***MGMT* promoter methylation in patients with glioblastoma: is methylation-sensitive high-resolution melting superior to methylation-sensitive polymerase chain reaction assay?**

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OBJECTIVE The methylation status of the O6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter is a prognostic factor in adults with glioblastoma (GBM); it also yields information that is useful for clinical decision-making in elderly GBM patients. While pyrosequencing is the gold standard for the evaluation of the methylation status of *MGMT*, methylation-sensitive polymerase chain reaction (MS-PCR) assay continues to be used widely. Although MS-PCR results exhibited a good correlation with the prognosis of patients with GBM treated under the Stupp protocol, interpretation of the bands is based on subjective judgment, and the assay cannot be used to analyze heterogeneously methylated samples. We assessed whether methylation-sensitive high-resolution melting (MS-HRM) is an alternative to MS-PCR.

METHODS The authors prepared 3 primer sets that covered CpG 72–89 for MS-HRM analysis to determine the methylation levels of 6 human glioma cell lines. The results were validated by bisulfite sequencing of cloned alleles. The authors also subjected surgical samples from 75 GBM patients treated with temozolomide (TMZ) to MS-HRM to assess the *MGMT* methylation status and compared the findings with MS-PCR results using receiver operating characteristic (ROC), univariate, and multivariate analyses.

RESULTS There was a strong correlation between the methylation levels of the 6 glioma cell lines evaluated by MS-HRM and by bisulfite sequencing; with primers 1 and 2, the correlation was significant ($r = 0.959$ and $r = 0.960$, respectively, $p < 0.01$). Based on log-rank analysis, MS-HRM was significantly better than MS-PCR for predicting progression-free survival (PFS) and overall survival (OS) based on the methylation status of the *MGMT* promoter (PFS predicted by MS-HRM and MS-PCR = 0.00023 and 0.0035, respectively; OS = 0.00019 and 0.00028, respectively). ROC analysis showed that the area under the curve was larger with MS-HRM than with MS-PCR (PFS: 0.723 vs 0.635; OS: 0.716 vs 0.695). Based on multivariate Cox regression analysis, MS-HRM was significantly better than MS-PCR for predicting the treatment outcome (MS-HRM vs MS-PCR: PFS, $p = 0.001$ vs 0.207; OS, $p = 0.013$ vs 0.135).

CONCLUSIONS The authors' findings show that MS-HRM is superior to MS-PCR for the detection of *MGMT* promoter methylation. They suggest MS-HRM as an alternative to MS-PCR for assessing the prognosis of patients with GBM.

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KEYWORDS O6-methylguanine-DNA methyltransferase; *MGMT*; methylation-sensitive high-resolution melting; methylation-sensitive polymerase chain reaction; temozolomide; glioblastoma; oncology

GLIOMAS account for 70% of human malignant primary brain tumors, and their prognosis is poor.¹⁴ Patients diagnosed with glioblastoma (GBM), the most malignant type of glioma (WHO grade IV), survive for an average of 14.6 months, even with the current stand-

ard treatment that includes surgery followed by the combined use of radiotherapy and temozolomide (TMZ).^{16,17} TMZ increases the level of O6-methylguanine and thereby elicits DNA damage-induced apoptosis.⁸ The cytotoxic effects of TMZ are weakened by O6-methylguanine-DNA

ABBREVIATIONS AUC = area under the curve; GBM = glioblastoma; KPS = Karnofsky Performance Status; *MGMT* = O6-methylguanine-DNA methyltransferase; MS-HRM = methylation-sensitive high-resolution melting; MS-PCR = methylation-sensitive polymerase chain reaction; OS = overall survival; PFS = progression-free survival; ROC = receiver operating characteristic; TMZ = temozolomide.

