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

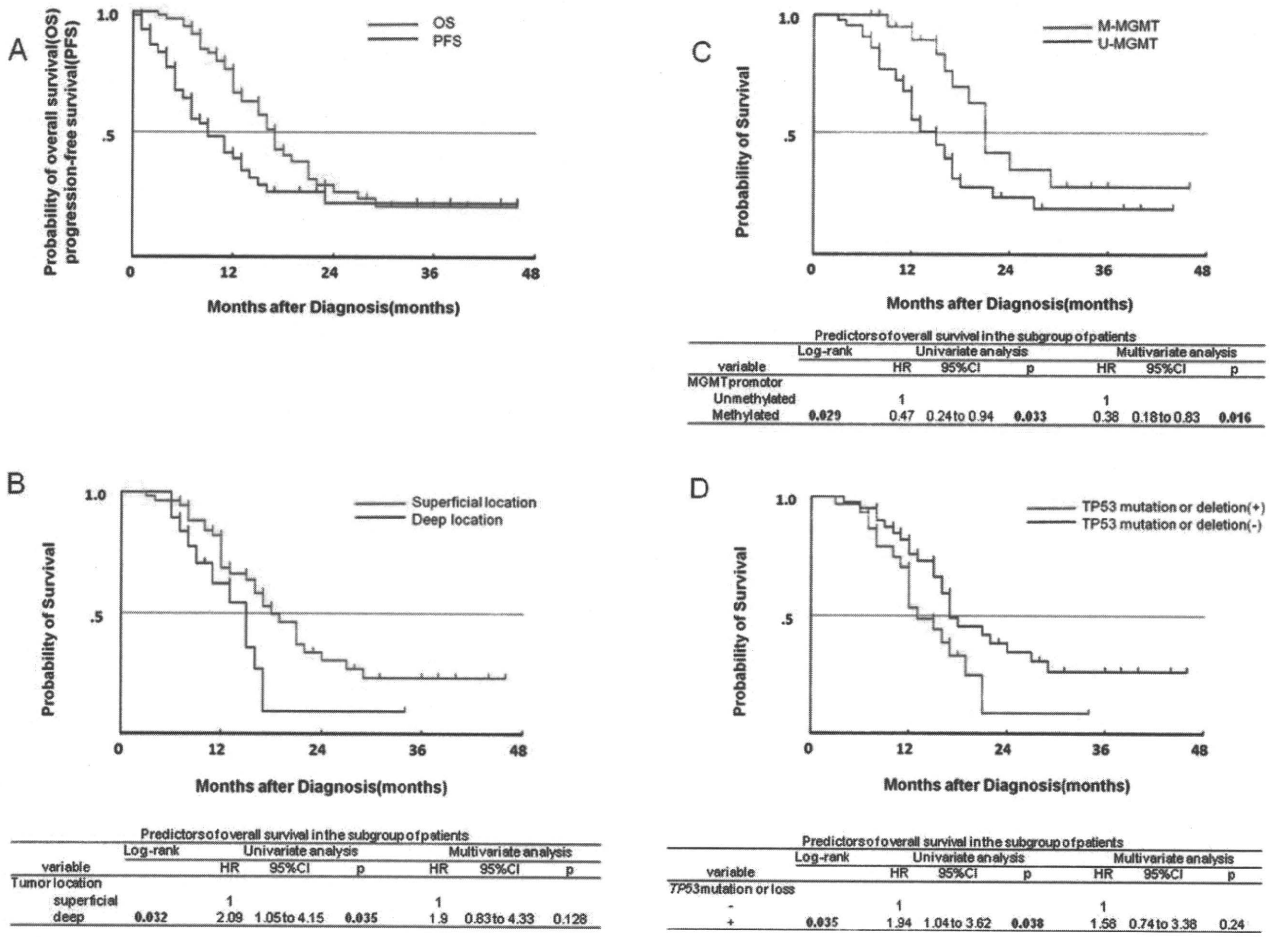
Next, we sought to determine the subpopulation that had benefited from the use of the IFN-β combination treatment. It is well known that patients with GBM containing the methylated MGMT promoter benefit from TMZ, but those with the unmethylated MGMT promoter show no such benefits.[1,2] Consistently, 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	0.029	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

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	0.128

Predictors of overall survival in the subgroup of patients

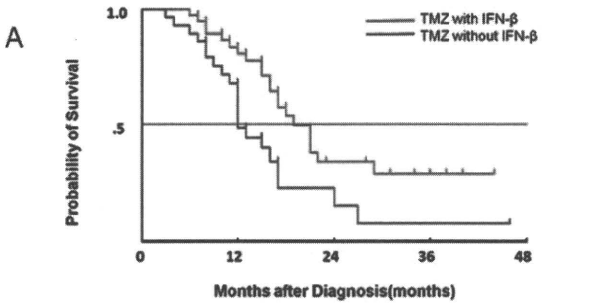
variable	Log-rank	Univariate analysis HR	95%CI	p	Multivariate analysis HR	95%CI	p
TP53 mutation or loss							
-		1			1		
+	0.035	1.94	1.04 to 3.62	0.038	1.58	0.74 to 3.38	0.24

