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

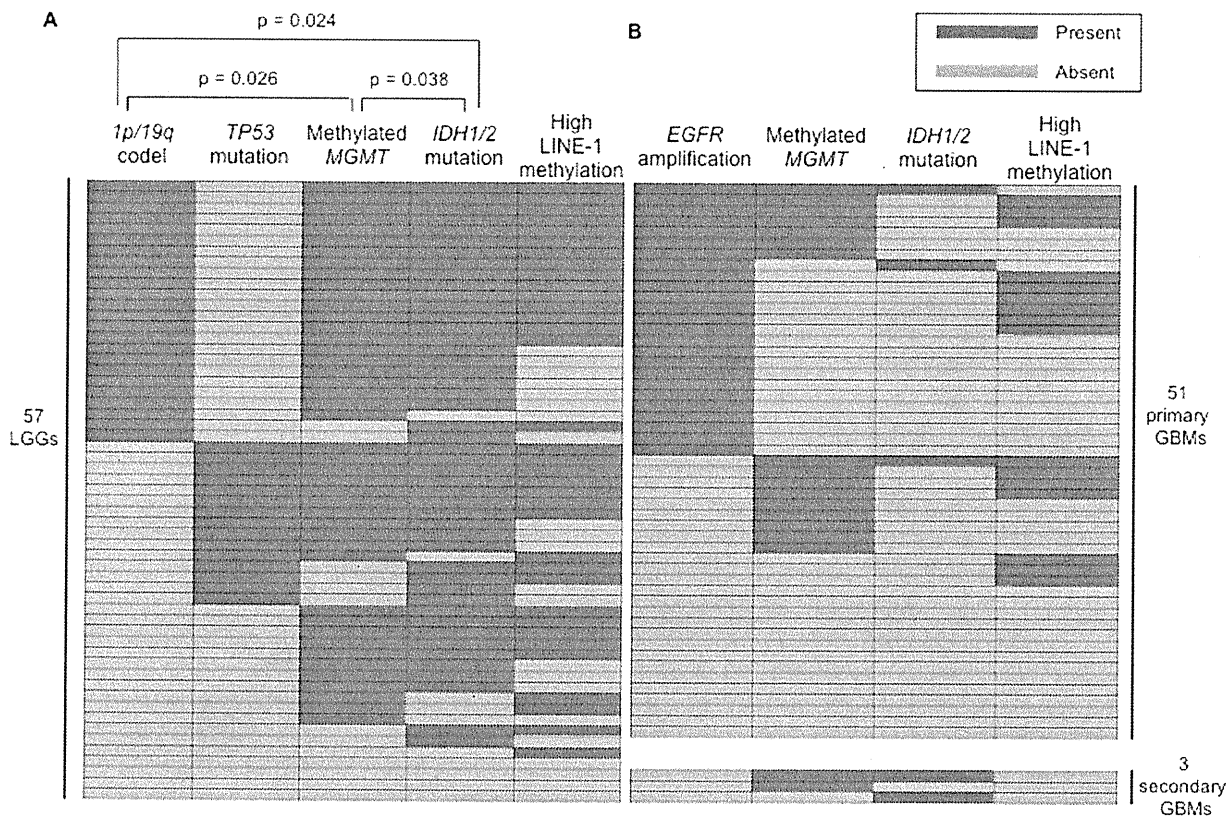

Figure 1. Correlations between the methylation status of the *MGMT* promoter, *IDH1/2* mutations, and *1p/19q* deletions, higher LINE-1 methylation in low-grade gliomas (LGGs), *EGFR* amplification, *MGMT* promoter, *IDH1/2* mutations, high LINE-1 methylation in primary and secondary GBMs. Using the 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

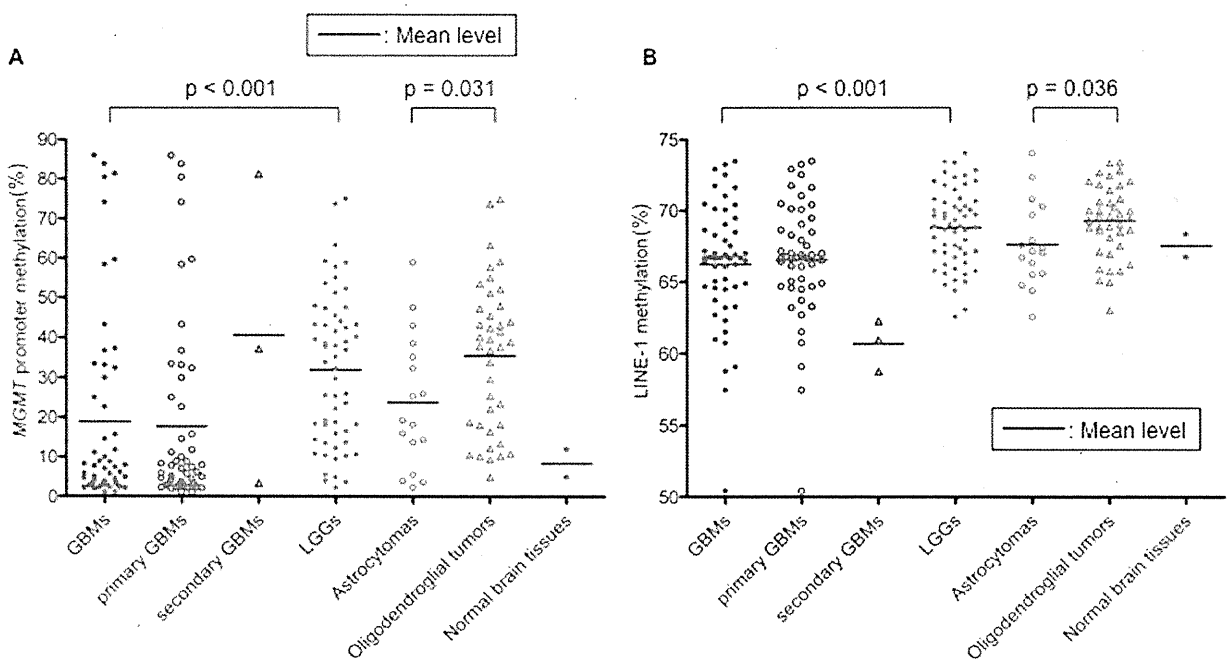

Figure 2. Differences in the methylation levels of *MGMT* promoter and LINE-1 between low-grade gliomas (LGGs) and glioblastoma multiforme (GBM), and between grade 2 astrocytomas and oligodendroglial tumors. A higher proportion of LGGs including astrocytoma, oligodendroglioma, and oligoastrocytoma, exhibited 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

