The Korean Journal of Pathology 2011; 45: 455-462
http://dx.doi.org/10.4132/KoreanJPathol.2011.45.5.455

MGMT Gene Promoter Methylation Analysis by Pyrosequencing of Brain Tumour

Young Zoon Kim · Young Jin Song[1]
Ki Uk Kim[1] · Dae Cheol Kim[2]

Department of Neurosurgery and Division of Neurooncology, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon; Departments of [1]Neurosurgery and [2]Pathology, Dong-A University College of Medicine, Busan, Korea

Received: May 22, 2011
Accepted: August 30, 2011

Corresponding Author
Dae Cheol Kim, M.D.
Department of Pathology, Dong-A University College of Medicine, Dongdaesin-dong 3-ga, Seo-gu, Busan 602-714, Korea
Tel: +82-51-240-2878
Fax: +82-51-243-7396
E-mail: freehand@dau.ac.kr

*This work was supported by the Dong-A University research fund.

Background: The aim of this study was to determine whether pyrosequencing (PSQ) might be useful to achieve O6-methyl guanine methyltransferase *(MGMT)* promoter methylation using 1- to 13-year-old archival tissues as a clinical biomarker in routine practice. **Methods:** The study included 141 formalin-fixed paraffin-embedded (FFPE) glial tumors from the archives of the Pathology Department from 1997-2010. **Results:** The average percentage of methylation (MP) of the 141 cases was 14.0±16.8%, and methylated cases were 32.3±14.9%. The average MP of each year did not show a linear increasing or decreasing pattern according to the age of the FFPE block ($p=0.771$). The average MP of methylated glioblastomas was 35.8±14.7%, 31.8±15.5% for anaplastic astrocytomas, and 22.4±15.1% for astrocytoma. A tendency was observed toward an increasing pattern of average MP with World Health Organization (WHO) grade ($p=0.063$) in astrocytic tumors. A correlation was observed between average MP and WHO grade ($p=0.038$) and a bimodal distribution was observed between the methylated and unmethylated cases, using a 9% cut-off value ($p<0.001$). **Conclusions:** The results showed that a quantitative approach for *MGMT* promoter methylation yielded a 100% success rate for FFPE tissues from archives. PSQ can be used in a retrospective trial, but the cut-off value and calculation method should be further validated.

Key Words: Glioma; MGMT; Pyrosequencing; Biomarker; Methylation

Gliomas are the most common type of primary brain tumors, and glioblastoma (GBM) is the most frequent malignant brain tumor, accounting for approximately 12-15% of all intracranial neoplasms and 60-75% of astrocytic tumors. An analysis of 5,692 patients diagnosed with primary central nervous system (CNS) tumors in 2005 in the Republic of Korea showed that GBM accounted for 5.9% of all CNS tumors and 30.4% of all neuroepithelial tumors.[1] Although the overall incidence rate of primary brain tumors is very low among all human cancers, GBMs are a highly invasive and aggressively growing tumor that responds poorly to radiation therapy and most forms of chemotherapy. The prognosis is correspondingly poor, with most patients dying within 1 year after diagnosis. Compared to treatment of other types of cancer, treatment for GBM is very difficult, because drugs must cross the blood brain barrier to reach the tumor and avoid protein efflux. The mainstream treatment for GBM is surgical removal where possible, followed by radiotherapy. Hegi *et al.*[2] investigated the O6-methyl guanine methyltransferase (*MGMT*) promoter methylation status in a large cohort of patients with GBM by comparing patients who received radiotherapy alone or radiotherapy combined with temozolomide (TMZ) with patients with methylated *MGMT* who benefitted from adding TMZ. A clinical trial (EORTC 26981/22981 and NCIC CE.3) of concurrent TMZ and radiotherapy followed by adjuvant TMZ for newly diagnosed patients with GBM showed a considerable advance, achieving a median survival of 14.6 months and a 2-year survival of 26%.[3] TMZ is an alkylating chemotherapeutic agent that causes DNA damage by transferring alkyl groups at several sites within DNA, including the O6 position of guanine. The *MGMT* gene plays a fundamental role maintaining genomic integrity by encoding for a DNA repair protein that removes alkyl groups from O6-guanine.[4] Epigenetic silencing by promoter methylation results in decreased *MGMT* expression and correlates with improved survival in patients with glioma treated with alkylating agents.[5] Although *MGMT* promoter methylation is used as a prognostic/predictive marker in patients with GBM, no consensus exists on the method to be applied. Methylation-specific poly-