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methyltransferase (*MGMT*), a suicide-repair enzyme that reverses DNA damage by removing alkylating adducts from guanines.^{8,22} Because *MGMT* gene expression is highly regulated at the epigenetic level via CpG island-promoter methylation, the *MGMT* methylation status can help to predict the effectiveness of TMZ treatment.^{1,2}

Methylation-sensitive polymerase chain reaction (MS-PCR) assay has been used to detect the *MGMT* methylation status. Although a good correlation between the methylation status and GBM prognosis has been reported, MS-PCR results are evaluated subjectively based on the interpretation of electrophoretic bands. Also, the CpG island of *MGMT* is heterogeneously methylated, and heterogeneously methylated samples cannot be assayed by MS-PCR.^{9,12,13,23} Since MS-PCR primers are designed to specifically amplify only fully methylated sequences in the primer region, the effect of heterogeneous methylation on the results is unpredictable.^{3,11} Furthermore, not all commonly used MS-PCR primers cover the region crucial for silencing *MGMT* transcription. The human *MGMT* gene is reported to harbor a 762-base-pair CpG island that contains 98 CpG sites in the promoter region (−531 to +231 from ATG). According to Malley et al.,⁹ CpG 73–90 is the region critically involved in transcription. Although most commonly used primers for MS-PCR target this region (forward: CpG 76–80; reverse: CpG 84–87), not all CpG sites of interest can be evaluated.⁴ Consequently, MS-PCR is not the ideal method for assessing the *MGMT* methylation status of the *MGMT* promoter.

To overcome the limitations of MS-PCR, Quillien et al.¹³ proposed pyrosequencing. Although it is a robust and accurate method for detecting the *MGMT* methylation status, it is expensive and requires special instrumentation not available in all laboratories. Therefore, we examined whether methylation-sensitive high-resolution melting (MS-HRM) is an alternative to MS-PCR. MS-HRM is based on the principle that the melting temperature of a PCR product depends on the methylation status of the locus at which the PCR product originates. Therefore, the methylation status of loci of interest can be quantified by comparing the melting profiles of unknown PCR products with known standard samples. As MS-HRM analysis is less expensive, faster, and less laborious than pyrosequencing,²¹ we examined its potential as an alternative for the detection of the methylation status of the *MGMT* promoter.

To test the reliability of MS-HRM for the quantitative assessment of the *MGMT* methylation level, we used 6 human glioma cell lines. The results were validated by bisulfite sequencing of cloned alleles, the gold standard for quantitatively evaluating methylation levels. Next, we obtained the methylation level of tumor samples from 75 GBM patients treated with TMZ and compared MS-HRM and MS-PCR findings using receiver operating characteristic (ROC), univariate, and multivariate analyses. Here, we report our results and discuss factors underlying the difference between MS-HRM and MS-PCR findings.

Methods

Patients and Tumor Samples

Our study was approved by the institutional review

board of Miyazaki University Hospital, Japan. All patients gave their prior written informed consent for the use of their tissues in this study. We included 75 newly diagnosed GBM patients who had undergone surgery at our institution between February 2008 and July 2015. They received basically standard-care treatment consisting of the concomitant delivery of TMZ and irradiation (Stupp protocol). The general characteristics of the patients, including their age, sex, Karnofsky Performance Status (KPS), and the extent of resection, were obtained from their medical records and are shown in Table 1. The median follow-up was 17 months; the median progression-free survival (PFS) was 9 months (95% CI 6.0–11.1 months), and the median overall survival (OS) was 17 months (95% 13.0–25.1 months).

Tissue specimens (n = 75) obtained at surgery were divided into 2 parts: one part was examined histologically, and the other was frozen in liquid nitrogen and stored at −80°C until DNA extraction.

Cell Culture

The established glioma cell lines (LN18, LN229, U87MG, U251, A172, and T98G) were a gift from the Department of Pathology of the University of Miyazaki. LN18 and LN229 were incubated in DMEM+L-glutamax supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin (37°C, 5% CO₂). The other cell lines were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (37°C, 5% CO₂).