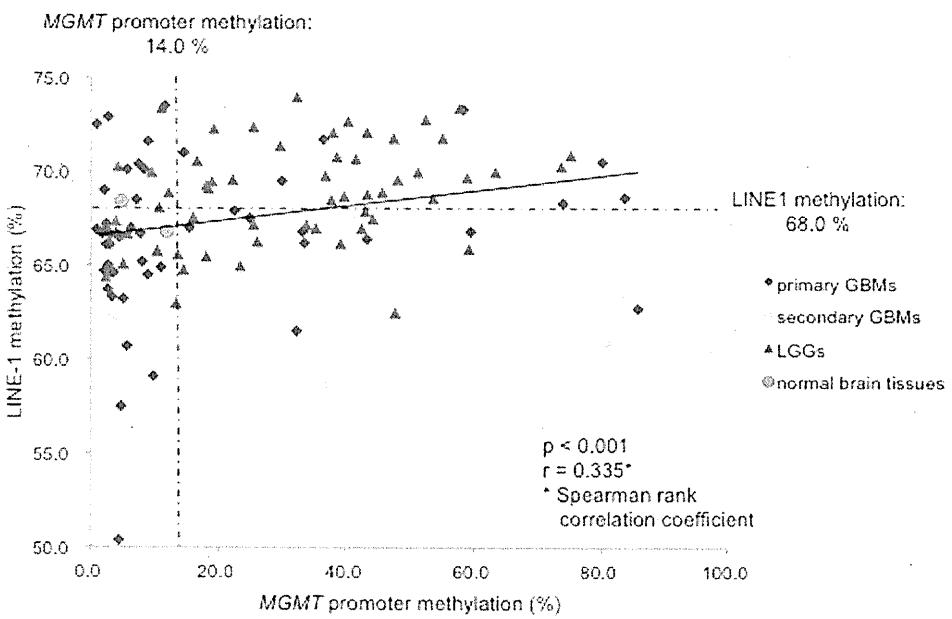

Figure 3. LINE-1 methylation is directly proportional to *MGMT* promoter methylation in gliomas. *MGMT* promoter methylation level was directly proportional to LINE–1 methylation in a statistically significant manner ($r = 0.335$, $p<0.001$) for all samples quantified, including LGGs, primary and secondary 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

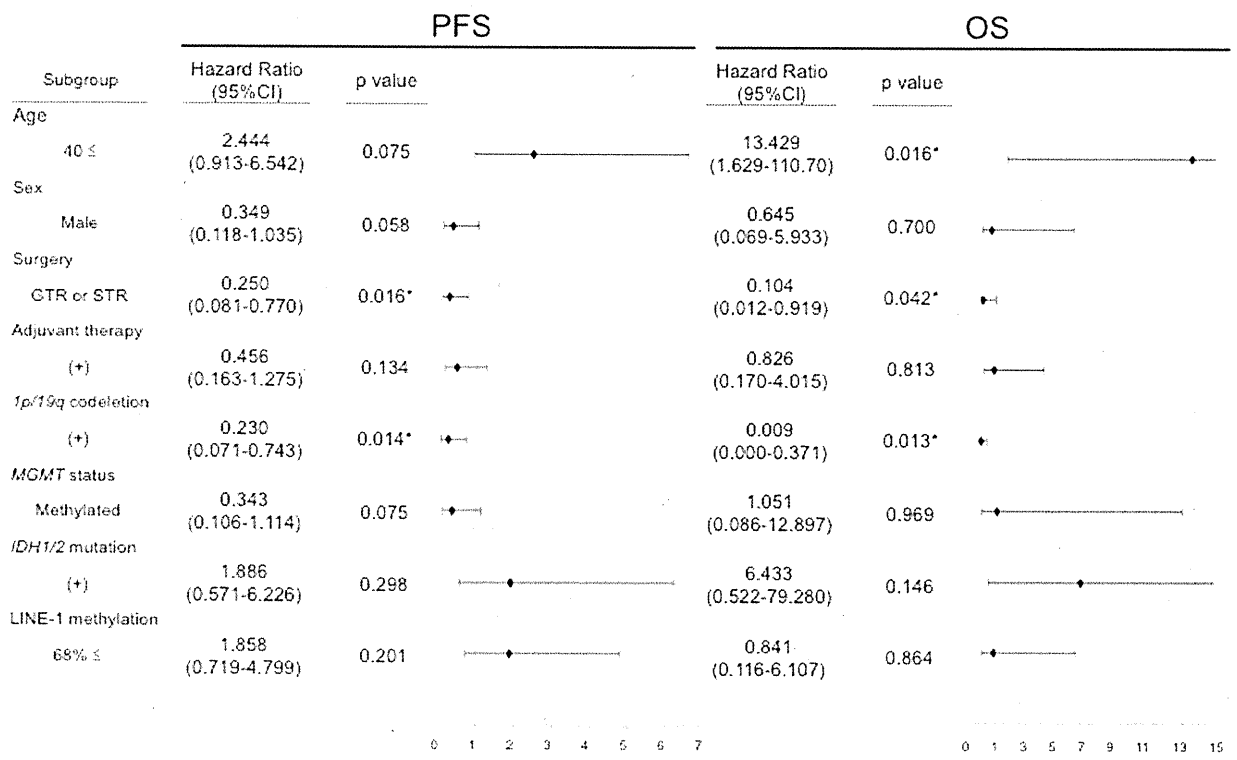

Figure 4. Clinical, genetic, and epigenetic parameters in correlation with progression-free survival (PFS) and overall survival (OS) in low-grade glioma patients. The presence of *1p/19q* codeletion and the extent of resection were independently correlated with prolonged PFS, as shown with multivariate 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).

