Relevance of MSP assay for the detection of *MGMT* promoter hypermethylation in glioblastomas

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Abstract. *O^6-Methylguanine-DNA methyltransferase* (*MGMT*) promoter hypermethylation has recently emerged as a powerful determinant of chemotherapy sensitivity in glioblastomas. To adapt such an important epigenetic biomarker to routine application in the clinical setting, we validated the conventionally used methylation-specific polymerase chain reaction (MSP) assay for its relevance in the determination of *MGMT* methylation status. *MGMT* promoter hypermethylation analysis employing MSP was performed on 25 primary glioblastoma samples and 7 cell lines, and compared with the more robust direct promoter sequencing that profiled the methylation status of 27 CpG sites within the *MGMT* promoter. In addition, the MGMT expression at the protein level was evaluated in the primary tumor samples using immunohistochemistry and in the cell lines using Western blotting analysis. Our MSP analyses yielded reproducible results, which were identical to the bisulfite sequencing data in all except one primary tumor that was negative on MSP. A poor correlation existed between the immunohistochemical staining results and the methylation status of the *MGMT* promoter in primary glioblastoma samples. Neither MSP-*MGMT* methylation nor immunohistochemical MGMT expression had prognostic implications in this small and non-uniform group of patients. In all of the cell lines with loss of MGMT expression, signals of methylated DNA were detected by MSP. Our data support the feasibility and reliability of MSP analysis, which could be routinely implemented in the diagnostic setting.

Introduction

Glioblastoma (WHO grade IV) is the most frequent and malignant neoplasm of the human nervous system. Despite optimal treatment with cytoreductive surgery and radiation therapy, median patient survival does not exceed 1 year from initial diagnosis. There have been numerous attempts to achieve improved results through the use of adjuvant chemotherapy. Chloroethylating agents such as the nitrosoureas have been the main chemotherapeutic agents employed as the first adjuvant modality and have exhibited a modest activity against malignant gliomas (1). More recently, the novel methylating agent, temozolomide, has been shown to prolong median survival significantly, albeit for a few months (2). The effectiveness of such alkylating agents is frequently hampered by inherent or acquired drug resistance.

The major determinant of resistance to the alkylating chemotherapeutic agents including nitrosourea and temozolomide is the activity of O^6-methylguanine-DNA methyltransferase (MGMT) which directly and specifically removes cytotoxic alkyl adducts at the *O^6* position of guanine formed by these alkylating agents (3,4). MGMT activity is controlled by a promoter, and methylation of the cytosine phosphate guanosine (CpG) islands in the promoter region prevents transcription of the gene (5). Recent clinical studies, including work at our institution, have demonstrated that the presence of *MGMT* hypermethylation was associated with an increased sensitivity to adjuvant therapy and/or a better survival in patients with glioblastomas who were treated with nitrosourea or temozolomide in conjunction with radiation therapy (6-8). Such observations highlight the importance of *MGMT* methylation as a specific predictive biomarker for responsiveness to alkylating agent chemotherapy.

In the preponderant publications dealing with assessment of the methylation status of the *MGMT* promoter, the most commonly used method is methylation-specific polymerase chain reaction (MSP) assay. The MSP requires small quantities of DNA that can be obtained from frozen stereotactic biopsy specimens or paraffin-embedded tissue samples, and enables easy-to-use and cost-efficient analysis (9-11). The protocol is based on bisulfite treatment of isolated genomic DNA. The bisulfite treatment results in conversion of unmethylated cytosine residues to uracil, leaving methylated cytosine protected. The methylated and unmethylated sequences can be detected using either sets of primers, one specific for a methylated promoter and one specific for an unmethylated promoter, respectively. Recently, several researchers have cast doubt on the authenticity of MSP for determining the

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MGMT promoter methylation status, e.g. non-quantitative information, a lower number of assessable CpG dinucleotides (usually around 5 in number), and a misleading risk of false-negativity largely due to low quality or quantity of amplifiable DNA (12-14). An alternative method for methylation analysis is direct promoter sequencing which is currently regarded as the golden standard for the analysis of methylation profiles. This method is widely employed in basic research, but it is too complex, time-consuming, and expensive for routine application in the clinical setting (14).