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

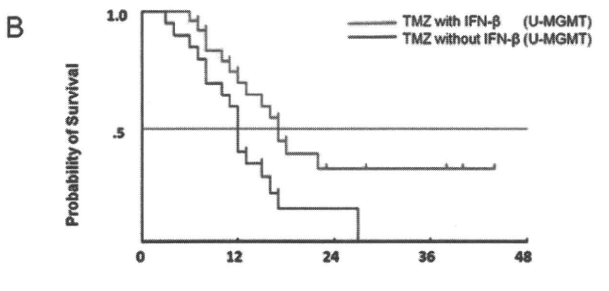


Predictors of overall survival in the subgroup of patients

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

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

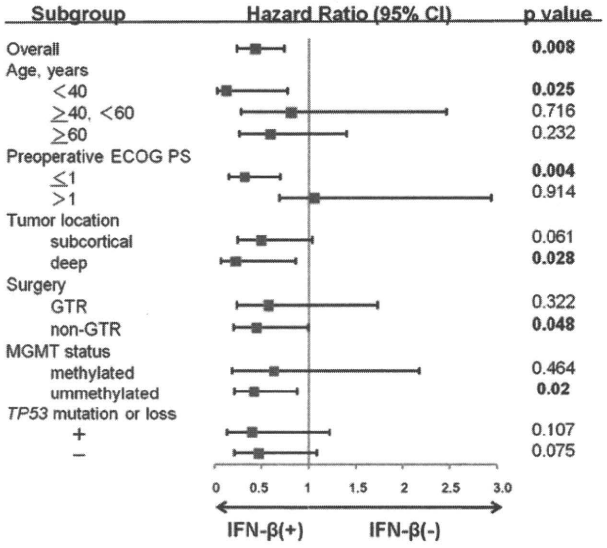


Figure 4. Estimated effect of temozolomide (TMZ) with interferon-β (IFN-β) versus TMZ without IFN-β on the hazard of overall survival (OS), according to baseline characteristics. The hazard ratio was computed using a proportional hazard model by selected factors. There were significant associations under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

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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ORIGINAL ARTICLE

Efficient delivery of liposome-mediated MGMT-siRNA reinforces the cytotoxity of temozolomide in GBM-initiating cells

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Glioblastoma multiforme (GBM) is one of the most formidable brain tumors with a mean survival period of approximately 12 months. To date, a combination of radiotherapy and chemotherapy with an oral alkylating agent, temozolomide (TMZ), has been used as first-line therapy for glioma. However, the efficacy of chemotherapy for treating GBM is very limited; this is partly because of the high activity levels of the DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT) in tumor cells, which creates a resistant phenotype by blunting the therapeutic effect of alkylating agents. Thus, MGMT may be an important determinant of treatment failure and should be considered as a suitable target for intervention, in an effort to improve the therapeutic efficacy of TMZ. In this study, we showed that small-interfering RNA (siRNA)-based downregulation of MGMT could enhance the chemosensitivity of malignant gliomas against TMZ. Notably, TMZ-resistant glioma-initiating cells with increased DNA repair and drug efflux capabilities could be efficiently transduced with MGMT-siRNA by using a novel liposome, LipoTrust. Accordingly, such transduced glioma-initiating cells could be sensitized to TMZ in both in vitro *and* in vivo *tumor models. Taken together, this study provides an experimental basis for the clinical use of such therapeutic combinations.*

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Keywords: *glioma; MGMT; siRNA; TMZ*

Introduction

Glioblastoma multiforme (GBM) is the most lethal form of primary glioma, and the median survival time for patients with GBM is less than 12 months, despite the administration of various therapies, including surgical resection, radiotherapy and chemotherapy.[1] A combination of radiotherapy and chemotherapy with an oral alkylating agent, temozolomide (TMZ), has been used as first-line therapy for glioma. However, the efficacy of TMZ for treating GBM is often very limited because of inherent or acquired resistance. The main determinant of the 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.[2,3] Thus, the downregulation of MGMT may enhance the chemosensitivity of malignant gliomas to TMZ.

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For many years, gliomas, including GBM, were considered to be heterogeneous bulk tumors composed of differentiated and undifferentiated cells with self-renewal and partial differentiation capabilities.[4] Therefore, glioma treatment failure was attributed to certain undifferentiated tumor cells that were responsible for regrowth. Tumors have been reported to harbor small cell populations possessing growth-sustaining and tumorigenesis capacities. These cells, called cancer stem cells or cancer-initiating cells, have been identified in leukemia, multiple myeloma, breast cancer and glioma. In solid tumors, they show many properties of normal stem cells such as self-renewal and multi-potency, and can also initiate tumor formation. Glioma-initiating cells (GICs) also support the cancer stem cell paradigm. They express genes associated with neural stem cells and differentiate into phenotypically diverse populations, including neuronal, astrocytic and oligodendroglial cells.[5] Moreover, GICs have been reported to contribute to radioresistance and chemoresistance through a preferential checkpoint response and the overexpression of DNA repair genes.[6,7] Indeed, we previously showed that compared with established glioma cell lines (for example, T98G), neurosphere-forming GICs expressed higher levels of MGMT, due to which these cells were far more resistant to TMZ.[8] Consequently, efforts to sensitize

cancer stem-like cells to chemotherapy can be directed at inhibiting molecular pathways involved in stem cell regulation. In addition to the increased DNA repair capacity, cancer stem-like cells may contribute to cytotoxic drug resistance through the expression of proteins associated with drug efflux, such as ATP-binding cassette (ABC) transporters. Normal stem cells and small subpopulations of rat and human glioma cells with stem cell-like properties have previously been identified by their ability to expel the fluorescent dye Hoechst 33342, a compound known to be effluxed by ABC transporters.[9–11] Efflux properties, such as those of the ABC transporters, have been suggested to be significant in maintaining neural stem cells in an undifferentiated state, and in protecting them from toxic substances *in vivo*. Thus, it is crucial to develop a new strategy to target GBM stem cells having drug resistance capacity. Liposome-mediated delivery could bypass the ABC transporters and overcome the drug resistance conferred by these transporters.

In this study, we delivered small-interfering RNA (siRNA) for MGMT encapsulated in cationic liposomes. RNAi therapy requires the development of clinically appropriate, safe and effective drug delivery systems. To this end, we used a novel liposome (LipoTrust EX Oligo) as an *in vivo* delivery system; this liposome has recently been described as an efficient *in vivo* delivery system.[12] In this study, we aimed to examine whether siRNA-based downregulation of MGMT could enhance the chemosensitivity of GICs for TMZ, and to provide an experimental basis for the clinical use of such therapeutic combinations.

Results

MGMT-siRNAs suppress MGMT expression in glioma cell lines

We first elucidated the knockdown effects of three siRNAs for MGMT (types A, B and C). Two MGMT-expressing glioma cells, T98G and U251 nu/nu, were transduced with these siRNAs by using LipoTrust EX Oligo according to the instruction manual. Reverse transcriptase–polymerase chain reaction (RT–PCR) revealed that all three MGMT-siRNA constructs could efficiently downregulate the expression of MGMT (Figure 1a). We thus used mixed siRNA for further *in vitro* and *in vivo* experiments.