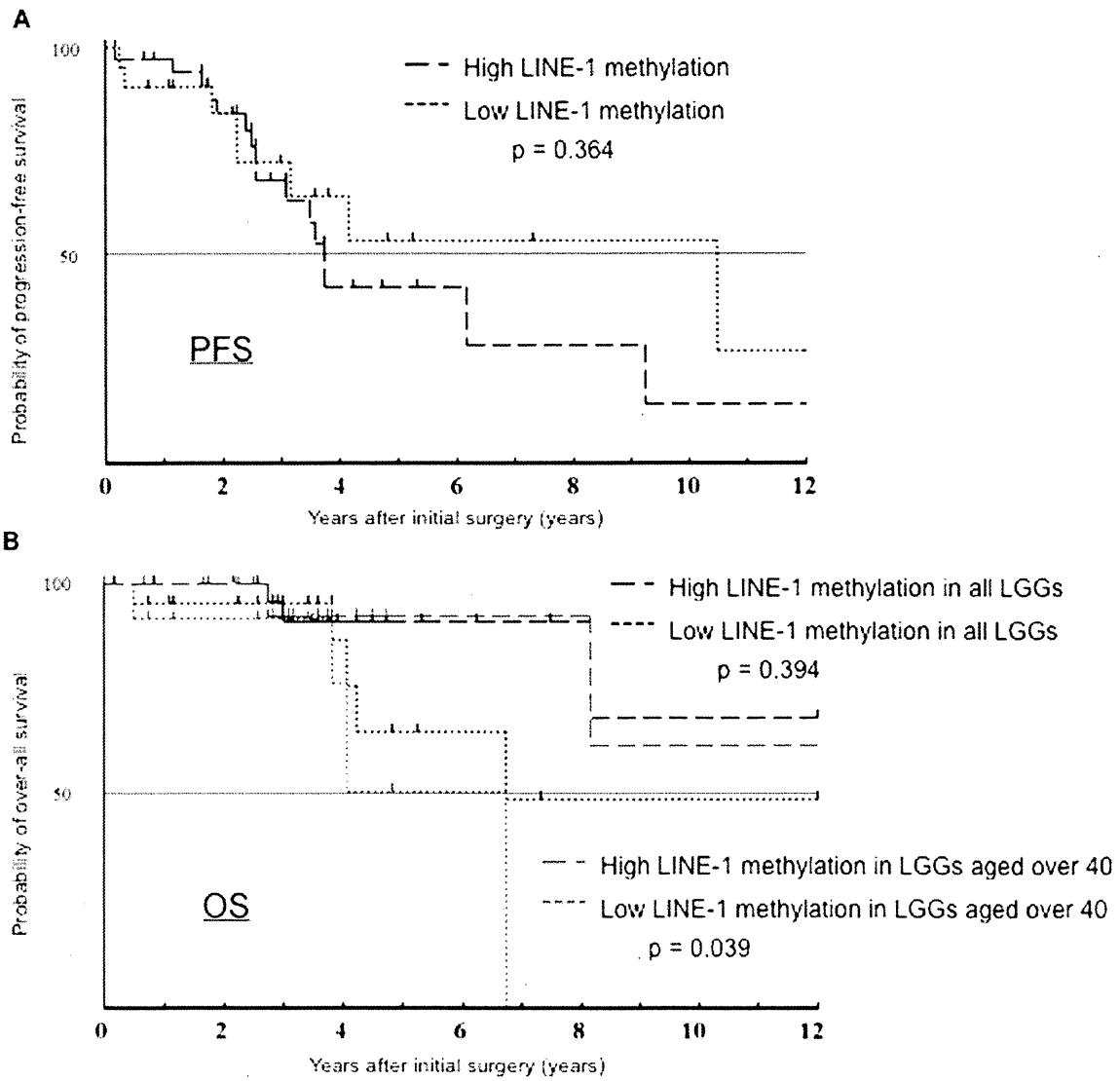

Figure 5. High LINE-1 methylation status in correlation with progression-free survival (PFS) and overall survival (OS) in low-grade glioma patients. In the Kaplan-Meier survival curve of patients with LGGs, High LINE-1 methylation status was not correlated with PFS in LGGs, using log-rank test (p = 0.364); (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).
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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

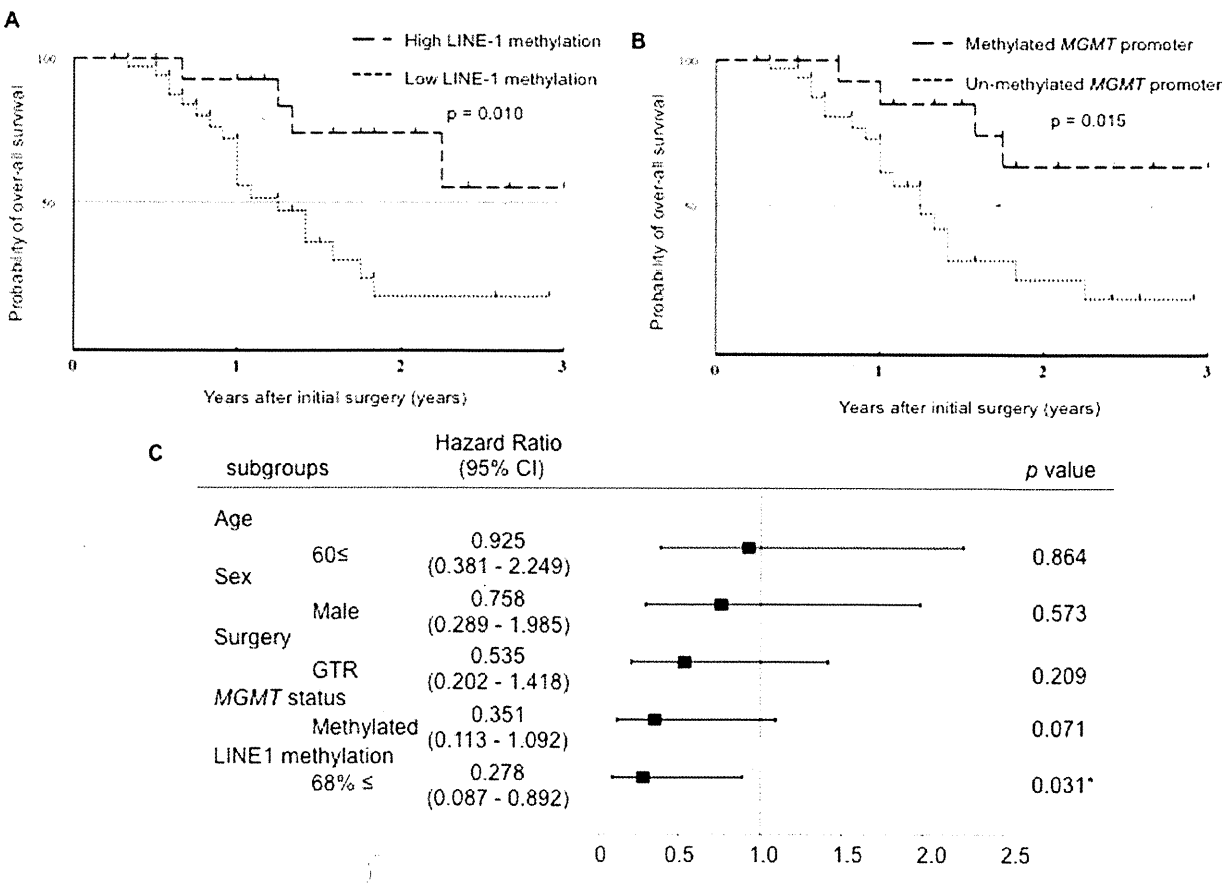

Figure 6. LINE-1 methylation is a better prognostic indicator in primary GBMs. In the Kaplan-Meier survival curve of patients with primary GBM, univariate analysis indicated a lower p value in the comparison of <68% and ≥68% of LINE-1 methylation (A) than in the comparison of <14% and ≥14% of *MGMT* 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).

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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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Original Article

Benefits of Interferon-β and Temozolomide Combination Therapy for Newly Diagnosed Primary Glioblastoma With the Unmethylated MGMT Promoter

A Multicenter Study

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BACKGROUND: The aim of the current study was to catalog genomic and epigenomic abnormalities in newly diagnosed glioblastoma patients and determine the correlation among clinical, genetic, and epigenetic profiles and clinical outcome. **METHODS:** This study retrospectively included 68 consecutive patients who underwent surgical treatment and received standard radiotherapy with temozolomide (TMZ)-based chemotherapy. Of a total of 68 patients, 39 patients (57.4%) received interferon (IFN)-β in combination of TMZ. **RESULTS:** The genetic and epigenetic 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%). Multivariate analysis revealed that methylated MGMT promoter and the combination of TMZ and IFN-β were independent prognostic factors associated with survival. The median survival time (MST) of the patients who received the combination of IFN-β and TMZ was significantly greater with 19.9 months as compared to the TMZ alone group (12.7 months). Notably, in even patients whose tumors had unmethylated MGMT promoter, the MST prolonged to 17.2 months when receiving TMZ with IFN-β, compared to 12.5 months in those receiving TMZ without IFN-β. **CONCLUSIONS:** Taken together, addition of IFN-β for newly diagnosed primary GBM achieved a favorable outcome, particularly in patients with unmethylated MGMT promoter. ***Cancer* 2011;117:1721–30.** © *2010 American Cancer Society.*

KEYWORDS: IDH1, MGMT methylation, glioblastoma, interferon-β, temozolomide.