DNA Extraction and Bisulfite Treatment

Genomic DNA was isolated from the cell lines and the frozen surgical specimens using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA quantity was measured using NanoDrop 2000 (Thermo Fisher Scientific). Genomic DNA (1 µg) was bisulfite-treated using the EpiTect (Qiagen) or the Innu CONVERT bisulfite basic kit (Analytik Jena AG) according to the manufacturer's instructions to convert all unmethylated cytosine to uracil, leaving 5-methylcytosine unaltered. The DNA methylation status of the *MGMT* promoter was analyzed by MS-PCR and MS-HRM.

Analysis of *MGMT* Promoter Methylation by MS-PCR

Converted DNA was subjected to MS-PCR using 2 primer pairs designed for the amplification of methylated and unmethylated alleles of the *MGMT* promoter. The primer sequence for unmethylated reactions was 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (forward), 5'-AACTCCACACTCTTCCAAAAACAAAA CA-3' (reverse); for methylated reactions it was 5'-TTTC GACGTTTCGTAGGTTTTTCGC-3' (forward), 5'-GCACT CTTCGAAAACGAAACG-3' (reverse).⁵ PCR was performed in a 2720 thermal cycler (Applied Biosystems). The PCR conditions were an initial melting step (10 minutes at 95°C); 30 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 45 seconds at 72°C; and a final elongation (4 minutes at 72°C). Amplified products were loaded on 16% polyacrylamide gels and visualized under ultraviolet light using ethidium bromide staining.

TABLE 1. Characteristics of the 75 patients from whom the GBM samples were obtained

Variable	Value
Median age at op in yrs (range)	64 (32–84)
Sex	
F	29
M	46
Type of op	
GTR	50
Partial	23
Biopsy	2
KPS score	
90–100	18
70–80	34
<70	23
<i>IDH1</i>	
Mutated	5 (6.8)
Wild type	69 (93.2)
<i>TERT</i>	
Mutated	51 (79.7)
Wild type	13 (20.3)

GTR = gross-total resection.

Values are presented as the number of patients (%) unless stated otherwise.

Analysis of *MGMT* Promoter Methylation by MS-HRM Curve Analysis

For sequential PCR amplification and HRM analysis, we used the StepOne system (Thermo Fisher Scientific). PCR was performed in a total volume of 20 μ l (10 μ l of MeltDoctor HRM master mix [Applied Biosystems], 1.2 μ l of each primer [10 μ M]) and 20 ng of bisulfite-converted DNA. The primer sets were 5'-GC GTTTCGGATATGTTGGGATAGT-3' (forward), 5'-C CTACAAAACCACTCGAAACTACCA-3' (reverse) primer 1;²¹ 5'-GGATATGTTGGGATAGTT-3' (forward), 5'-CCCAAACACTCACGAAAT-3' (reverse) primer 2,¹⁸ and 5'-CGTTTGCGATTTGGTGAGTGTT-3' (forward), 5'-CCTACAAAACCACTCGAAACTACCA-3' (reverse) primer 3.²¹

The PCR conditions were 2 minutes at 95°C; 45 cycles of 10 seconds at 95°C, 30 seconds at 54°C, and 15 seconds at 72°C; and a final HRM step of 30 seconds at 95°C, 60 seconds at 60°C, 10 seconds at 70°C, and continuous acquisition to 90°C at 1 acquisition per 0.2°C. DNA extracted from normal lymphocytes was treated with CpG methylase M.sssI (New England Biolabs) to represent fully methylated *MGMT* alleles. Untreated DNA from normal lymphocytes represented fully unmethylated alleles. DNA was bisulfite-converted and mixed with fully unmethylated alleles to obtain 0%, 5%, 10%, 30%, 50%, and 100% methylation standards. MS-HRM data were analyzed using HRM software (version 3.0.1, Thermo Fisher Scientific). Output plots were produced as aligned melting curves (Supplemental Fig. 1). The area under the curve (AUC) was calculated from the aligned melting curves

using ImageJ (NIH); linear regression was applied to interpolate unknown samples from the standards (Supplemental Figs. 2 and 3). All measurements were performed in triplicate.