merase chain reaction (PCR) (MSP) has been widely used, and this method enables cost-efficient analysis of *MGMT* promoter methylation. However, it is not a quantitative method and has the risk of false-positive or false-negative results, particularly when the DNA quality and/or quantity are low. In the EORTC 26981/22981 and NCIC CE.3 trials, Hegi *et al.*[2] only collected data from 67% of the samples analyzed, representing 36% of cases from their archives. The success rate of MSP on formalin-fixed paraffin-embedded (FFPE) tumor samples was highly variable and the median success rate was 75.0% (range, 0 to 100%). They recommended that diagnostic *MGMT* testing requires sufficient and optimally preserved tumor tissue such as cryopreserved tumor specimens; thus, avoiding fixation-related deterioration of tumor DNA quality. An alternative technique to overcome the problem with MSP is pyrosequencing (PSQ), which is based on the sequencing-by-synthesis principle and involves a simple technique for accurate and quantitative analysis of DNA sequences. PCR followed by PSQ is one such sensitive, highly reproducible, and cost-effective method[6] for DNA methylation analyses. It provides absolute quantitative information based on each interrogated CpG site, which is not possible with most other methods. Additionally, unlike other methods, the assay design allows inclusion of internal controls to address inaccuracies resulting from incomplete bisulfite conversion. Mikeska *et al.*[7] compared and optimized three quantitative *MGMT* promoter methylation techniques and concluded that the PSQ assay provides the most accurate and is robust when applied to archival samples including those of GBM. They[7] described a comparison of the programs obtained from matched snap-frozen and FFPE tissues, and the overall methylation score led to the same molecular diagnostic decision in all cases. The objective of this study was to determine whether PSQ might be useful to determine *MGMT* promoter methylation status using 1- to 13-year-old archival tissue samples of glial tumors as a clinical biomarker in routine practice. We discuss our results with applicability to molecular diagnostics.

MATERIALS AND METHODS

Sample selection

The study set included FFPE brain tumor tissue from 162 patients at the Department of Pathology archives of Dong-A University Medical Center from 1997 to 2010, of which all were diagnosed with glial tumors. All patients underwent surgical removal or biopsy sampling of their tumors. Adjuvant conventional radiotherapy and/or chemotherapy were performed after the pathological diagnosis in all patients. All hematoxylin and eosin slides were reviewed (World Health Organization Classification 2007) and selected by two pathologists without any information regarding clinical and pathological parameters. Tumors were excluded if the tissue was almost entirely necrotized or tumor volume of the section was $<80\%$.[7] Finally, 141 patients were selected. This study was reviewed and approved by the Dong-A University Ethical Committee (10-10-189).

DNA extraction and bisulfite conversion

Genomic DNA was extracted from three (10 μm thickness) slices of FFPE material using the QIAamp DNA FFPE extraction kit and the QIAcube automated DNA extraction machine (Qiagen, Hilden, Germany) and quantified by UV absorption (Nanodrop, Thermo Scientific, Wilmington, DE, USA), typically yielding a total >1 μg of gDNA per specimen. gDNA (200 ng) was used in the bisulfite conversion reactions in which unmethylated cytosine was converted to uracil with the EpiTect bisulfite kit (Qiagen), according to the manufacturer's instructions. In short, DNA was mixed with water. The DNA was protected by the buffer and bisulfite mix, and the conversion was run on an ABI 2727 Thermocycler (Applied Biosystems, Foster City, CA, USA) under the recommended cycle conditions. Converted DNA was purified and eluted in two steps in a total of 40 μL buffer EB and further diluted into 20 μL aliquots of 1,000 cell-equivalents/μL (the cell calculations assumed 6 pg DNA per diploid cell).