Glioblastoma multiforme (GBM) is one of the most frequent primary brain tumors in the central nervous system in adults and is highly malignant, with a median survival time of about one year from diagnosis. This is despite aggressive treatment, surgery, postoperative radiotherapy, and adjuvant chemotherapy. An international randomized trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) comparing radiotherapy alone and concomitant radiotherapy and temozolomide (TMZ) clearly attested the benefits of adjuvant TMZ chemotherapy for GBM patients.[1] Since then, TMZ has been the current first-line chemotherapeutic agent for GBM.

A subanalysis in this trial showed the effectiveness of epigenetic silencing of the MGMT gene by promoter methylation with longer survival in patients with primary GBM; it also suggested the benefits of combining chemotherapy using TMZ with radiotherapy.[2]

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Furthermore, there have been recent attempts to comprehensively profile GBM genes by The Cancer Genome Atlas (TCGA) project and other groups.[3,4] Some genetic aberrations in GBM, such as *TP53* mutation or deletion, *NF1* deletion or mutation, and *ERBB2* mutation, have been found to be more common than previously reported. In addition, novel molecular markers, such as frequent mutations of the *IDH1* and *IDH2* genes in secondary GBM have been discovered.[5-7] These findings on mutations, genomic and epigenomic aberrations, and transcriptomal features in GBM might aid in understanding the classification of GBM and its further potential clinical implications.

However, the TCGA project included GBM patients who received surgical treatment, and detailed information on adjuvant chemoradiotherapy was not provided. Therefore, the close relationship between the gene profile provided by TCGA and chemotherapy regimens remains unknown.[3]

In this current study, we aimed to determine the correlation between clinical, genetic, and epigenetic profiles, and clinical outcome in newly diagnosed GBM patients who received TMZ-based chemotherapy. Interestingly, we found a significant beneficial outcome in patients receiving TMZ in addition to IFN-β. Moreover, our study discovered that GBM patients with the ummethylated O^6-methylguanine-DNA methyltransferase (MGMT) promoter, in particular, showed benefits from IFN-β.

MATERIALS AND METHODS

Patient population

We retrospectively reviewed 68 consecutive patients with newly diagnosed primary GBM who underwent surgical treatment at several academic tertiary-care neurosurgical institutions: Nagoya University Hospital, Hamamatsu University Hospital, Oita University Hospital, and Shizuoka Cancer Center from May 2006 through June 2010 after TMZ was approved as the treatment agent for malignant gliomas by the National Ministry of Health and Welfare of Japan. The diagnosis of GBM was established by histological confirmation according to the WHO guidelines[8,9] independently by at least two expert neuropathologists. The clinical, operative, and hospital course records were reviewed. Information collected from clinical notes included patient demographics, pre- and postoperative neuroimaging, and adjuvant therapy. Preoperative Eastern Cooperative Oncology Group performance status (ECOG PS) scores were assigned by the clinician at the time of evaluation and were available in the chart for review for all patients. The study was approved by the institutional review board at each participating hospital and complied with all provisions of the Declaration of Helsinki.

Treatment

Radiotherapy

After undergoing surgery, the patients received focal external-beam radiotherapy by conventional radiation planning to approximately 60 Gray (Gy) (±5% total dose), with daily concurrent TMZ at 75 mg/m^2 throughout the course of radiotherapy.

Chemotherapy

All patients received the standard Stupp regimen.[1] In the absence of grade 3 or 4 hematological excessive toxicity, TMZ administration was continued until clinical or radiological evidence of disease progression was observed. Of these 68 patients, 39 patients (57.4%) received adjuvant IFN-β treatment (Table 1). Patients in Nagoya University and Oita University received chemotherapy consisting of IFN-β. There were no significant differences in any of the clinical parameters and genetic, epigenetic parameters (i.e., age, sex, preoperative PS, tumor location, extent of resection, genetic and epigenetic alterations between the institutions using regimen with and without IFN-β. The IFN-β chemotherapy regimen comprised 3 million international units (MIU)/body administered intravenously on alternate days during radiotherapy and TMZ-induction chemotherapy.[10,11] At the end of the induction period, after a 4-week interval, the patients were administered 3 MIU/body of IFN-β on the first morning every 4 weeks during TMZ maintenance chemotherapy. In the case of tumor progression, salvage or second-line therapy was administered at the investigators' discretion; most patients received additional chemotherapy.

Response Evaluation During Treatment

Both radiological and clinical findings were used to evaluate the response. Follow-up magnetic resonance imaging (MRI) was performed for alternate cycles. If the MRI showed continued increase in enhancement, the case was considered as tumor progression. If re-resection was performed for a recurrent mass lesion, histological interpretation formed the basis for definitive diagnosis (treatment-related necrosis vs recurrent tumor).

Table 1. Clinical Characteristics[a]{TC}

Parameter	No. of Patients	%
	n=68	
Age(y)		
Median	55.0	
Range	12-84	
<40	12	17.6
≥40, <60	24	35.3
≥60	32	47.1
Sex		
Male	41	60.3
Female	27	39.7
Preoperative ECOG performance status		
Median	1	
Range	0-3	
Preoperative ECOG performance status		
≤1	45	66.2
>1	23	33.8
Tumor location		
Superficial	50	73.5
Deep	18	26.5
Surgery		
GTR	24	35.3
Non-GTR	44	64.7
Chemotherapy		
TMZ only	29	42.6
TMZ+ IFN-β	39	57.4

ECOG indicates Eastern Cooperative Oncology Group; PS, performance status; GTR, macroscopic (gross) total removal; TMZ, temozolomide.

Tumor Samples and DNA Extraction

All patients provided their written informed consent for molecular studies of their tumor, and the protocol was approved by the ethics committee at each center. Sixty-eight brain tumor specimens were obtained at the time of first surgical resection.

Tumor tissue samples were immediately frozen and stored at −80°C until the extraction of genomic DNA. DNA was prepared using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Placental DNA was used as the normal control. 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 for the determination of allelic losses and gains of the gene 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).[12-15] 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). Duplicate experiments were performed to obtain accurate MLPA values. 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, Courtaboeuf Cedex, France). Pyrosequencing technology was used to determine the methylation status of the CpG island region of MGMT as described previously.[16,17] We used the touchdown PCR method. The primer sequences used were the MGMT forward primer, 5′-TTGGTAAATTAAGGTATAGAGTTTT-3′, and the MGMT biotinylated reverse primer, 5′-AAA CAATCTACGCATCCT-3′. PCR 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. 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 0.2 N NaOH solution and 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 was 5′-GGAAGTTGGGAAGG-3′. Methylation quantification was performed using the provided software.