The aim of the present study was to validate the reliability of MSP assay for assessment of the methylation status of the *MGMT* promoter. We examined *MGMT* hypermethylation using conventional MSP in 32 glioblastoma samples including 7 cell lines, and compared the data obtained to the more rigorous bisulfite sequencing that determined the methylation status of 27 CpG sites within the *MGMT* promoter. The putative relationship between *MGMT* promoter hypermethylation and MGMT protein expression was also assessed.

Materials and methods

Tumor samples. Primary brain tumor samples were collected from a total of 25 patients with a new histological diagnosis of supratentorial glioblastomas classified according to the World Health Organization-2000 criteria (15), undergoing surgery at Nihon University School of Medicine between April 2002 and November 2006. The patients comprised 16 men and 9 women aged 24-74 years (median, 58 years). They had been enrolled in a prospective study designed to evaluate the efficacy of individualized chemotherapy based on *MGMT* methylation status, combining interferon and radiation therapy, as reported previously (16): 8 patients with MSP-positive tumors were treated with a procarbazine, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-2(2-chloroethyl)-3-nitrosourea (ACNU), and vincristine regimen, while 17 patients with MSP-negative tumors were treated with a carboplatin and etoposide regimen. The study protocol was approved by the Clinical Research Ethics Committee. Genomic DNA was extracted from frozen tissues using a QIAamp® DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA) following the manufacturer's instructions. To exclude contamination with necrotic debris and normal brain tissue, selected tissues were subjected to careful examination by hematoxylin and eosin staining of corresponding sections.

Human malignant glioma U-87MG and U-138MG cells were purchased from American Type Culture Collection (Manassas, VA, USA), and A-172, AM-38, T98G, U-251MG, and YH-13 were purchased from Health Science Research Resources Bank (Sennann, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle medium (D-MEM, GIBCO™, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen) in a standard humidified incubator at 37˚C in a 5% CO_2 - 95% air atmosphere. DNA was extracted using a QIAamp® DNA Mini kit.

MSP assay. Sodium bisulfite modification was performed with a CpGenome™ DNA Modification Kit (CHEMICON™, Millipore Corporation, Billerica, MA, USA) as described previously (17,18). The primer sequences of *MGMT* for the methylated and unmethylated reactions were as described originally by Esteller *et al* (5). The PCR was carried out as described previously (8,19). Amplified products were electrophoresed on 3% agarose gels, and were visualized with ethidium bromide. CpGenome Universal Methylated DNA (CHEMICON™, Millipore Corporation) and normal blood DNA were included in each set of the PCR as methylated and unmethylated controls, respectively.

Sodium bisulfite sequencing. Sequencing of the *MGMT* promoter was performed using the primers established by Mikeska *et al* (14) that amplify a fragment of 266 base pairs encompassing 27 CpG dinucleotides (GeneBank accession number AL355531, nucleotides 46891 to 47156). The respective primers were the forward: 5'-GGATATGTTG GGATAGTT-3' and the reverse: 5'-AAACTAAACAACA CCTAAA-3'. The PCR was carried out with a final volume of 30 μl containing 11.25 pmol of each primer, 250 μmol/l of dNTP, 2 mmol/l of $MgCl_2$, 1.5 U of Platinum® Taq DNA Polymerase (Invitrogen), and 3 μl of bisulfite-treated genomic DNA as template. Amplification was carried out with initial denaturing at 97˚C for 10 min followed by 37 cycles of denaturing at 95˚C for 1 min, annealing for 1 min at 50˚C, and extension for 1 min at 72˚C, and then a final extension for 10 min at 72˚C. The PCR products were purified and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and Applied Biosystems 3730*xl* DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Based on the criteria described by Grasbon-Frodl *et al* (10), 'partial methylation' was defined as the cytosine and thymine peaks being equally sized or the thymine peak being twice as high compared to the corresponding cytosine peak; 'weak methylation' was defined as the cytosine peak being as small as 10-50% of the thymine peak. Both completely and partially, but not weakly, methylated positions were considered as 'methylated'.