MGMT-siRNAs enhance the efficacy of TMZ in MGMT-expressing glioma cell lines

The glioma cells were treated with an MGMT-siRNA and LipoTrust complex before the addition of TMZ, and the viable cell numbers were measured by the WST assay. MGMT-negative U251SP cells were very sensitive to TMZ alone, and showed no additional toxicity with MGMT-siRNA. In contrast, TMZ alone reduced the number of T98G and U251 nu/nu cells by a mere 13 and 35%, respectively, compared with the dimethyl sulfoxide (DMSO) control. MGMT-siRNA alone showed minimal reduction in T98G cell growth. However, MGMT-siRNA in combination with TMZ induced dramatic cytotoxicity in both cell lines (Figure 1b).

Efficient RNAi liposome-mediated delivery in GBM-initiating cells

We established tumor-initiating cell lines, also known as tumor stem cells, from tumor tissues obtained from GBM patients. Tumor stem cells possibly contain many similar properties to normal stem cells, which may confer a long life span, including relative quiescence, resistance to drugs and toxins through the expression of several ABC transporters,[13,14] and an active capacity for DNA repair.[7] First, we investigated whether LipoTrust efficiently delivers siRNA to GICs that are known to express proteins that have a physiological role in the export of drugs. As shown in Figure 2a, almost all the GICs were successfully transfected with FAM-labeled siRNA by LipoTrust. Previous studies have reported that GICs express abundant levels of MGMT, which could help explain their tremendous capacity for DNA repair.[7,8] The results of conventional RT–PCR as well as real-time PCR confirmed that the MGMT-siRNA/LipoTrust complex effectively downregulated MGMT expression in both GIC lines (Figure 2b).

MGMT-siRNA enhances the efficacy of TMZ in GBM-initiating cells

Glioma-initiating cells were resistant to TMZ alone; this result is in agreement with that of a previous study.[8] MGMT-siRNA in combination with TMZ exerted the greatest antitumor effect. It should be noted that the most substantial cytotoxicity was observed in GICs treated with a combination of MGMT-siRNA and TMZ (Figure 2c).

Liposome-mediated siRNA delivery in in vivo *tumors*

One of the limiting factors in the clinical use of siRNA technology is the delivery to target cells *in vivo*. We investigated whether intratumoral administration of siRNA/LipoTrust complex is efficient in delivering siRNA in subcutaneous glial tumors derived from GICs. First, we injected 80 pmol of an FAM-labeled siRNA/LipoTrust complex intratumorally once a day for 5 days. FAM-siRNA was distributed diffusely within the tumor (Figure 3a). Next, the knockdown of MGMT expression was investigated by using an MGMT-siRNA/LipoTrust complex. MGMT was expressed in 99% of the tumor cells administered with control siRNA, compared with only 7% of cells in MGMT-siRNA-administered tumors (Figure 3b).

Treatment of subcutaneous tumor models with a combination of MGMT-siRNA and TMZ

Subcutaneous glial tumors in nude mice were treated with either TMZ alone, MGMT-siRNA alone or a combination of the two. Mice were separated into four groups (five animals each) (control group, TMZ group, siRNA group and TMZ plus siRNA group) when the diameter of the tumors reached 10 mm. The mice were given intratumoral injections of 80 pmol of siRNA complex or phosphate-buffered saline (PBS) followed by intraperitoneal (i.p.) injections of 50 mg kg^{-1} TMZ or DMSO once every day from days 1–5. The tumor growth was not affected in mice administered MGMT-siRNA alone. Mice that were administered TMZ alone showed slight tumor reduction, whereas those received the combination of TMZ and siRNA showed a significant