PCR and PSQ

Primer sets with one biotin-labeled primer were used to amplify the above bisulfite-converted DNA samples. The PyroMark Q96 CpG *MGMT* kit[8,9] (Ensembl ID: OTTHUMT00000051009) (Qiagen) is available as a ready-to-use research kit, and this kit was able to detect the level of methylation at exon 1 positions 17-39 of the *MGMT* gene, containing 5 CpGs. A cytosine not followed by a guanine, which is not methylated, served as an internal control and was programmed automatically by the PSQ96MA 2.1 software (Biotage, Uppsala, Sweden) to verify the efficiency of bisulfite conversion. Theoretically, the internal control must be zero, because all cytosines not followed by a guanine are converted to uracil during bisulfite conversion, then, uracil is converted to thymine after PCR. Usually $<5\%$

was an acceptable value for the internal control according to the manufacturer's protocol. If the value of the internal control was >5%, all procedures were repeated again (from bisulfite conversion to PSQ).

PCR was performed using a converted gDNA equivalent of approximately 5,000 cells and using the PyroMark PCR kit (Qiagen). Briefly, 12.5 μL master mix, 2.5 μL Coral red, 5 pmol of each primer, 7 μL of water, and 2 μL sample were mixed for each reaction and run under the following thermal cycling conditions: 95°C for 15 minutes and then 45 cycles for 30 seconds at 94°C; 30 seconds at the optimized primer-specific annealing temperature; 30 seconds at 72℃, and a final extension for 10 minutes at 72℃. The correct amplified DNA was confirmed by electrophoresis on a 2% low melting point agarose gel (Sigma-Aldrich, Steinheim, Germany) in tris-borate-EDTA buffer. A standard PSQ sample preparation protocol was applied.[10] Streptavidin beads (3 μL; GE Healthcare, Buckinghamshire, UK), 37 μL PyroMark binding buffer (Qiagen, Hilden, Germany), 20 μL PCR product, and 20 μL water were mixed and incubated for 10 minutes on a shaking table at 1,300 rpm. Amplicons were separated, denatured, washed, and added to 45 μL annealing buffer containing 0.33 μM of PSQ primer using the Biotage Q96 Vacuum Workstation. Primer annealing was performed by incubating the samples at 80℃ for 2 minutes, which were allowed to cool to room temperature prior to PSQ. PyroGold reagents were used for the PSQ reaction, and the signal was analyzed using the PSQ 96MA System (Biotaqe, Uppsala, Sweden). Target CpGs were evaluated by PSQ96MA 2.1 instrument software (Biotaqe, Uppsala, Sweden) which converts the pyrograms to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio. Methylation values ≤5% were considered potential background noise signals of questionable significance, as shown in other studies.[11-13] Unmethylated (300 ng; Qiagen, Hilden, Germany) and hypermethylated DNA (Millipore, Billerica, MA, USA) were used as standard controls, and bisulfite was converted as described above.

Data analyses

The main analyses converted individual C/T ratio data into mean values of the five CpGs analyzed in a gene segment. The percentage of the mean value of five CpGs were considered methylated if the percentage was >9%.[14] The chi-square test was used for parametric comparisons to analyze associations between markers. Differences in the averages between groups were tested by a t-test or analysis of variance. Significance was accepted at p-values<0.05. Statistical analyses were performed with SPSS ver. 14.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

In total, 141 patients were analyzed by the PSQ method, and Table 1 shows the overall details of the data set. The age of the patients ranged from 1 to 77 years (mean age, 43.4 years). Seventy-seven cases were males (mean age, 44.8 years), and 64 were females (mean age, 41.8 years). The data set showed a male predominance, and females tended to be diagnosed at a younger age than males; however it was not statistically significant. Seventy-five cases (53.2%) were diagnosed with GBM (mean age, 51.1 years), 22 cases (15.6%) were diagnosed with anaplastic astrocytoma (AA) (mean age, 47.0 years) and 19 cases (13.5%) were diagnosed with grade II astrocytoma (mean age, 33.9 years). The mean age of patients with an astrocytic tumor increased significantly according to the World Health Organization (WHO) grading system (p<0.01). Nine patients (6.4%) were diagnosed with anaplastic oligodendroglioma (AO) (mean age, 34.5 years) and two patients (1.4%) were diagnosed with oligodendroglio-