Analysis of *MGMT* Promoter Methylation by Cloning-Based Bisulfite Sequencing

The 3' and 5' sequences of PCR products derived from MS-HRM using primer 1 were digested with *Bam*HI and *Eco*RI and ligated into a pBluescript II plasmid using 2 $\times$ ligation mix (Wako); subcloning-efficient DH5 α -competent cells (Invitrogen) were used for transformation. Primer 1 was the sequencing primer. PCR products derived from 25 colonies of each sample were used for the sequencing reaction using BigDye Terminator v1.1 (Applied Biosystems) according to the manufacturer's instructions. The cycling conditions were 60 seconds at 96°C; and 25 cycles of 30 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. The PCR products were cleaned using Centri-Sep Spin Columns (Thermo Fisher Scientific) and then sequenced on an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Analysis of *IDH1* and *TERT* Mutations

IDH1 mutations were confirmed by immunohistochemical analysis using either R132H antibody (1:20 dilution, H09, Dianova) or direct sequencing. The primer sequence for *IDH1* was 5'-GGCTTGTGAGTGGATGGGTA-3' (forward), 5'-GCAAAATCACATTATTGCCAAC-3' (reverse). Analysis was as previously described.¹⁵

TERT mutations were confirmed by direct sequencing. A fragment of the *TERT* promoter was amplified with a primer (5'-GTCCTGCCCCCTTCACCTT-3' [forward], 5'-GCACCTCGCGGTAGTGG-3' [reverse]) that contained the C228T and C250T mutation sites. The PCR conditions were initial denaturation at 95°C for 60 seconds; and 35 cycles of 95°C denaturation for 30 seconds, 58°C annealing for 30 seconds, and elongation for 20 seconds at 72°C. Sequencing was performed with BigDye Terminator v1.1 on an ABI PRISM 310 genetic analyzer.

Statistical Analysis

ROC curves were produced to assess the sensitivity and specificity of MS-HRM and MS-PCR for predicting PFS $\geq$ 10 months and OS $\geq$ 18 months. Data obtained by univariate analysis and on survival curves were analyzed with the Kaplan-Meier method and compared using the log-rank test. For multivariate analysis, we applied multiple Cox regression analysis. To evaluate the correlation between methylation levels determined by MS-HRM and by cloning-based bisulfite sequencing, we used the Pearson test. Differences of $p < 0.05$ were considered statistically significant. Statistical analyses were performed with StatMate III software (ATMS) and Prism 6 (GraphPad).

Results

Glioma Cell Lines: Region of Interest in the *MGMT* Promoter and MS-HRM Analysis

The promoter region of the *MGMT* gene harbors 98

FIG. 1. Glioma cell cultures. Region of interest in the MGMT promoter and MS-HRM analysis of glioma cell lines. **A:** MGMT promoter methylation was analyzed by MS-HRM and MS-PCR. For MS-HRM, we designed 3 primer sets to evaluate CpG sites 72–89. **B:** Dot-plot of the methylation level of CpG sites analyzed by MS-HRM using primers 1, 2, and 3 and by cloning-based bisulfite sequencing. With primers 1 and 2 there was a strong correlation with the results of cloning-based bisulfite sequencing (primer 1: $r = 0.959$; primer 2: $r = 0.960$; both $p < 0.01$, primer 3: $r = 0.649$, $p = 0.163$). **C:** Cloning-based bisulfite sequencing results of the MGMT promoter region in U87. Black and white indicate methylated and unmethylated CpG dinucleotides, respectively. The methylation level of each CpG site was calculated from the methylation status of 25 clones. The CpG sites analyzed by MS-HRM primers 1 (red), 2 (blue), and 3 (green) are identified at the bottom of the illustration. **D:** Western blots showing a strong LN18 band, a faint T98G band, and no band for the 4 other cell lines. The expression patterns were inversely correlated with the methylation levels evaluated by MS-HRM using primers 1 and 2. Figure is available in color online only.