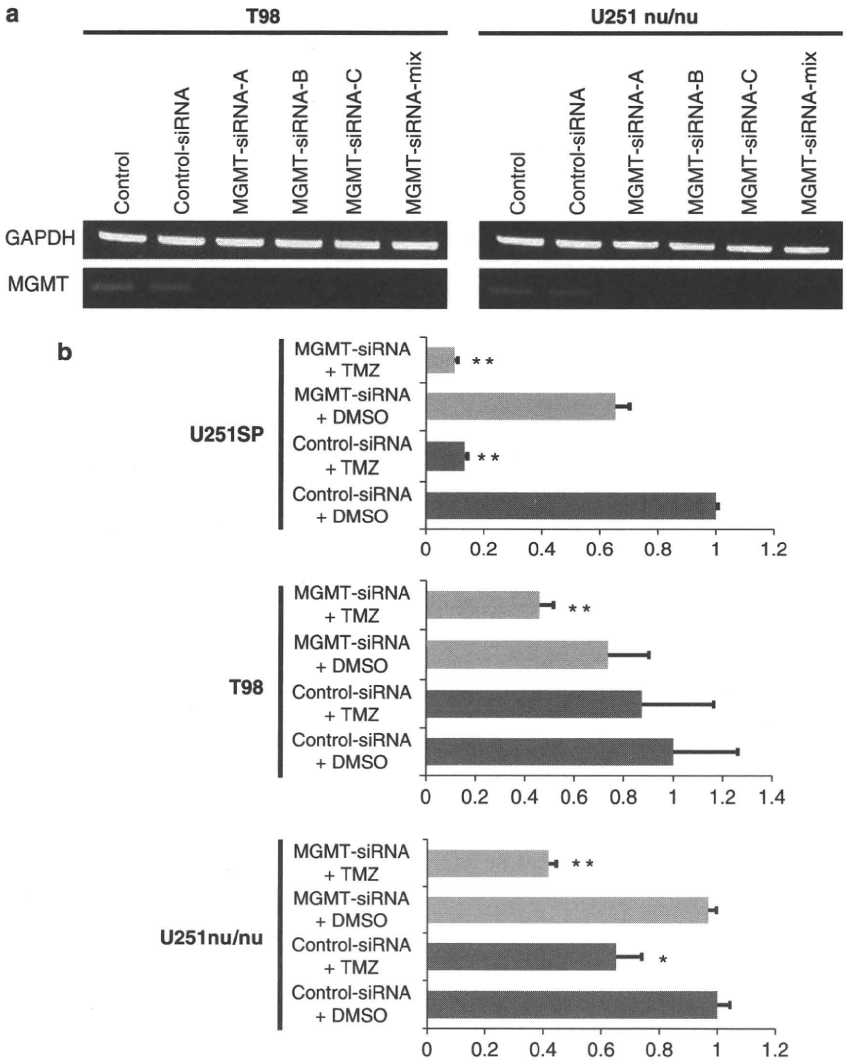

Figure 1 MGMT suppression by liposome/siRNA complex enhances the efficacy of TMZ in MGMT-expressing glioma cell lines. (**a**) Two MGMT-expressing glioma cells, T98G and U251 nu/nu, were transduced with three siRNAs for MGMT (types A, B and C) by using LipoTrust. RT–PCR revealed that three MGMT-siRNA constructs could efficiently downregulate the expression of MGMT. (**b**) The glioma cells were treated with MGMT-siRNA and LipoTrust complex before the addition of TMZ, and the viable cell numbers were measured by the WST assay. MGMT-negative U251SP cells were very sensitive to TMZ alone, and showed no additional toxicity with MGMT-siRNA and TMZ. In contrast, TMZ alone reduced the number of T98G and U251 nu/nu cells by a mere 13 and 35%, respectively, compared with the dimethyl sulfoxide (DMSO) control. MGMT-siRNA alone showed minimal reduction in T98G cell growth, but MGMT-siRNA in combination with TMZ induced dramatic cytotoxicity in both cell lines. $^{**}P<0.01$, $^{*}P<0.05$ compared with control siRNA+DMSO.

decrease in tumor growth (Figure 4). There was no difference in tumor size between mice that were intratumorally injected with siRNA and control mice that were intratumorally injected with PBS (data not shown).

Treatment of brain tumor models in NOD/SCID mice by a continuous drug delivery system

Intracranial inoculation of GICs can generate infiltrative tumors that phenocopy features of parental human GBM tumors, while glioma cell lines tend to form well-demarcated tumors in the brains of mice.[4,5,8] The brain tumor model generated from GICs is ideal to investigate the efficacy of our strategy.

We used an osmotic pump as the drug delivery system, which could supply the siRNA complex at a flow rate of 0.5 $\mu l\ h^{-1}$ for a week. We transplanted 10^5 cells GICs in the right frontal lobe of NOD-SCID mice, and simultaneously implanted an osmotic pump containing the MGMT-siRNA/LipoTrust complex. TMZ was administered i.p. for 5 consecutive days starting from day 2. Although mice treated with TMZ alone showed longer survival than untreated mice, Kaplan–Meier analysis revealed that mice receiving MGMT-siRNA had a significantly better response to TMZ (Figure 5). This result suggests that inhibiting MGMT expression using MGMT-siRNA sensitizes mice to TMZ treatment in the brain tumor model. This result is similar to that observed in subcutaneous tumor models.

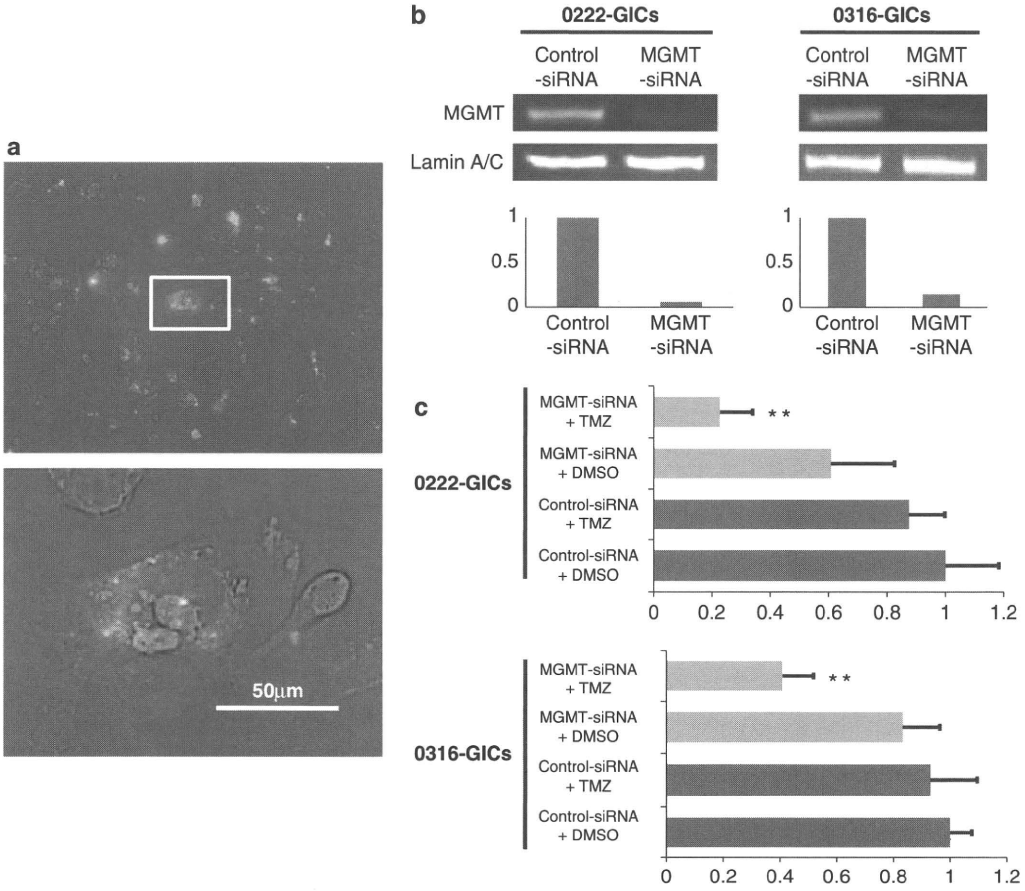

Figure 2 siRNA for MGMT enhances the efficacy of TMZ in GBM-initiating cells. (**a**) GBM-initiating cells (GICs) were transfected with FAM-labeled siRNA by LipoTrust. The lower panel shows high magnification of the inset in the upper panel. Almost all the GICs were transfected with FAM-labeled siRNA by LipoTrust. (**b**) The conventional RT–PCR (upper) as well as real-time PCR (lower) shows that the MGMT-siRNA/LipoTrust complex effectively downregulates the MGMT expression in 0222- and 0316-GIC lines. (**c**) 0222- and 0316-GICs were treated with MGMT-siRNA and LipoTrust complex before the addition of TMZ, and the viable cell numbers were measured by the WST assay. MGMT-siRNA in combination with TMZ was observed to exert the greatest antitumor effect. $^{**}P<0.01$ compared to control-siRNA+DMSO.

