respectively ($p < 0.0001$). The use of multiple chemotherapeutic regimens after WBRT was found to be associated with better survival after WBRT in multivariate analysis ($p < 0.0001$). Among 95 patients with pre-irradiation KPS ≥ 70, 59 patients (62 %) received chemotherapy, whereas 5 patients (13 %) with KPS < 70 received chemotherapy. Among patients with KPS ≥ 70, the MST of the patients who received none, single and multiple regimens after WBRT were 4.5, 7.9 and 16.4 months, respectively ($p < 0.0001$). Overall, 95 % of the patients included in this study received chemotherapy either before or after WBRT.

The effect of molecular-targeted therapy after WBRT

A total of 34 patients (25 %) received molecular-targeted therapy after WBRT for 1 month or more. Of these patients, the sites of primary disease were lung in 28, breast