FIG. 2. MS-HRM analysis of surgical samples. ROC analysis was applied to evaluate the sensitivity and specificity for predicting OS ≥ 18 months. MS-HRM was with primers 1, 2, and 3 to assess the *MGMT* promoter methylation status. The AUC was largest with primer 1.

CpG sites; CpG 73–90 has been reported to be the critical region for transcriptional control.⁹ To evaluate this region by MS-HRM, we used 3 primer sets that covered CpG 72–89 (primer 1), CpG 72–83 (primer 2), and CpG 84–89 (primer 3) (Fig. 1A).^{18,21} For MS-PCR we applied primers commonly used for detecting the methylation status (forward primer CpG 75–78, reverse primer CpG 84–86). The primer sets for MS-HRM revealed sufficient differences in the methylation level among the 6 standard samples (methylation 0%, 5%, 10%, 30%, 50%, and 100%) to permit its evaluation in tumor cell and cell line samples (Supplemental Fig. 3).

The *MGMT* methylation status of the 6 GBM cell lines was quantitatively evaluated by MS-HRM. As shown in Fig. 1B, data obtained with primers 1 and 2 revealed a similar trend; LN18 showed no methylation, T98G showed some, and the other cell lines manifested higher methylation than T98G. To validate the accuracy of these findings, we compared them with the results of cloning-based bisulfite sequencing. The methylation level of each CpG site was calculated from the methylation status of 25 clones. The average score of CpG sites covered by each primer set was recorded as the methylation level (Fig. 1C, Supplemental Figs. 4–8). Regression analysis indicated that the results of MS-HRM obtained with primers 1 and 2 exhibited a good correlation with those of cloning-based bisulfite sequencing ($r = 0.959$ and $r = 0.960$ for primers 1 and 2, respectively; Fig. 1B). When we compared our MS-HRM findings with the *MGMT* protein expression on Western blots, we found a good inverse correlation (Fig. 1D). These findings suggest that MS-HRM performed with primers 1 and 2 is useful for the quantitative evaluation of *MGMT* promoter methylation levels.

Patient Samples: MS-HRM Analysis

MGMT promoter methylation of samples from 75 GBM patients was retrospectively assessed by MS-HRM and

MS-PCR; all analyses were performed in a blinded fashion. To identify the optimal primer set for MS-HRM and the cutoff value for discriminating between the methylated and unmethylated *MGMT* promoter, we plotted ROC curves to predict PFS of ≥ 10 months (Supplemental Fig. 9) and OS of ≥ 18 months (Fig. 2). For both PFS and OS, primer 1 yielded the largest ROC AUC, suggesting that it was the most suitable for evaluating the *MGMT* methylation level. Calculation of the Youden index indicated that 10.65% was the optimal cutoff value for the *MGMT* methylation level to predict PFS and OS (Supplemental Figs. 10 and 11). To validate this cutoff value, we used Kaplan-Meier estimates to compare 5 cutoff values (5%, 8%, 10%, 12%, and 15%). The log-rank test confirmed that a cutoff value of 10% was appropriate (Table 2, Supplemental Figs. 12 and 13). Based on these findings, we used primer 1 and a cutoff value of 10% for analyzing the *MGMT* promoter methylation status by MS-HRM.