Discussion

The principal finding of this study is that liposome-mediated *in vivo* delivery of MGMT-siRNA efficiently enhanced the cytotoxicity of TMZ in GICs. Notably, although glioma stem cells are considered to have extensive resistance to chemotherapeutic agents due to the high expression of DNA repair-related molecules, our study showed that liposomes were able to deliver the siRNA into glioma stem cells effectively, thus inducing susceptibility to TMZ.

Overcoming TMZ resistance due to MGMT

MGMT is capable of counteracting the cytotoxicity induced by O^6-alkylating agents, and the increasing MGMT expression level correlates well with *in vitro* and *in vivo* glioma resistance to TMZ.[15–18] However, in this process, MGMT is rapidly degraded through the ubiquitin/proteasome pathway after receiving alkyl groups from DNA, and the repletion of cellular MGMT pools depends on resynthesis of the molecule.[19] This makes it a suitable target for intervention in an effort to improve the therapeutic efficacy of TMZ. O^6-Benzylguanine (O^6-BG) is a potent inhibitor that irreversibly inactivates MGMT by covalent transfer of its benzyl group to the MGMT active site cysteine residues.[20] As a result, O^6-BG enhances TMZ cytotoxicity in MGMT-proficient glioma cells both *in vitro* and *in vivo*, but not in MGMT-deficient cells.[21] Because patients with MGMT overexpression in the tumors respond more poorly to alkylating agents, co-administration of O^6-BG to deplete the tumor pools of MGMT to enhance drug cytotoxicity has been previously attempted in clinical settings.[22,23] However, systemic delivery of O^6-BG increased the myelotoxicity caused by MGMT depletion in bone marrow cells, and therefore the dose of alkylating agents was reduced to a subtherapeutic level. Consequently, none of the patients responded to this drug combination. Therefore, the therapeutic potential of adding O^6-BG to enhance TMZ cytotoxicity in tumor cells has thus far been discouraging. In this regard, intratumoral delivery of MGMT-siRNA may be more advantageous as compared to O^6-BG.

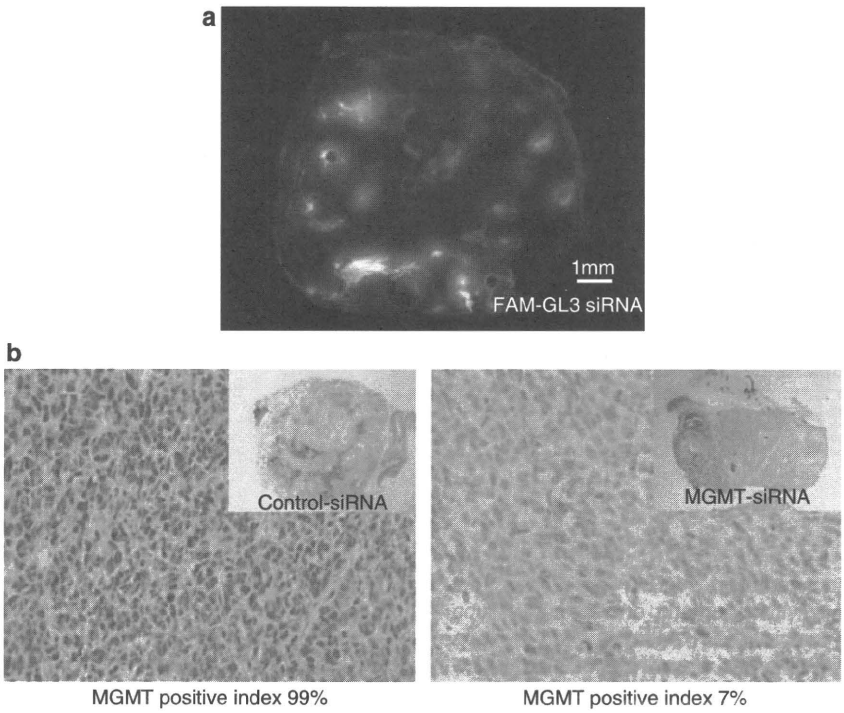

Figure 3 Liposome-mediated siRNA delivery in *in vivo* tumors. (**a**) FAM-labeled siRNA/LipoTrust complex (80 pmol siRNA) was injected intratumorally once a day for 5 days. The frozen sections were observed by a fluorescence microscope. FAM-siRNA was distributed diffusely within the tumor. (**b**) The knockdown by MGMT-siRNA in the subcutaneous tumor was investigated. MGMT was expressed in 99% of the tumor cells injected with control siRNA, whereas MGMT positivity was greatly suppressed to only 7% of MGMT-siRNA-injected tumors.

Use of liposomes to deliver nucleic acids could be an efficient strategy in stem cells that have ABC transporters that render them drug resistant. Unilamellar and multilamellar liposomes are commonly used as pharmaceutical delivery vehicles. In an aqueous environment, one set of polar head groups can form the outer surface of the nanocomplex, whereas another set of polar head groups orients itself to face the interior hydrophilic core, which contains the nucleic acid payload. Liposomes have been used for the delivery of nucleic acids for more than 20 years: Felgner and co-workers[24,25] detailed the ability of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride to deliver both DNA and RNA into mammalian cell lines. The LipoTrust liposomes used in this study are cationic liposomes suitable for the *in vivo* delivery of siRNAs, antisense DNAs and microRNAs. Recently, Sato *et al*.[26] used vitamin-A-coupled LipoTrust liposomes to deliver anti-gp46 siRNA to fibrogenic hepatic cells for the treatment of liver cirrhosis. LipoTrust was introduced as an *in vivo* siRNA delivery agent in a recent review article.[12] Bypassing the ABC transporters on the cell surface, LipoTrust coupled with MGMT-siRNA can direct intracellular synthesis of the MGMT molecule.