TP53 and IDH1/IDH2 Sequencing

Direct sequencing of the *TP53* exons 5 to 8 and *IDH1/IDH2* was performed as previously described.[7,18,19] The primer sequences are listed in Table 2.

Table 2. List of Primer Sequences for Direct DNA Sequencing{TC}

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;.

For IDH sequencing, a fragment 129 bp in length, spanning the sequence encoding the catalytic domain of *IDH1*, including codon 132, and a fragment 150 bp in length spanning the sequence encoding the catalytic domain of *IDH2*, including codon 172, were amplified. We applied touchdown PCR, using the standard buffer conditions: it comprised 5 ng of DNA and AmpliTaq Gold DNA Polymerase (Applied Biosystems) run for 16 cycles with denaturation at 95°C for 30 s, annealing at 65 to 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 μl and add 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, ending with at 72°C for 7 min to complete extension.

Direct sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

Statistical analysis was performed using the statistical software SPSS for Windows, version 17.0 (SPSS Inc, Chicago, Ill). The Mann-Whitney U test, χ^2 test, and Fisher exact test were used to test for association of clinical variables and molecular markers. Survival was estimated by using the Kaplan-Meier method, and survival curves were compared by using the log-rank test. Progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow up. Overall survival (OS) was calculated from the day of first surgery until death or the end of follow up. Univariate and multivariate analyses were performed to test the potential influence of baseline characteristics on survival. The effect of each single molecular marker on PFS and OS was investigated using the Cox proportional hazards model, adjusting for the major clinical prognostic factors, including age at diagnosis (<40 vs ≥40, <60 vs ≥60 years), ECOG performance status score (ECOG PS; ≤1 vs >1), extent of resection (macroscopic [gross] total resection [GTR] vs non-GTR), tumor location (superficial vs deep), MGMT promoter methylation status, chromosome 1p loss of heterozygosity (LOH), 19qLOH, *PTEN* loss, *CDKN2A* loss, *TP53* loss and mutation, *ERBB2* amplification, *EGFR* amplification, *IDH1* and *IDH2* mutation, and adjuvant therapy (with IFN-β vs without IFN-β). Factors with no significant association with survival, at a level of more than 0.05 in the multivariate analysis, were eliminated. The remaining factors in the multivariate proportional hazard model ($P < .05$) were considered to be independent predictors of survival.

To assess for the treatment effects of TMZ with IFN-β versus TMZ without IFN-β for overall survival (OS), the hazard ratio was computed using a proportional hazard model by baseline characteristics in stratified analysis.

RESULTS

Clinical Parameters

Between May 2006 and June 2010, 68 consecutive patients newly diagnosed with primary GBM were registered in this study. Their clinical characteristics are summarized in Table 1. This study group comprised 41 men and 27 women aged 12-84 years (median, 55). The median preoperative ECOG PS score at diagnosis was 1 (range, 0-3); the preoperative ECOG PS score was <1 in 45 patients (66.2%). All tumors were located in the supratentorial region: 50 tumors were located in the superficial area (cortical or subcortical area), and 18 were located in deep anatomical structures such as the basal ganglia and corpus callosum. No tumor was noted in the optic nerve, olfactory nerve, and pituitary gland on pretreatment MRI. No tumor dissemination was detected by MRI. Surgical GTR was achieved in 24 patients (35.3%), and 44 patients underwent non-GTR (64.7%). None of the patients had concurrent active malignancy, and the baseline organ function before chemotherapy was as follows: absolute WBC ≥3000/mm^3 or neutrophil count ≥1,500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥8.0 g/dl, AST less than 2.5 × the upper limit of normal (ULN), total bilirubin 2 × ULN, and creatinine 2 × ULN, and electrocardiogram showing no serious

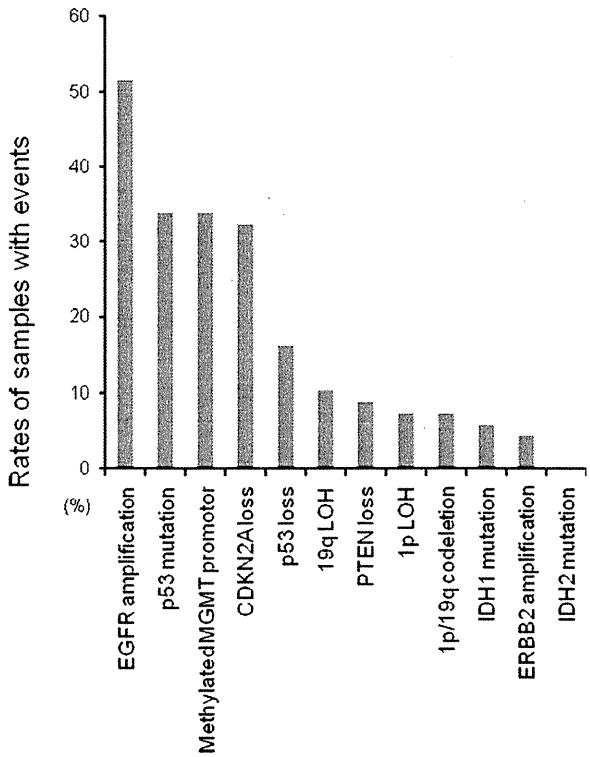


Figure 1. Frequency and pattern of genetic and epigenetic alterations in newly diagnosed primary glioblastoma multiforme (GBM).