Table 1. Clinicopathological data of the 141 glial tumor cases

		No. of cases	%
Sex	Male	77	54.6
	Female	64	45.4
Age (yr)	≤40	49	34.8
	>40	92	65.2
Diagnosis	Glioblastoma	75	53.2
	Anaplastic astrocytoma	22	15.6
	Astrocytoma	19	13.5
	Anaplastic oligodendroglioma	7	5.0
	Oligodendroglioma	1	0.7
	Pilocytic astrocytoma	9	6.4
	ETC[a]	8	5.7
WHO Grade	Grade 1	13	9.2
	Grade 2	24	17.0
	Grade 3	29	20.6
	Grade 4	75	53.2
Status of methylation	Methylated	52	36.9
	Unmethylated	89	63.1
	Total	141	100.0

[a]Three ependymomas, a juvenile pilomyxoid astrocytoma, a dysembryoplastic neuroepithelial tumor, an infantile desmoplastic astrocytoma, a pleomorphic xanthoastrocytoma, and a primitive neuroectodermal tumor.
ETC, et cetera; WHO, World Health Organization.

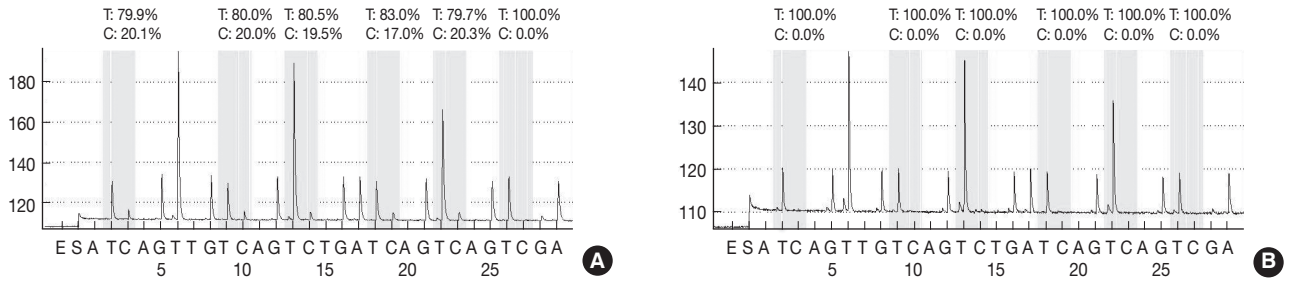

Fig. 1. Two representative glioblastoma pyrograms are shown. Five consecutive positions are analyzed for CpGs and the last one is an internal control for bisulfite treatment. Each C peak in a yellow background represents the methylation percentage for each CpG. (A) Methylated glioblastoma. (B) Unmethylated glioblastoma.

Table 2. Average percentage of methylation in total and the methylated cases from 1997 to 2010

Year of block	No. of cases (%)	MP average (%)	SD (%)	No. of methylated cases (%)	MP average (%)	SD (%)
1997	17 (12)	8.5	11.7	3 (17.6)	29.0	17.4
1998	4 (3)	17.0	16.3	3 (75.0)	21.0	17.4
1999	8 (6)	11.5	15.3	3 (37.5)	27.7	13.7
2000	10 (7)	14.8	16.3	4 (40.0)	33.0	7.5
2001	10 (7)	10.7	14.2	3 (30.0)	30.3	9.1
2002	11 (8)	17.0	17.5	5 (45.5)	32.2	15.0
2003	5 (4)	19.0	27.4	2 (40.0)	44.5	29.0
2004	9 (6)	15.3	17.8	4 (44.4)	30.5	16.7
2005	9 (6)	9.6	10.4	2 (22.2)	27.0	7.1
2006	10 (7)	12.3	14.1	4 (40.0)	25.8	13.7
2007	16 (11)	12.8	16.0	6 (37.5)	29.0	16.1
2008	7 (5)	13.9	18.0	3 (42.9)	29.3	18.6
2009	13 (9)	14.4	19.8	4 (30.8)	40.0	17.2
2010	12 (9)	24.8	23.6	6 (50.0)	45.8	13.0
Total (%)	141 (100)	14.0	16.8	52 (36.9)	32.3	14.9

MP average, average percentage of methylation; SD, standard deviation.

ma (mean age, 40.5 years). Seven patients (5.0%) had pilocytic astrocytoma (PA) (mean age, 17.7 years). Three cases were an ependymoma, a juvenile pilomyxoid astrocytoma, and a dysembryoplastic neuroepithelial tumor. Infantile desmoplastic astrocytoma, pleomorphic xanthoastrocytoma, and a primitive neuroectodermal tumor were included in the et cetera (ETC) category.