Future directions and conclusions

Although liposomes are among the most popular nucleic acid delivery agents, there remain some concerns regarding their safety and delivery efficiency for therapeutic use. The toxicity of certain cationic lipid particles has been reported both *in vitro* and *in vivo*, and certain synthetic agents have been found to induce a gene signature of their own that might increase the off-target effects of the siRNA. In this study, LipoTrust itself showed *in vitro* cytotoxicity, but no remarkable toxicity was observed *in vivo*. A direct intracerebral approach called convection-enhanced delivery (CED) may be used as a strategy to address these issues.[27–29] CED uses a positive pressure generating a local pressure gradient to distribute agents in the extracellular space. Unlike diffusion delivery, CED is not significantly affected by the concentration, molecular weight or particle size of the agent. Further, CED ensures high concentrations and homogenous distribution of the drug throughout a given target tissue. Further studies should investigate the efficacy of the LipoTrust/MGMT-siRNA complex by the CED in a clinical setting.

Materials and methods

Glioma cell lines

The human glioma cell lines U251SP, T98G and U251 nu/nu were obtained from the Memorial Sloan-Kettering Cancer Institute (New York, NY, USA). They were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 5 mM L-glutamine, 2 mM nonessential amino acids, and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. TMZ was supplied by the Schering-Plough Research Institute (Kenilworth, NJ, USA). TMZ was dissolved in DMSO.

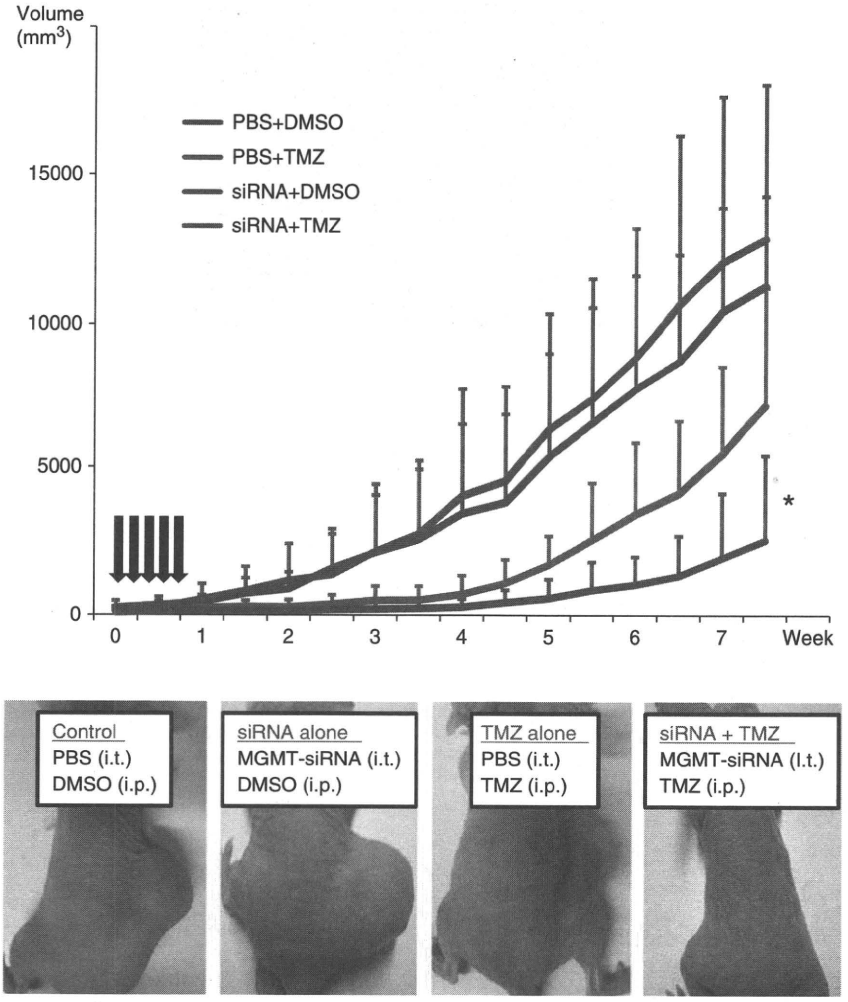

Figure 4 Treatment of subcutaneous tumor models with a combination of MGMT-siRNA and TMZ. Subcutaneous glial tumors in nude mice were treated with either TMZ alone, MGMT-siRNA alone or a combination of the two. Mice were separated into four groups (control group, TMZ group, siRNA group and TMZ plus siRNA group) when the diameter of the tumors reached 10 mm. The mice were given intratumoral injections of 80 pmol of siRNA complex or PBS followed by intraperitoneal injections of 50 mg kg^{-1} TMZ or DMSO once every day from days 1 to 5. The tumor sizes were then measured using calipers two times every week. Tumor volume was calculated using the following formula: volume (mm^3) = 0.5236 *ddD*, where *D* is the longest diameter and *d* the shortest diameter. The tumor growth did not decrease in mice administered MGMT-siRNA alone. Mice that were administered TMZ alone showed slight tumor reduction, whereas the combination of TMZ and siRNA was found to decrease tumor growth significantly. *$P < 0.05$ compared with PBS+DMSO.

GBM-initiating cells

Glioblastoma-multiforme-initiating cells were established according to the protocol described previously.[8]

Tissue collection. Tumor samples were obtained after consent from the patients (0222 and 0316); the procedure was approved by the Department of Neurosurgery, Nagoya University Hospital, Nagoya, Japan.

Primary sphere culture. Tumors were washed and acutely dissociated in PBS (pH 7.4) with 0.1% trypsin (Worthington Biochemical, Freehold, NJ, USA) and 0.04% DNaseI, type II (Sigma-Aldrich, St Louis, MO, USA). The cells were purified using a 70-μm cell strainer (BD Falcon, San Jose, CA, USA) followed by density-gradient centrifugation with Ficoll–Paque Premium (GE Healthcare, Waukesha, WI, USA). The purified cells were then cultured in a 100 × 20 mm Petri dish (BD Falcon) in neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 2 mM L-glutamine, 0.5% N_2 supplement (Gibco), 1% B27 supplement (Gibco), 20 ng ml^{-1} recombinant human epidermal growth factor (rh-EGF; R&D Systems, Minneapolis, MN, USA), and 20 ng ml^{-1} rh-basic fibroblast growth factor (bFGF; R&D Systems) in 5% CO_2 at 37 °C.