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

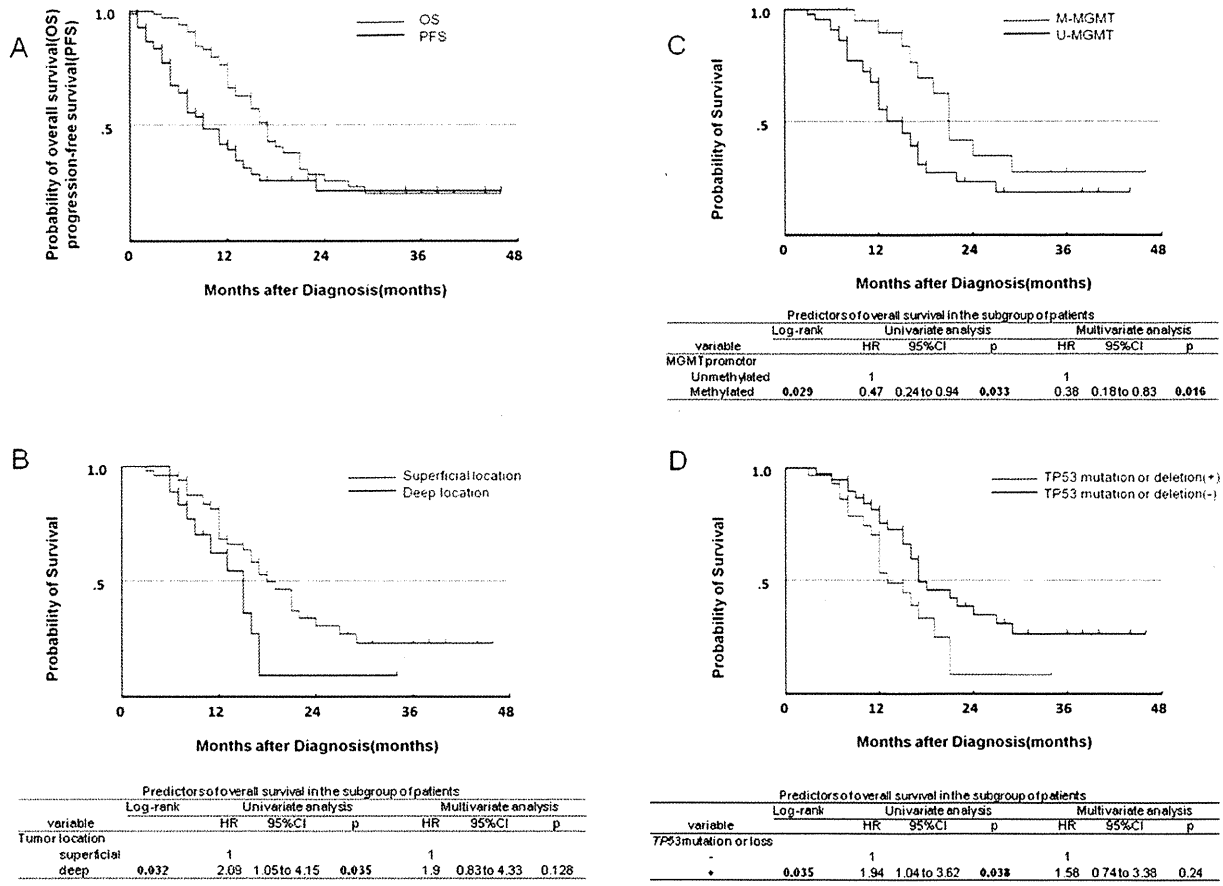
Next, we sought to determine the subpopulation that had benefited from the use of the IFN-β combination treatment. It is well known that patients with GBM containing the methylated MGMT promoter benefit from TMZ, but those with the unmethylated MGMT promoter show no such benefits.[1,2] Consistently, 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

	Log-rank	Univariate analysis			Multivariate analysis		
variable		HR	95%CI	p	HR	95%CI	p
MGMT promotor							
Unmethylated		1			1		
Methylated	0.029	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

	Log-rank	Univariate analysis			Multivariate analysis		
variable		HR	95%CI	p	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	0.128

Predictors of overall survival in the subgroup of patients

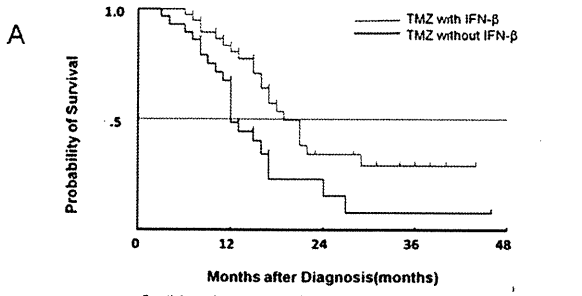
	Log-rank	Univariate analysis			Multivariate analysis		
variable		HR	95%CI	p	HR	95%CI	p
TP53 mutation or loss							
-		1			1		
+	0.035	1.94	1.04 to 3.62	0.038	1.58	0.74 to 3.38	0.24

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

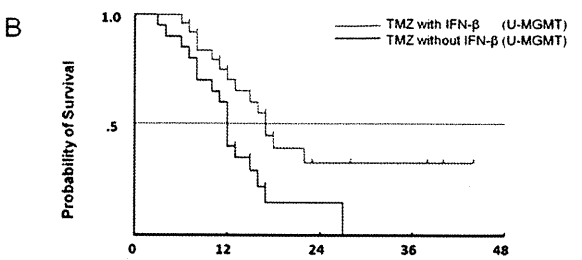


Predictors of overall survival in the subgroup of patients

	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.

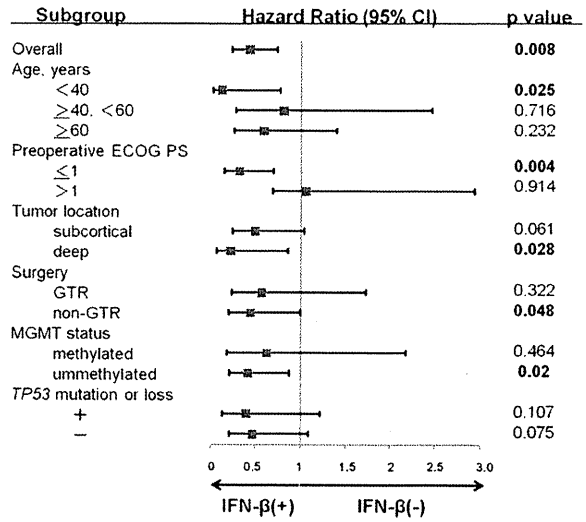


Figure 4. Estimated effect of temozolomide (TMZ) with interferon-β (IFN-β) versus TMZ without IFN-β on the hazard of overall survival (OS), according to baseline characteristics. The hazard ratio was computed using a proportional hazard model by selected factors. There were significant associations under 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

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CLINICAL INVESTIGATION **Thoracic Cancer**

PHASE I STUDY OF CONCURRENT HIGH-DOSE THREE-DIMENSIONAL CONFORMAL RADIOTHERAPY WITH CHEMOTHERAPY USING CISPLATIN AND VINORELBINE FOR UNRESECTABLE STAGE III NON-SMALL-CELL LUNG CANCER

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Purpose: To determine the maximum tolerated dose in concurrent three-dimensional conformal radiotherapy (3D-CRT) with chemotherapy for unresectable Stage III non–small-cell lung cancer (NSCLC).

Patients and Methods: Eligible patients with unresectable Stage III NSCLC, age ≥20 years, performance status 0–1, percent of volume of normal lung receiving 20 GY or more (V_{20}) ≤30% received three to four cycles of cisplatin (80 mg/m^2 Day 1) and vinorelbine (20 mg/m^2 Days 1 and 8) repeated every 4 weeks. The doses of 3D-CRT were 66 Gy, 72 Gy, and 78 Gy at dose levels 1 to 3, respectively.

Results: Of the 17, 16, and 24 patients assessed for eligibility, 13 (76%), 12 (75%), and 6 (25%) were enrolled at dose levels 1 to 3, respectively. The main reasons for exclusion were V_{20} >30% (n = 10) and overdose to the esophagus (n = 8) and brachial plexus (n = 2). There were 26 men and 5 women, with a median age of 60 years (range, 41–75). The full planned dose of radiotherapy could be administered to all the patients. Grade 3–4 neutropenia and febrile neutropenia were noted in 24 (77%) and 5 (16%) of the 31 patients, respectively. Grade 4 infection, Grade 3 esophagitis, and Grade 3 pulmonary toxicity were noted in 1 patient, 2 patients, and 1 patient, respectively. The dose-limiting toxicity was noted in 17% of the patients at each dose level. The median survival and 3-year and 4-year survival rates were 41.9 months, 72.3%, and 49.2%, respectively.