Western blotting analysis. To determine the protein levels of MGMT in the cell lines, soluble protein lysates of sub-confluent glioma cells were obtained employing cell lysis buffer (Medical & Biological Laboratories, Woburn, MA, USA) for 20 min on ice. These proteins (5 μg) were loaded and separated by 12.5% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) for 30 min at 10 V with a Bio-Rad transblot (Bio-Rad Laboratories). Non-specific binding was blocked with wash buffer (PBS/0.05% Tween-20) containing 5% ECL blocking agent (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK) for 60 min at room temperature. The primary antibodies and dilutions used for immunoblotting were MGMT (1:200) (MT 3.1: Neomarkers, Lab Vision Corporation, Fremont, CA, USA) for 120 min and ß-actin (1:5000) (AC-15: Sigma-Aldrich, St. Louis, MO, USA) for 60 min. The secondary antibodies employed were biotinylated anti-mouse IgG (H and L) (1:1000) (BA-2000: Vector Laboratories, Burlingame, CA, USA) for 60 min. The immunoblotted complex was

Table I. Summary of data for 7 glioblastoma cell lines and 25 primary glioblastoma samples.

	Age/ Sex	MSP	Promoter sequencing (%)	Western blotting	Immunohisto-chemistry (%)
Cell lines					
A-172		M	77.8	-	
AM-38		M	81.5	-	
T98G		M, U	100	+	
U-87MG		M	100	-	
U-138MG		U	0	+	
U-251MG		M, U	66.7	-	
YH-13		U	0	+	
Patients (case no.)					
1	54/M	U	14.8		9.6
2	58/F	U	59.3		0.3
3	65/M	U	NA		5.4
4	61/F	M, U	85.2		28.1
5	66/F	U	NA		26.0
6	64/M	U	7.4		6.4
7	53/F	M, U	59.1		3.8
8	32/M	U	29.6		56.9
9	60/F	U	0		37.8
10	24/F	U	0		52.4
11	35/M	U	0		77.7
12	63/M	U	14.8		14.5
13	62/M	M, U	88.9		0.5
14	55/M	U	29.6		14.2
15	62/M	M, U	70.8		24.6
16	71/F	U	NA		3.1
17	74/F	U	0		NA
18	40/M	M, U	88.5		64.9
19	45/F	M, U	NA		31.2
20	29/M	U	4.2		27.5
21	57/M	U	11.1		15.7
22	61/M	M, U	88.9		13.8
23	56/M	U	0		60.1
24	65/M	M, U	92.6		15.2
25	45/M	U	0		NA

MSP, methylation-specific polymerase chain reaction; M, methylated; U, unmethylated; NA, not available.

visualized with the aid of an ECL Western blotting analysis system (GE Healthcare UK Ltd.).

Immunohistochemical analysis. Staining for MGMT protein on primary tumor samples was performed using anti-MGMT antibody clone MT3.1 (CHEMICON™, Millipore Corporation). The materials were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 10% H_2O_2 in phosphate-buffered saline. For antigen retrieval, the sections were incubated in sodium citrate buffer using a microwave. Skimmed milk was applied to prevent non-specific bindings. After deparaffinization and rehydration, the sections were incubated for 1 h at 4˚C with an anti-MGMT antibody (1:100 dilution). For the MGMT immunostaining, only nuclear staining was considered as retention of MGMT protein; cytoplasmic reactivity may be non-specific. Endothelial staining was employed as an internal positive control. To calculate the percentage of MGMT-positive cells, the number of immunoreactive tumor cells was determined for at least 1,000 cells in 10 randomly chosen fields.

Statistical analysis. The Kaplan and Meier method was used to calculate the progression-free survival (PFS) and overall survival (OS). We designated the PFS as the time period from the start of adjuvant therapy to the point when radiographic evidence of disease progression was noted. When disease progression was evident at the time of the first evaluation, the PFS was set at zero. The OS was defined as the interval between the start of adjuvant therapy and the date of death or the most recent evaluation. The log-rank test was employed to assess the degree of significance of differences in different subgroups. The relationships between the various parameters were analyzed statistically using Pearson's correlation coefficient test, the χ^2 test, Fisher's exact test, Student's t-test, or the Mann-Whitney U-test as appropriate. All values are expressed as the means ± standard deviation. The significance level chosen was $P<0.05$, and all tests were two-sided.

Results

MGMT promoter methylation. For the 7 cell lines, MSP analysis was performed in triplicate, and this produced reproducible results (Table I). PCR amplicon in the methylated reaction was found in 5 out of the 7 cell lines (71.4%). A-172, AM-38, and U-87MG cells showed only the methylated band with no unmethylated *MGMT*, while both methylated and unmethylated sequences were detected in T98G and U-251MG cells (Fig. 1).