Patient Samples: Comparison of MS-HRM and MS-PCR Findings

To evaluate the usefulness of MS-HRM for analyzing the *MGMT* promoter methylation level, we compared MS-HRM and MS-PCR findings. As shown in Fig. 3A, for the prediction of PFS and OS, MS-HRM was superior to MS-PCR. We confirmed these findings by ROC analysis. Comparison of the ROC curves depicting the sensitivity and specificity of the *MGMT* promoter methylation status obtained by MS-HRM and MS-PCR for PFS ≥ 10 months and OS ≥ 18 months showed that the AUC was larger with MS-HRM. This suggests that MS-HRM produces fewer false-positive and false-negative results than MS-PCR for predicting survival (Fig. 3B). Univariate and multivariate Cox regression analyses using factors such as the patient age, sex, KPS score, surgery, *IDH1*, and the *MGMT* methylation status determined by MS-PCR and MS-HRM *MGMT* were performed to identify independent variables for PFS and OS. As shown in Tables 3 and 4, only data obtained by MS-HRM yielded significant prognostic indicators (MS-HRM vs MS-PCR: for PFS $p = 0.001$ and $p = 0.207$; and for OS $p = 0.013$ and 0.135 , respectively). Survival analysis stratified by the extent of resection also showed that MS-HRM yielded strong prognostic indicators (Supplemental Fig. 14). These findings indicate that MS-HRM was superior to MS-PCR for predicting the prognosis of patients with GBM.

To determine why MS-PCR was inferior to MS-HRM for predicting survival, we focused on the relationship between the methylation status evaluated by MS-PCR and MS-HRM using a dot-plot graph (Fig. 3C). While 3 of 31 (9.7%) MS-HRM-positive samples were MS-PCR negative, more than twice as many MS-HRM-negative samples were MS-PCR positive (10 of 44, 22.8%). The MS-PCR-positive, MS-HRM-negative samples manifested significantly higher methylation levels than MS-PCR-negative, MS-HRM-negative samples ($p < 0.01$, Fig. 3C). These findings suggest that the 38 MS-PCR-positive samples were obtained with lower methylation cutoff values than the 31 MS-HRM-positive samples. In fact, when we analyzed our standard methylation samples prepared for MS-HRM by MS-PCR assay, we found that those with 5%

FIG. 3. Surgical samples. Comparison of results obtained with MS-HRM and MSP. **A:** Kaplan-Meier estimates of PFS and OS of 75 GBM patients based on the *MGMT* promoter methylation status determined by MS-HRM and MSP. Based on the log-rank test for PFS the MS-HRM and MS-PCR values were significantly different (MS-HRM: $p = 0.00023$; MS-PCR: $p = 0.0035$), as were the values for OS (MS-HRM: $p = 0.00019$; MS-PCR: $p = 0.00028$). **B:** ROC curves were calculated on the basis of *MGMT* promoter methylation determined by MS-HRM and MS-PCR (MSP) for PFS ≥ 10 months and OS ≥ 18 months. For both PFS and OS, the AUC was larger with MS-HRM than with MS-PCR. **C:** Scatterplot showing the methylation status of 75 patients classified as MS-PCR negative/MS-HRM negative ($n = 34$), MS-PCR positive/MS-HRM negative ($n = 10$), MS-PCR negative/MS-HRM positive ($n = 3$), and MS-PCR positive/MS-HRM positive ($n = 28$). Among MS-HRM-negative samples, the methylation level was significantly higher in MS-PCR-positive samples than in MS-PCR-negative samples ($p < 0.01$). **D:** Standard samples prepared to evaluate the methylation level by MS-HRM were analyzed by MS-PCR assay. Samples with 5% methylation featured a faint positive band, suggesting that the appropriate cutoff value for MS-PCR is 5%. M = methylated; U = unmethylated. Figure is available in color online only.

methylation featured a faint positive band (Fig. 3D). This suggests that the cutoff value for MS-PCR was around 5%. Together, our observations suggest that some methylation-positive samples identified by MS-PCR included samples with a poor prognosis and that this explains the inferiority of MS-PCR with respect to the survival of GBM patients.