Conclusions: 72 Gy was the maximum dose that could be achieved in most patients, given the predetermined normal tissue constraints. © 2012 Elsevier Inc.

Lung cancer, Chemotherapy, Radiotherapy, High dose, Conformal.

INTRODUCTION

Approximately one third of patients with non–small-cell lung cancer (NSCLC) present with locally advanced Stage III disease at the initial diagnosis (1). Of this category, Stage IIIA disease with bulky N2 and Stage IIIB disease without pleural effusion are characterized by a large primary lesion and/or involvement of the mediastinal or supraclavicular lymph nodes. In addition, the majority of these patients have occult systemic micrometastases. Concurrent thoracic radiotherapy and chemotherapy has been the standard care for these patients with unresectable disease (2, 3). A platinum doublet with a third-generation anticancer agent combined with thoracic radiotherapy was reported to yield a median overall survival time (OS) of more than 2 years and long-term survivors (4–6), but the effect of platinum-based chemotherapy has reached a plateau.

The failure pattern in patients with Stage III NSCLC treated by concurrent chemoradiotherapy was roughly local recurrence alone in one third of the patients, both local and distant recurrence in another third of patients, and distant metastasis without local failure in the remaining third of patients (2, 5).

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Thus, improvement of local control and suppression of distant metastasis are essential for prolongation of patient survival.

The conventional total dose of thoracic radiotherapy in patients with inoperable NSCLC has been 60 Gy administered in 30 fractions. This dose was established in 1987 by randomized Radiation Therapy Oncology Group trials that demonstrated better 3-year survival with a radiation dose of 60 Gy than with lower doses (7). In these trials, two-dimensional treatment planning was used, wherein the tumor volume was defined on kilovoltage radiographs (7). Thereafter, the standard initial target volume included the primary tumor, metastatic lymph nodes, and adjacent uninvolved ipsilateral hilar and mediastinal regions (elective nodal irradiation: ENI). Except for selected patients, excessive toxicity hampered an increase of the total dose to over 60 Gy in patients with locally advanced NSCLC.

It is, however, time now to reconsider the optimal dose of thoracic radiotherapy using new techniques in patients with locally advanced NSCLC, for the following reasons. First, positron emission tomography (PET) provides more accurate diagnosis of mediastinal lymph node metastases (8) and more accurate quantification of the tumor volumes, especially when atelectasis is present (9). Second, three-dimensional conformal radiation therapy (3D-CRT) enables radiation oncologists to delineate the tumor and adjacent normal tissue more sharply and to choose beam angles to maximize tumor coverage with minimum irradiation of normal tissues (10). Third, omission of the ENI resulted in improvement of radiation-associated toxicity without worsening the local control rate of the tumor (11, 12). Thus, by use of these new techniques, the optimal dose of thoracic radiation could exceed the conventional 60 Gy.

Two dose escalation studies in patients with locally advanced NSCLC showed that the total dose of thoracic radiotherapy could be increased up to 90 Gy in concurrent chemoradiotherapy using the 3D-CRT technique combined with weekly carboplatin and paclitaxel chemotherapy (13, 14). In these trials, chemoradiotherapy was administered after induction chemotherapy. However, it remained unclear whether these doses could be delivered safely to the majority of patients with locally advanced NSCLC, because it is not known how many patients were screened for the trials and how many of them were actually registered, and because some of the registered patients were excluded from the chemoradiotherapy phase after induction chemotherapy. The total number of patients evaluated in the two trials was also limited. Furthermore, chemotherapy other than weekly carboplatin and paclitaxel has not been evaluated in the setting of combined chemotherapy with high-dose thoracic radiotherapy, to our knowledge. The objectives of the current study were (1) to evaluate the toxicity of concurrent high-dose 3D-CRT without ENI with cisplatin and vinorelbine for unresectable Stage III NSCLC, (2) to determine the maximum tolerated dose (MTD) of thoracic radiotherapy, and (3) to observe the antitumor effects of this regimen.

PATIENTS AND METHODS

Study design

This study was designed as a Phase I study at the National Cancer Center Hospital. The protocol and consent form were approved by the Institutional Review Board of the National Cancer Center on July 28, 2005. We planned to treat 12 patients at a dose level and follow them up at least 6 months, and then escalate to the next level if 67% of the patients did not experience dose-limiting toxicity (DLT). We followed widely accepted normal tissue dose constraints. Patients with percent volume of the normal lung receiving 20 Gy or more (V_{20}) of greater than 30% were excluded and treated outside the study. Other dosimetric constraints were applied at the discretion of the treating radiation oncologist. Maximum doses exceeding 50 Gy to the spinal cord, 66 Gy to the esophagus, or 66 Gy to the brachial plexus were generally excluded.

Patient selection

Previously untreated patients with locally advanced NSCLC without effusion were screened for entry into this study. The eligibility criteria were (1) histologically or cytologically proven NSCLC, (2) unresectable Stage IIIA or IIIB disease confirmed by both computed tomography (CT) and PET, (3) no previous treatment, (4) measurable disease, (5) $V_{20} \leq 30\%$, (6) age ≥ 20 years, (7) Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, and (8) adequate bone marrow function (white blood cell [WBC] count $\geq 4.0 \times 10^9$/L, hemoglobin ≥ 9.5 g/dL, and platelet count $\geq 100 \times 10^9$/L), liver function (total bilirubin ≤ 1.5 mg/dL and transaminase ≤ 80 IU/L), renal function (serum creatinine ≤ 1.5 mg/dL), and pulmonary function ($PaO_2 \geq 70$ Torr under room air). Patients were excluded if (1) they had malignant pleural or pericardial effusion or (2) they had a concomitant serious illness such as uncontrolled angina pectoris, myocardial infarction in the previous 3 months, heart failure, uncontrolled diabetes mellitus, uncontrolled hypertension, interstitial pneumonitis or lung fibrosis identified by a chest x-ray, infection, or other diseases contraindicating chemotherapy or radiotherapy, or (3) they were pregnant or breast feeding. All patients gave their written informed consent.

Pretreatment evaluation

The pretreatment assessment included a complete blood cell count and differential count, routine chemistry determinations, creatinine clearance, blood gas analysis, electrocardiogram, lung function testing, chest x-rays, chest CT scan, brain CT scan or magnetic resonance imaging, abdominal CT, and PET.

Treatment schedule

Chemotherapy consisted of cisplatin 80 mg/m^2 on Day 1 and vinorelbine 20 mg/m^2 on Days 1 and 8, repeated every 4 weeks for three to four cycles. Cisplatin was administered by intravenous infusion for 60 minutes with 2,500 to 3,000 mL of intravenous fluid for hydration and prophylactic antiemetic therapy consisting of a 5-hydroxytriptamine-3 antagonist on Day 1 and a corticosteroid on Days 1 to 5. Vinorelbine, diluted in 50 mL of normal saline, was administered intravenously.