The *MGMT* methylation as analyzed by MSP in 9 patients has been reported previously (16). In all of the 25 patient cases, *MGMT* MSP analysis was performed at least 3 times for each biopsy specimen; when the 3 attempted MSP procedures did not reveal PCR amplicons in the methylated reaction with the initial specimens, subsequent specimens were collected from different sites whenever possible. Our MSP assays yielded identical results in all cases except one (Table I). In this latter patient (case no. 7), the initial sample presented no evidence of a methylated sequence, while a methylation-positive PCR was detected in an additional sample. Such divergent findings could be attributable to the fact that the initial biopsy materials largely contained extensive necrosis with a scarcity of viable tumor cells. Overall, MSP showed *MGMT* hypermethylation in 8 (32%) of the 25 tumors. Unlike with the cell lines, the methylated primary tumors always displayed evidence of signals of unmethylated DNA, probably indicating some contamination of normal cells within the specimen.

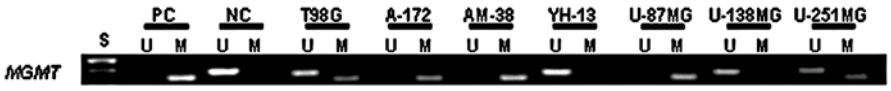

Figure 1. MSP of *MGMT* promoter in glioblastoma cell lines. *MGMT* methylation is present in T98G, A-172, AM-38, U-87MG and U-251MG cells, and absent in YH-13 and U-138MG cells. A-172, AM-38 and U-87MG cells showed only the methylated band with no unmethylated *MGMT*, while both methylated and unmethylated sequences were found in T98G and U251-MG cells. S, molecular size marker; U, PCR product amplified by unmethylated-specific primers; M, PCR product amplified by methylated-specific primers; NC, normal control; PC, positive control.

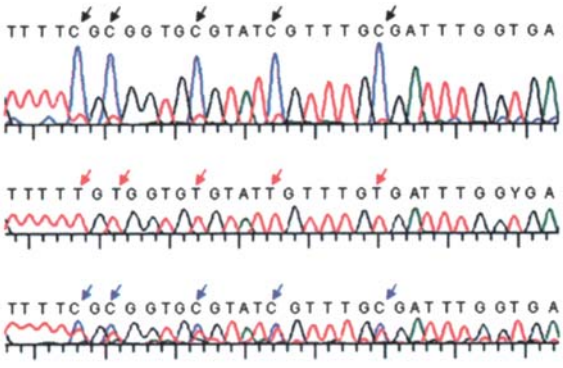

Figure 2. Promoter methylation status of the *MGMT* gene by bisulfite sequence analysis in representative completely methylated (upper), unmethylated (middle), and partially methylated tumors (lower). Parts of the forward sequencing are shown. Arrows point to the CpG sites.

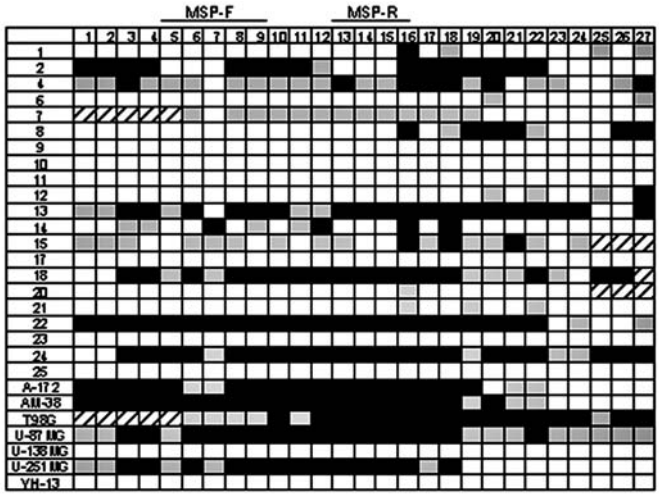

Figure 3. Methylation profiles of 27 CpG islands in 7 analyzable glioblastoma cell lines and 21 primary glioblastoma samples. Black rectangle, completely methylated CpG island; gray rectangle, partially methylated CpG island; open rectangle, unmethylated CpG island; oblique rectangle, unable to be evaluated.

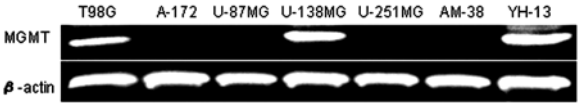

Figure 4. Western blotting analysis of MGMT in glioblastoma cell lines. A-172, U-87MG, U-251MG and AM-38 cells showed loss of MGMT protein expression. While T98G, U-138MG and YH-13 cells retained MGMT expression.