Discussion

The methylation status of *MGMT* is recognized as a strong prognostic factor in GBM patients treated with TMZ, which alkylates DNA in the O6-position of guanine.^{6,7,8} Moreover, studies on elderly patients with malignant astrocytoma or GBM have shown that those with *MGMT* promoter methylation derived similar survival benefits from TMZ monotherapy or radiotherapy alone.^{10,20} Consequently, information on the *MGMT* methylation status not only is a predictor of the patient response to TMZ,

but also can be useful for selecting appropriate treatments. Although MS-PCR has been widely used to determine *MGMT* methylation, its limitations^{9,12,23} led us to investigate whether MS-HRM might be a superior method.

We analyzed the special region of *MGMT* promoter CpG sites (CpG 72–89), which Malley et al. have shown as the critical region for transcriptional control, by MS-HRM with 3 primer sets.⁹ Our study showed that the use of primer 3, reported as the most sensitive and useful primer for detecting *MGMT* promoter methylation in colorectal cancers by MS-HRM, exhibited a poor correlation with comparative findings made by cloning-based bisulfite sequencing. Differences in tumor types, raw-data analysis, and PCR conditions, especially differences in the annealing temperature, may account for this discrepancy. At lower annealing temperatures, the PCR primers bind both methylated and unmethylated templates, where PCR biases may favor the amplification of unmethylated

TABLE 2. Validation of the optimal cutoff value

Cutoff (%)	No. of Samples		p Value
	Methylated	Unmethylated	
PFS			
5	40	34	0.0026*
8	33	41	0.0016*
10	30	44	0.00023*
12	29	45	0.0092*
15	28	46	0.00038*
OS			
5	40	35	0.00178*
8	35	40	0.0034*
10	31	44	0.00019*
12	30	45	0.00025*
15	28	47	0.0140*

PFS data were missing for 1 patient. p values were determined using the log-rank test.

* Statistically significant.

sequences.¹¹ In fact, the use of primer 3 for MS-HRM assay of our 6 glioma cell lines resulted in the detection of methylation levels that were lower than those detected by cloning-based bisulfite sequencing. Optimized annealing temperatures may yield better results when primer 3 is used for MS-HRM.

To identify the best primer set, we subjected our surgical samples to ROC analysis of the MS-HRM results. The AUC was largest with primer 1. Switzeny et al.¹⁸ reported that *MGMT* promoter methylation levels determined by MS-HRM with primer 2 exhibited the best inverse correlation with *MGMT* activity. Again, differences in instrumentation, reagents, and software used for the analysis of raw data may explain their findings, as may differences in the CpG sites covered by each primer. Unlike primer 1, which covered almost the full length of the region critical for transcriptional control (CpG islands 72–89), primer 2 yielded only partial coverage (CpG islands 72–83). Because a pyrosequencing study by Quillien et al.¹³ found that the methylation level

of CpG 89 and the mean methylation level of CpG 84–88 were strongly correlated with the patient outcome, we think that it is advantageous to use primer 1 for evaluating critical CpG regions.

We found that while 5% is the cutoff value for MS-PCR, for MS-HRM the optimal cutoff is 10%. According to Quillien et al.,¹³ the cutoff value for MS-PCR is 2.5%; their methylation-positive rate in GBM patients was 60% with MS-PCR and 33% with MS-HRM. They showed that MS-HRM is superior to MS-PCR for predicting PFS, and their findings agree with ours. With respect to the cutoff values in studies predicting survival, Switzeny et al.¹⁸ identified 5% for MS-HRM; Quillien et al.¹³ used 8% for pyrosequencing. This supports the notion that the *MGMT* promoter region involved in transcriptional control requires some methylation levels to extend survival. Therefore, the suitable *MGMT* methylation status for predicting survival should be determined by quantitative methods such as pyrosequencing and MS-HRM with an optimized cutoff value.