MGMT promoter methylation analysis

PSQ data were obtained from all 141 samples. Fig. 1 shows the pyrograms, representing cases of methylated and unmethylated GBM. Table 2 shows the average methylation percentages (MPs) for all cases and the averages of the methylated cases for 13 years by year. The mean of all cases was 14.0±16.8%, and that of methylated cases was 32.3±14.9%. The MP values for each year were not significantly different ($p=0.771$) and did not show a increasing or decreasing linear pattern according to the age of the FFPE block.

Thirty-one (41.3%) of 75 GBMs were methylated. The average MPs of the methylated and unmethylated cases were 35.8±14.7% and 3.2±1.8%, respectively ($p<0.001$). Eight (36.4%) of 22 AA were methylated, and the average MP of AA was 31.8±15.5%. Eight (42.1%) of 19 astrocytomas were methylated, and the average MP was 22.4±15.1%. We observed a tendency for an increasing pattern of average MP with WHO grade ($p=0.063$) in astrocytic tumors. Four of seven cases (57.4%) with AO were methylated, and the average MPs were 30.0±8.5% and 4.7±1.1% in the methylated and unmethylated cases, respectively. One oligodendroglioma was methylated, and 20.0±0.0% was the average MP. Five of eight cases (75.0%) of oligodendroglial tumor were methylated, and the average MPs of the methylated and unmethylated oligodendroglial tumors were 28.0±8.1% and 4.7±1.2%, respectively ($p=0.024$). Nine PAs were unmethylated, and the average MP was 3.0±1.3%. The ETC group was unmethylated as well with an average MP of 3.8±2.1% (Table 3).

Table 4 shows the overall MPs of methylated glial tumors according to WHO grade. Grade 1 tumors were entirely unmethylated. We observed a correlation between average MP and WHO grade ($p=0.038$) and a bimodal pattern, which divided the methylated and unmethylated populations clearly using a 9% cut-off value ($p<0.001$).

DISCUSSION

Locus-specific hypermethylation, mostly at the CpG island (CGI) promoters, is frequent in patients with GBM. CGIs are regions of ~500 bp to 1 kb in which CpG nucleotides are approximately five times more abundant compared to the rest of the genome.[15] CGI promoter hypermethylation occurs at genes

Table 3. Summary of the average methylation percentage of methylated and unmethylated cases, categorized by diagnosis

	Methylated		Unmethylated		Total (%)	p-value
	No. of cases (%)	Mean ± SD (%)	No. of cases (%)	Mean ± SD (%)		
Glioblastoma	31 (41.3)	35.8 ± 14.7	44 (58.7)	3.2 ± 1.8	75 (100)	< 0.001
Anaplastic astrocytoma	8 (36.4)	31.8 ± 15.5	14 (63.6)	3.3 ± 2.2	22 (100)	< 0.001
Astrocytoma	8 (42.1)	22.4 ± 15.1	11 (57.9)	3.4 ± 2.6	19 (100)	0.001
Anaplastic oligodendroglioma	4 (57.1)	30.0 ± 8.5	3 (42.9)	4.7 ± 1.1	7 (100)	0.005
Oligodendroglioma	1 (100)	20.0 ± 0.0	0	0	1 (100)	
Pilocytic astrocytoma	0	0	9 (100)	3.0 ± 1.3	9 (100)	
ETC[a]	0	0	7 (100)	2.7 ± 1.1	7 (100)	
Total	52	32.3 ± 14.9	89	3.3 ± 1.9		

[a]Three ependymomas, juvenile pilomyxoid astrocytoma, a dysembryoplastic neuroepithelial tumor, an infantile desmoplastic astrocytoma, a pleomorphic xanthoastrocytoma, and a primitive neuroectodermal tumor.
SD, standard deviation; ETC, et cetera.