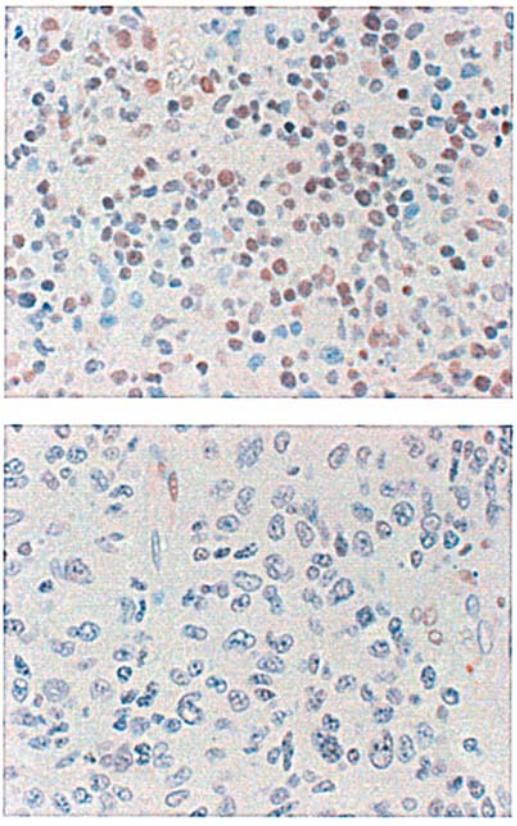

Figure 5. Immunohistochemical staining of MGMT in primary glioblastoma samples. A tumor with unmethylated-*MGMT* promoter shows nuclear expression of MGMT in a heterogeneous pattern (upper), while a tumor with methylated-*MGMT* promoter shows a complete lack of MGMT expression (lower). Original magnification, x200.

Sequence analysis was successfully performed in all cell lines and 21 (84%) out of the 25 primary tumor samples (Figs. 2 and 3). In 4 tumor samples, sequencing data were not analyzable, mainly due to technical problems. Notably, a majority of CpG sites analyzed was completely methylated in the A-172, AM-38 and U-87MG cells that displayed evidence of only methylated gene by MSP. In contrast, the T98G and U-251MG cells with signals of both methylated and unmethylated DNAs on MSP had a considerable degree of diversely methylated CpG positions, probably indicating regional variations in methylation within the CpG site rather than the presence of unmethylated normal alleles.

We next examined the correlation between the MSP results and the data obtained by direct sequencing. The

extent of methylation was measured as a percentage of total methylation. The percentage of methylated CpG sites within the investigated *MGMT* promoter fragment was significantly higher in MSP-positive tumors than in MSP-negative tumors. MSP-positive tumors had a median percentage of methylated CpG positions of 88.5% as opposed to one of 5.8% in MSP-negative tumors (P=0.0003 by the Mann-Whitney U test). The MSP assay used in the present study covers CpG positions 5 to 9 and 13 to 16 (14). In all of the MSP-positive tumors, at least half of these CpG sites were methylated. When tumors with in excess of 50% of methylated CpG sites were defined as methylation-positive cases, the sequence analysis and MSP displayed concordant results, with one exception (case no. 2). This case had methylation at 59% of sequenced CpG sites, but was negative on MSP analysis.

MGMT protein expression. The 7 cell lines were subjected to Western blotting to evaluate the MGMT expression at the protein level (Fig. 4). This revealed an absence of MGMT expression in 4 cell lines, all of which were classified as hypermethylated based on both the MSP and sequencing analysis. Among 2 cell lines with methylated and unmethylated sequences on MSP, U-251MG showed loss of expression, whereas T98G retained MGMT expression.

We examined the primary tumor samples for immunohistochemical MGMT protein expression (Fig. 5). The immunohistochemistry was successful in 23 out of the 25 samples; 2 tumors could not be evaluated for their expression, because the nuclear staining was too weak to differentiate positive and negative cells, as described by Capper *et al* (20). The percentage of immunoreactive tumor cells ranged widely from 0.3% to 77.7% (mean, 25.6±22.6%; median, 15.7%). We next attempted to match the methylation data to the immunohistochemical staining results, but failed to observe any significant correlation. The median percentage of MGMT-positive cells was 19.9% in MSP-positive tumors and 15.7% in MSP-negative tumors, respectively (P=0.8465 by the Mann-Whitney U test). When 5, 10, 20, 30, or 35% was defined as the cut-off point for distinguishing between expression-positive and -negative groups, no correlation existed in any case (data not shown). Similarly, the extent of methylation as assessed by direct sequencing revealed no correlation with the immunohistochemical staining (P=0.1976 and R=0.311 by Pearson's correlation coefficient test).