While our study documented the advantage of MS-HRM over MS-PCR, MS-HRM has some disadvantages. Unlike MS-PCR, it requires a real-time PCR machine and special analysis software that may not be available in all laboratories. Moreover, there is no standardized method for estimating methylation levels with MS-HRM. We used the AUC of the normalized melting curves to generate standard curves and linear regression analysis to calculate the methylation level, as did Switzeny et al.¹⁸ Tse et al.,¹⁹ on the other hand, employed both the peak height and the AUC from normalized, temperature-shifted difference curves for generating a standard curve,¹⁹ and Wojdacz and Dobrovic²¹ proposed a semiquantitative estimate method based on similarities in MS-HRM patterns without a mathematical approach. Because data obtained from different real-time PCR machines may involve specific analysis methods, standardized methods for estimating *MGMT* methylation levels and a universal cutoff value applicable to these methods are needed for the wider acceptance of MS-HRM to predict the survival of GBM patients based on the methylation status of the *MGMT* promoter. We are in the process of assembling a large cohort of GBM patients to validate our current findings.

TABLE 3. Univariate and multivariate analyses of different prognostic parameters for PFS of 74 GBM patients

Variable	Univariate			Multivariate		
	p Value	HR	95% CI	p Value	HR	95% CI
Age (<65 vs ≥65 yrs)	0.129	0.702	0.399–1.125			
Sex (F vs M)	0.549	1.153	0.695–1.982			
KPS score (<80 vs ≥80)	0.026*	0.591	0.333–0.934	0.588	0.847	0.465–1.543
Surgery (GTR vs other)	<0.001*	0.372	0.123–0.450	<0.001*	0.237	0.126–0.444
<i>IDH1</i> (mutated vs wild type)	0.159	0.458	0.228–1.274			
MS-PCR <i>MGMT</i> (methylated vs unmethylated)	0.0035*	0.519	0.259–0.767	0.207	0.671	0.324–1.163
MS-HRM <i>MGMT</i> (methylated vs unmethylated)	0.00023*	0.432	0.217–0.627	0.001*	0.349	0.215–0.832

PFS data were missing for 1 patient.

* Statistically significant.

TABLE 4. Univariate and multivariate analyses of different prognostic parameters for OS of 75 GBM patients

Variable	Univariate			Multivariate		
	p Value	HR	95% CI	p Value	HR	95% CI
Age (<65 vs ≥65 yrs)	0.065	0.636	0.345–1.032			
Sex (F vs M)	0.254	1.334	0.798–2.350			
KPS score (<80 vs ≥80)	0.641	0.888	0.520–1.495			
Surgery (GTR vs other)	<0.001*	0.103	0.002–0.013	<0.001*	0.363	0.208–0.633
IDH1 (mutated vs wild type)	0.059	0.288	0.192–1.031			
MS-PCR MGMT (methylated vs unmethylated)	0.00028*	0.424	0.198–0.616	0.135	0.614	0.324–1.163
MS-HRM MGMT (methylated vs unmethylated)	0.00019*	0.399	0.207–0.613	0.013*	0.423	0.215–0.832

* Statistically significant.

Conclusions

Our study showed that MS-HRM is superior to MS-PCR for the identification of GBM patients likely to respond to TMZ treatment. As its results are reliable, we suggest MS-HRM as an alternative to MS-PCR for assessing the prognosis of patients with GBM.

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Disclosures

The authors report no conflict of interest concerning the materi-

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Author Contributions

Conception and design: Yamashita, Yokogami. Acquisition of data: Yamashita, Matsumoto, Saito, Mizuguchi. Analysis and interpretation of data: Yamashita, Yokogami. Drafting the article: Yokogami, Takeshima. Reviewed submitted version of manuscript: Yamashita, Yokogami, Takeshima. Approved the final version of the manuscript on behalf of all authors: Yamashita. Statistical analysis: Yamashita. Study supervision: Yokogami, Ohta, Takeshima.

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