Radiation therapy started on Day 1 of the first cycle of chemotherapy and was delivered with megavoltage equipment (6–10 MV) once daily for 5 days a week. The total dose was 66 Gy in 33 fractions at level 1, 72 Gy in 36 fractions at level 2, and 78 Gy in 39 fractions at level 3. All patients underwent a 3D treatment planning CT 3 to 7 days before the start of the treatment, and the eligibility was finally confirmed based on evaluation using the

dose–volume histogram (DVH). The gross tumor volume (GTV) was defined as the primary tumor delineated on pulmonary windows of the chest CT or on the diagnostic PET scans. Atelectasis or secondary changes in the peripheral lung region of the primary tumor were not included. Metastatic lymph nodes defined as nodes of 1 cm or larger visualized on mediastinal windows of the CT images or PET-positive lymph nodes were also included in the GTV. The clinical target volume (CTV) was equivalent to the GTV. Uninvolved mediastinum or supraclavicular fossae were not included in the CTV. The planning target volume (PTV) was determined as the CTV plus 1.0 cm for the anterior, posterior, medial, and lateral margins and a 1.0 to 2.0 cm for the superior and inferior margins, taking account of setup variations and internal organ motion. The spinal cord dose was typically limited to 44 Gy, but a maximum of 50 Gy was allowed. The lung V_{20} was limited to 30% in all patients. The maximum dose to the brachial plexus and esophagus did not exceed 66 Gy. The 100% dose was prescribed to the reference point located in the central part of the PTV, and the entire PTV was covered with 95–107% of the prescribed dose principally, but variation of ±10% was allowed. Lung heterogeneity corrections using the equivalent path length algorithm were applied in all patients.

Toxicity assessment and treatment modification

Complete blood cell counts and differential counts, routine chemistry determinations, and a chest x-ray were performed once a week during the course of treatment. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE v3.0). The lung toxicity grade was defined as the highest grade among cough, dyspnea, obstruction/stenosis of airways, pneumonitis/pulmonary infiltrates, and pulmonary fibrosis in the pulmonary/upper respiratory section (15).

Vinorelbine administration on Day 8 was omitted if any of the following were noted: WBC count $<3.0 \times 10^9$/L, neutrophil count $<1.5 \times 10^9$/L, platelet count $<100 \times 10^9$/L, Grade 2–3 elevation of the serum hepatic transaminase level or total serum bilirubin levels, Grade 2–3 infection, Grade 2–3 pneumonitis, other ≥Grade 3 nonhematologic toxicity, body temperature ≥38°C, or PS of 2–3. Subsequent cycles of cisplatin and vinorelbine chemotherapy were delayed if any of the following toxicities were noted on Day 1: WBC count $<3.0 \times 10^9$/L, neutrophil count $<1.5 \times 10^9$/L, platelet count $<100 \times 10^9$/L, serum creatinine level ≥1.6 mg/dL, Grade 2–3 elevation of the serum hepatic transaminase level or total serum bilirubin levels, Grade 2–3 infection, Grade 2–3 pneumonitis, other ≥Grade 3 nonhematologic toxicity, body temperature ≥38°C, or PS of 2–3. If these toxicities did not recover within 6 weeks from Day 1 of the previous cycle of chemotherapy, subsequent cycles of chemotherapy were stopped. The dose of cisplatin was reduced by 25% in all subsequent cycles if the serum creatinine level rose to 2.0 mg/dL or higher. The dose of vinorelbine was reduced by 25% in all subsequent cycles if any of the following toxicities were noted: WBC count $<1.0 \times 10^9$/L, platelet count $<25 \times 10^9$/L, or Grade 3 infection or liver dysfunction. Thoracic radiotherapy was suspended if any of the following were noted: body temperature ≥38°C, Grade 3 esophagitis, PS of 3, or suspected radiation pneumonitis. Thoracic radiotherapy was terminated if any of the following were noted: Grade 4 esophagitis, Grade 3 or 4 pneumonitis, PS of 4, or duration of radiotherapy of over 62 days (level 1), 67 days (level 2), or 70 days (level 3). Any protocol-defined treatments were terminated if Grade 4 nonhematologic toxicities other than transient electrolyte disturbances or a PS of 4 was noted.

Dose-limiting toxicity and maximum tolerated dose

The DLT was defined as the following toxicities observed during a 6-month period from the start of treatment: (1) Grade 3 esophagitis, lung toxicity, myelitis, dermatitis associated with radiation, and cardiac toxicity associated with radiation, (2) Grade 4 nonhematologic toxicity, or (3) treatment termination due to prolonged toxicity. Twelve patients were enrolled at each dose level. All patients were followed up for at least 6 months to evaluate DLT. During the period, if none to 4 of the 12 patients experienced DLT, the next cohort of patients was treated at the next higher dose level. If 5 or more of the 12 patients experienced DLT, that level was considered to be the MTD. The recommended dose for Phase II trials was defined as the dose preceding the MTD.

Response evaluation

Objective tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) ver. 1.0 (16).

Follow-up

Patients who completed the protocol therapy were followed up to monitor toxicity, response, and recurrence. CT of the chest was performed every 2 to 4 months for 1 year, every 6 months for 2 years, and then yearly for 2 years. The relapse pattern was categorized into (1) local alone, including relapse from the primary site or the hilar, mediastinal, or supraclavicular lymph nodes, (2) distant metastasis alone, including pleural dissemination, pleural and pericardial effusions, and distant metastases, and (3) local and distant.

Statistical analyses

Progression-free survival time (PFS) and OS were estimated by the Kaplan-Meier method. The PFS was measured from the date of registration to the date of disease progression or death resulting from any cause or date of last follow-up. The OS was measured from the date of registration to the date of death resulting from any cause or date of last follow-up. Patients who were lost to follow-up without events were censored at the date of their last known follow-up. A confidence interval (CI) for the response rate was calculated by the method used for exact binomial CIs. The Dr. SPSS II 11.0 software package for Windows (SPSS Japan Inc., Tokyo, Japan) was used for the statistical analyses.

RESULTS

Registration and characteristics of the patients

From August 2005 to September 2008, 57 patients were deemed to initially be eligible. Of these, 3 patients were excluded because idiopathic interstitial pneumonitis ($n = 1$) and anemia ($n = 2$) developed. Explanation of the study using the consent form was given to 54 patients, and informed consent was obtained in 51 patients. The 51 patients underwent 3D treatment planning, and eligibility was finally confirmed in 31 patients. Those 31 were enrolled into this study. A total of 20 patients were excluded as a result of the DVH evaluation: because of V_{20} higher than 30% in 10 patients, overdose to the esophagus in 8 patients, and overdose to the brachial plexus in 2 patients. Eventually, of 17 patients assessed as to their eligibility for dose level 1, 16 patients for dose level 2, and 24 patients to dose level 3, 13 (76%), 12 (75%), and 6 (25%) patients were actually enrolled into levels 1 to 3, respectively (Fig. 1).