Survival analysis. At the end point of observation (December 2007), one patient was alive without disease, one was alive with disease, 22 were dead of the disease, and one with evidence of relapse was dead due to esophageal cancer. For the entire study population, the estimated median PFS was 7 months and the estimated median OS was 13 months.

In patients with MSP-positive tumors, the PFS (median, 12.5 months) and OS (median, 19.5 months) were longer as compared to those with MSP-negative tumors (median PFS, 6 months; median OS, 12 months), although this difference lacked statistical significance (P=0.1860 and P=0.7479 for the PFS and OS analyses, respectively). When the methylation status was determined on the basis of the direct sequencing analysis, patients with methylation-positive tumors (as defined by methylation of >50% of the sequenced CpG sites) had a somewhat longer survival. The median PFS was 6 and 12 months in methylation-negative and positive tumors, respectively (P=0.6995), and the median OS was 13 and 16.5 months in methylation-negative and -positive tumors, respectively (P=0.7438). With respect to the immunohistochemical MGMT protein expression, no prognostic value was found in either group with the cut-off point set at 5, 10, 20, 30, or 35% (data not shown).

Discussion

Among various molecular genetic alterations, the *MGMT* promoter methylation has recently been recognized as the most powerful determinant of chemotherapy sensitivity in patients with glioblastomas (6-8). The main goal of our study was thus to translate such an important epigenetic marker into a feasible and robust clinical diagnosis tool, which could stratify chemotherapeutic strategies for these patients in the future. Employing the MSP assay that has been conventionally used in many laboratories, we examined the methylation profile of the *MGMT* gene in 7 glioblastoma cell lines and 25 primary glioblastoma samples. Our MSP analyses yielded reproducible results, which were identical to the data obtained by direct promoter sequencing, in all of the assessable samples except for one primary tumor. These findings suggest that MSP analysis of *MGMT* promoter methylation is suitable as a reliable and widely applicable biomarker for the clinical setting.

To the best of our knowledge, only 3 studies in the literature have compared *MGMT* sequence analysis with MSP in diffuse gliomas. Möllemann *et al* (21) screened 52 oligodendroglial tumors and detected *MGMT* methylation (as defined by methylation of more than 50% of sequenced CpG sites) in 46 tumor samples. Among these 46 samples, 43 were also positive on MSP analysis, and only 3 were negative on MSP. In the present study, we made similar observations, i.e. identified only one false-negative case on MSP. In the study of Grasbon-Frodl *et al* (10), who screened 69 biopsy specimens from 25 patients with high-grade astrocytomas, sequence analysis and MSP yielded concordant findings in all of the samples tested. Coupled with data provided by us in the present study, these findings support the reliability of MSP for the determination of *MGMT* promoter methylation status. In the most recent study by Parkinson *et al* (22), who investigated 22 glioblastoma samples consisting of 10 primary tumors and 12 corresponding recurrent tumors, 2 recurrent tumors that had a fraction of methylated CpG amounting to <50% were positive on MSP. Although they neither mentioned nor explained these false-positive results, such an unusual observation draws the attention to the possibility that methylation-positive MSP might be observed under certain PCR performance conditions even if methylated cells represent only a minor portion of the tumor.

For further standardization of the *MGMT* MSP assay, some technical challenges arising during the testing steps need to be addressed. As Cankovic *et al* (9) and Grasbon-Frodl *et al* (10) advocated, MSP analysis should be repeated for several biopsy specimens from different sites. While *MGMT* methylation appears to be homogeneously distributed

within the same tumor, as recently demonstrated by two studies (10,22), we should bear in mind that significant contamination by necrotic tissue could lead to false-negative results, as exemplified by our case no. 2. Misleading results also occur in the case of incomplete DNA modification during the bisulfite treatment or DNA loss during the testing procedure. To assess the quality and quantity of DNA, a clearly visible unmethylated signal, which can always be detected even in methylated tumors, should serve as an internal amplification control. Regarding loss of DNA during bisulfite treatment, a newly developed bisulfite conversion kit could yield the best recovery of amplifiable DNA, as recently demonstrated by Cankovic *et al* (9).