Sphere formation. Approximately 2 weeks later, these cells became spherical, and the spherical cells that had been cultured for at least 2 months were defined as established GICs.

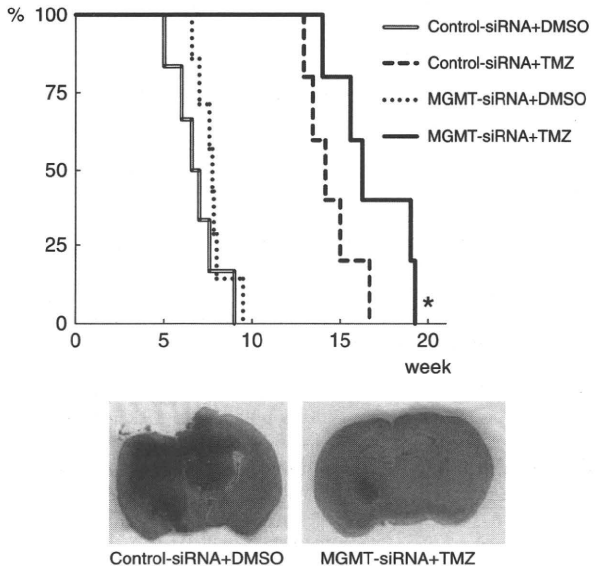

Figure 5 Treatment of brain tumor models in NOD/SCID mice by continuous drug delivery system. GICs (10^5 cells) in the right frontal lobe of NOD-SCID mice. Simultaneously, an Alzet osmotic pump (0.5 μl h^{-1} for 1 week) containing 80 pmol siRNA complex was implanted. From day 2, 50 mg kg^{-1} of TMZ or DMSO was administered intraperitoneally into the mice for 5 consecutive days. Survival analyses were performed using the Kaplan–Meier method. The mice receiving MGMT-siRNA had significantly better response to TMZ. The representative hematoxylin-eosin-stained coronal sections are shown. *$P<0.05$ compared with control-siRNA+TMZ.

Experimental procedure. One day before the experiments were performed, spherical GICs were dissociated using a NeuroCult chemical dissociation kit (StemCell Technologies, Vancouver, Canada).

Preparation of siRNAs

In this study, three siRNA formulations directed against MGMT (B-Bridge, catalog no, SYA21-325, name: 283SKSV_73, 283SKSV_284 and 283SKSV_481) were used. Sense and antisense strands were as follows: MGMT (283SKSV_73; sequence A) beginning at nt 73: sense, 5′-GAGCAGGGUCUGCACGAAATT-3′ and antisense, 5′-UUUCGUGCAGACCCUGCUCTT-3′; MGMT (283SKSV_284; sequence B) beginning at nt 284: sense, 5′-CCAGACAGGUGUUAUGGAATT-3′ and antisense, 5′-UUCCAUAACACCUGUCUGGTT-3′ and MGMT (283SKSV_481; sequence C) beginning at nt 481: sense, 5′-GGACUGGCCGUGAAGGAAUTT-3′ and antisense, 5′-AUUCCUUCACGGCCAGUCCTT-3′. FAM-labeled siRNA against GL3 luciferase, sense 5′-CUUACGCUGAUGACUUCGATT-3′; antisense, 5′-UCGAAGUACUCAGCGUAAGTT-3′. Negative control siRNA (B-Bridge, catalog no S5C-0600).

Formation of transfection complex

Small-interfering RNAs were transduced into glioma cells by using LipoTrust EX Oligo (Hokkaido System Science, Hokkaido, Japan). A vial of LipoTrust (1 μmol lipid) was reconstituted using 1 ml of nuclease-free water. For *in vitro* experiments, 10 μl of Opti-MEM (Gibco) containing 4 pmol siRNA was gently mixed with 0.5 μl of reconstituted LipoTrust solution (0.4 pmol siRNA/0.05 nmol lipid per μl; *in vitro* dose), and incubated for 20 min at room temperature. For animal experiments, 100 μl of Opti-MEM containing 80 pmol siRNA was gently mixed with 8 μl of reconstituted LipoTrust solution (0.8 pmol siRNA/0.08 nmol lipid per μl; *in vivo* dose). In a preliminary experiment, an *in vivo* dose of LipoTrust/MGMT-siRNA complex had a knock-down effect on MGMT after incubation for a week at 37 °C (data not shown).

Reverse transcriptase–polymerase chain reaction

We plated T98G and U251 nu/nu glioma cells in 100 μl Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at the density of 1×10^3 cells per well in a 96-well plate. On day 1, the cells were transfected with the transfection complex described above. Total RNA was extracted from cells by using Trizol reagent (Invitrogen) on day 4. GICs (cultured from patients 0222 and 0316) were plated in 100 μl Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at the density of 8×10^3 cells per well in a 96-well plate. Transfection of siRNAs and RNA extraction were performed on days 2 and 5, respectively. Total RNA (500 ng) was subjected to reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The MGMT forward and reverse primer sequences were 5′-GCAATGAGAGGCAATCCTGT-3′ and 5′-CAGTCCTCCGGAGTAGTTGC-3′, respectively. PCR was performed using GoTaq DNA Polymerase (Promega, Madison, WI, USA) with an initial denaturation step of 94 °C for 5 min; followed by 27 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and a final extension step at 72 °C for 7 min. Glyceraldehyde 3-phosphate dehydrogenase or Lamin A/C PCR products from the same RNA samples were amplified and used as internal controls. cDNA was quantified in a spectrophotometer and diluted to 500 ng μl^{-1} for use in quantitative real-time PCR. Real-time PCR was carried out using a LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). The PCR reaction was analyzed with a Roche LightCycler. Lamin A/C was used as the internal control.