In contrast to the powerful prognostic effect noted in previous studies using MSP (6-8), we failed to observe significant differences of survival according to *MGMT* methylation status as determined by MSP. This inconsistency is probably attributable to the limited number of patients investigated in our study. In the large analysis by Hegi *et al* (7) involving 206 glioblastomas, *MGMT* promoter methylation stood out as an independent favorable prognostic factor. Another factor that could possibly explain the lack of correlation between *MGMT* methylation and survival is the different chemotherapeutic modalities: either nitrosourea-based chemotherapy or platinum-based chemotherapy was selectively administered to our patients. This is in line with a previous report of Balaña *et al* (23) who demonstrated an association between *MGMT* methylation and survival in nitrosourea-treated patients but not in cisplatin-treated patients. In their recently published study on the largest series of 219 glioblastomas, Crinière *et al* (24) found a prognostic impact of *MGMT* methylation exclusively in patients who were treated with nitrosourea-based chemotherapy plus radiation therapy. Prospective randomized clinical investigations will be needed to provide further confirmation of the actual prognostic value of *MGMT* MSP assay in glioblastomas.

Immunohistochemistry is the most common and technically least complex method for the detection of MGMT protein expression, although implementation of such analysis in routine clinical diagnosis is constrained by interobserver variability. Few studies have evaluated a weak albeit statistically significant correlations between *MGMT* methylation and protein expression (5,9,25), and contradictory results were obtained in most previously published studies (10,12,13,21,26). Likewise, the prognostic value of immunohistochemical MGMT expression in diffuse gliomas remains debatable (20). In our small cohort of patients, MGMT protein expression was associated with neither *MGMT* methylation status nor survival, whenever examined with various cut-off values that have been employed previously to discriminate glioblastoma patients into different prognostic groups (20,27,28).

In contrast to the immunohistochemistry findings, Western blotting revealed that loss of protein expression was concordant with the presence of *MGMT* methylation, with one exception: T98G with methylated and unmethylated sequences on MSP retained protein expression. Interestingly, U-251MG with loss of expression also displayed signals of both unmethylated and methylated DNA by MSP. Such a discrepancy of protein expression between these two cell lines could possibly be due to the difference in proportions of methylated tumor cells, i.e. loss of MGMT expression may be related to the degree of methylation. In these two cell lines, mosaic methylation patterns with variable grades of methylation at each CpG position were observed, although the quantitative information provided by the sequence analysis used in the present study was not sufficient to confirm such an assumption. Another potential explanation is that modifications beside promoter hypermethylation could affect the functional protein expression. Indeed, it has been demonstrated that MGMT expression can be induced by glucocorticoids, ionizing radiation, and genotoxic agents including those employing during chemotherapy (29,30). Recently, p53 has been shown to regulate cellular MGMT independently of methylation status (31), indicating that promoter hypermethylation is not necessarily the primary mechanism of MGMT down-regulation.

Recently, several researchers have attempted to establish alternative experimental methods for the robust and reliable analysis of the methylation status of the *MGMT* promoter. Mikeska *et al* (14) compared and optimized three experimental techniques, i.e. combined bisulfite restriction analysis (COBRA), SNuPE ion pair-reverse phase high-performance liquid chromatography (HPLC), and pyrosequencing, in terms of their accuracy of detecting *MGMT* promoter methylation. In comparison with a comprehensive methylation profile in 22 glioblastomas and 3 normal brain tissues as determined by bisulfite sequencing, they concluded that pyrosequencing assay provides the most accurate and most robust *MGMT* methylation marker. Jeuken *et al* (13) validated methylation-specific multiplex ligation-dependent probe amplification (MLPA) which has the advantage of omitting the potentially troublesome bisulfite conversion of unmethylated cytosines and of providing semi-quantitative data. A significant correlation existed between the MSP and MLPA in the detection of *MGMT* methylation. They also found a trend toward longer survival with increasing *MGMT* hypermethylation ratio in a group of 20 glioblastoma patients receiving temozolomide chemotherapy. Further assessments and validations of these assays in comparison with the MSP, as well as of their reliability, cost, and applicability for implementation in routine clinical diagnosis, are needed in larger numbers of samples.

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