Cell viability assay

The relative cell numbers were determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), a WST-8 cell proliferation assay system, according to the manufacturer's instructions. Glioma cell lines (T98G, U251 SP, and U251 nu/nu cells; 1×10^3 cells per well), 0222-GICs and 0316-GICs (8×10^3 cells per well) were added to wells containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a 96-well plate. The transfection complex was added to the glioma cell lines on day 1 and to the GIC culture on day 2. After 2 days, 100 μM TMZ or DMSO was added. We then assessed the viability of glioma cell lines 2 days later, and that of GICs, 8 days later.

Subcutaneous xenograft tumor model

The 0316-GIC cells (1×10^5 cells) were transplanted subcutaneously in the right flank of BALB/c (nu/nu genotype) nude mice. The tumors were allowed to establish, and they were then passaged *in vivo*. When the subcutaneous tumors had reached a diameter of

10 mm, intratumoral injection of 80 pmol of siRNA/LipoTrust complex (*in vivo* use mixture) and i.p. perfusion of 50 mg kg^{-1} of TMZ was performed for 5 consecutive days. PBS administered intratumorally and i.p. perfusion of DMSO were used as the control. The tumor sizes were then measured using calipers two times every week. Tumor volume was calculated using the following formula: volume (mm^3) = $0.5236\ d^2 \times D$, where D is the longest diameter and d the shortest diameter. The volumes were measured by a single masked observer to prevent observer bias and to avoid inter-operator differences in caliper measurement of the tumors.

Intracranial xenograft tumor model

The 0316-GICs (1×10^5cells) were stereotactically injected into the frontal lobe of 6-week-old female NOD-SCID mice. The injection coordinates are as follows: 3 mm to the right of the midline, 2 mm anterior to the coronal suture and 3 mm in depth. Simultaneously, an Alzet osmotic pump (0.5 µl h^{-1} for 1 week; 1003D; Durect, Cupertino, CA, USA) containing 80 pmol siRNA complex (for *in vivo* use) was implanted. From day 2, 50 mg kg^{-1} of TMZ or DMSO was injected i.p. into the mice for 5 consecutive days. Survival analyses were performed using the Kaplan–Meier method.

Statistical analyses

The statistical significance of the observed difference was determined by analysis of variance (StatView software; SAS Institute, Cary, NC, USA), and subsequently, Bonferroni's correction for multiple comparisons was applied. Survival curves were generated using the Kaplan–Meier method. The log-rank statistic (StatView) was used to compare the distribution of the survival times. All reported P-values were two sided; a value less than 0.05 was considered to be statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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〔VI〕

VHL 病診断治療ガイドライン(案)

フォン・ヒッペル・リンドウ(VHL)病
診療ガイドライン（案）

平成２２年度 厚生労働科学研究費

難治性疾患克服研究事業研究奨励分野

「ﾌｫﾝ･ﾋｯﾍﾟﾙﾘﾝﾄﾞｳ病の病態調査と診断治療系確立の研究」班

(２０１１年３月)

厚生労働省科学研究費補助金難治性疾患克服研究事業
「フォン・ヒッペルリンドウ病の病態調査と診断治療系確立の研究」班

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VHL 病の歴史

フォン・ヒッペル・リンドウ （von Hippel-Lindau、VHL）病（あるいは症候群）（MIM ID#193300）は、常染色体優性遺伝性の疾患で、複数の臓器に腫瘍性あるいは嚢胞性病変を多発する。発症病変としては、網膜血管腫、中枢神経系（小脳、延髄、脊髄）の血管芽腫、膵臓の神経内分泌腫瘍・嚢胞、副腎褐色細胞腫、腎臓の癌・嚢胞、精巣上体嚢胞腺腫、さらに内耳リンパ嚢の腫瘍や女性の子宮広間膜の嚢胞腺腫なども報告されている。

歴史的には、ドイツの眼科医である Eugen von Hippel が網膜の多発血管腫例、家族例に注目し、19 世紀末から 20 世紀初頭にかけてこれらを報告している[1,2]。またスウェーデンの神経病理医である Arvid Lindau は、網膜のみでなく中枢神経系にも血管腫を多発する家族例の病理検索所見を報告した[3,4]。その後本疾患の臨床病態が、Melmon ら、さらに Lamiell らによって整理され、本疾患は先の二人の医師名を冠して von Hippel-Lindau 病と呼ばれるようになっている[5,6]。1988 年に Seizinger らは家系の連鎖解析により、ヒト染色体 3 番短腕上に原因遺伝子の局在を推定した[7]。その 5 年後に、米国 NIH/NCI のグループが中心となり、positional cloning 法により 3p25 領域より原因遺伝子の同定に成功し、von Hippel-Lindau 病（VHL）遺伝子として 1993 年に報告した[8]。

1．発症機構と VHL 蛋白の機能

VHL 遺伝子は癌抑制遺伝子（tumor suppressor gene）に分類され、Knudson が提唱した 2-hit の機構で 2 つのアレル（allele）に変異が起こることでその機能が消失し、細胞の腫瘍化が始まると考えられる。VHL 家系患者では、遺伝的変異（germline mutation）により、出生時に既に片側の VHL 遺伝子の不活性化が起こっており（1-hit)、その後対立 allele に体細胞変異（somatic mutation）が起こることで（2-hit)、遺伝子機能が完全に消失する。一方散発例の淡明細胞型腎癌などでも VHL 遺伝子の高頻度の変異、不活性化が検出されるが、この場合には、2 回の体細胞変異が起きている。臨床的に VHL 病と診断された家系患者においては 80～90%で、この遺伝子の遺伝的変異が検出できるので、この遺伝子変異を指標にした、いわゆる遺伝子診断（DNA test）が行われている。

VHL 遺伝子は 3 つの exon より構成されており、ヒトゲノム上では 3p25.3 上の約 13,000bp の領域に存在し、そこから全長約 4.5kb の mRNA が転写される[9]。mRNA の蛋白翻訳領域は 639 塩基であるが、アミノ酸 1 番と 54 番の 2 か所のメチオニンより翻訳が開始され、それぞれ 213 と 160 アミノ酸（約 30kd と 19kd のサイズ）の VHL 蛋白が作られ、両者とも腫瘍抑制機能を持っている[10,11]。