Table 4. Average methylation percentage of all cases and the methylated cases according to WHO grade

WHO grade	No. of total cases	Mean ± SD (%)	No. of methylated cases	Mean ± SD (%)
Grade 1	13	3.1 ± 1.3	-	-
Grade 2	24	10.6 ± 12.6	9	22.3 ± 14.1
Grade 3	29	14.9 ± 16.1	12	31.0 ± 13.2
Grade 4	75	16.6 ± 18.7	31	35.8 ± 14.7
Total	141	14.0 ± 16.8	52	32.3 ± 14.9

WHO, World Health Organization; SD, standard deviation.

with diverse functions related to tumorigenesis and tumor progression in patients with GBM, including cell cycle regulation, DNA repair, apoptosis, angiogenesis, invasion, and drug resistance. Genes involved in invasion and metastasis of gliomas can also be affected by promoter hypermethylation. Promoter hypermethylation can modulate sensitivity to drugs and radiotherapy in patients with GBM. One of the best known examples is *MGMT* promoter methylation and the response to DNA alkylating agents. *MGMT* encodes a DNA repair protein that removes alkyl adducts at the O6 position of guanine. *MGMT* expression protects normal cells from carcinogens; however, it can also protect cancer cells from chemotherapeutic alkylating agents. An association between *MGMT* promoter methylation status and clinical outcome of patients with GBM has been shown in many studies. Based on these results, *MGMT* promoter methylation is considered a promising molecular factor predictive of the chemotherapy response and longer survival in patients with GBM.

Hegi *et al.*[2] reported that 45% of GBMs are methylated in his randomized trial, and that the median success rate of MSP was 75% in FFPE samples. Our PSQ success rate was 100% on 141 samples from 1- to 13-year-old archive specimens, and 41.3% of the GBMs were methylated. Both methylation frequencies were very similar and, recently, Lee *et al.*[16] showed that 44.4% of GBMs are methylated of 27 cases of GBM. Overall, 36.9% of all gliomas were methylated, ranging from 36.4% to 57.1% in our study. Based on our results and several researcher's data, 40-50% of GBMs are potentially methylated, which might be of benefit during treatment with alkylating agents.

We demonstrated PSQ results from 1- to 13-year-old archive specimens. The percentage of methylation over the years were evenly distributed and no increasing or decreasing pattern of MP with specimen age was observed. This might be a huge improvement in the success rate when compared with Hegi *et al.*[2] Dunn *et al.*[14] obtained 264 of 287 tumor samples from 121 cases by PSQ. Failure may have occurred due to the small sample size (< 1 mm^3) with low DNA yield or due to poor DNA integrity in the FFPE samples. Despite some failures, the PSQ data were reproducible and showed a good correlation between duplicate PCR reactions from the same bisulfite modification and between two independent bisulfite modifications of the same DNA extract.[14] Therefore, the PCR followed by the PSQ method is a stable technique and produces consistent data. These results support the need for a large nationwide retrospective trial to evaluate *MGMT* promoter methylation status of old archives from multi-institutions.

The average percentage of methylation in the methylated cases increased significantly with WHO grade, suggesting that MP increases when tumors progress and that methylation status and MP must be analyzed together. Some cases with a high MP in grade II astrocytomas or a low percentage in GBMs may have different tumor progression behavior and responsiveness to alkylating agents. Dunn *et al.*[14] showed an interesting clustering analysis based on the extent of methylation. Progression-free survival was significantly different between low ($9\% < \text{MP} \leq 29\%$) and high ($> 29\%$) methylation groups of GBM and between high methylated and unmethylated cases. Log-rank tests showed significant differences in overall survival between un-

methylated and methylated groups and between cases with low vs high methylation. This result suggested that qualitative and quantitative data should be simultaneously obtained from the *MGMT* methylation analysis to expect progression-free survival, overall survival, or a prediction of chemo-responsiveness in clinical practice.

Alonso *et al.*[17] reported aberrant promoter methylation of multiple genes, including *MGMT* in oligodendrogliomas and ependymomas, using MSP. According to their data, *MGMT* presented with the highest methylation frequency among oligodendroglial tumors, 80% (33/41), showing similar rates in the three groups (oligodendroglioma, AO, and oligoastrocytoma). A significant *MGMT* promoter methylation rate (28%; 2/7) was found in grade II and III ependymomas. Everhard *et al.*[18] evaluated the frequency of *MGMT* promoter methylation in low grade gliomas and showed that 11% of grade II astrocytomas, 62% of grade II oligodendrogliomas, and 27% of grade II oligoastrocytomas were methylated by MSP. In our series, 42% of grade II astrocytomas and a case of oligodendroglioma were methylated. A large difference in methylation status, such as 11% and 80% by MSP and 42% by PSQ, was observed. A few clinical trials of low grade gliomas are ongoing to determine the status of *MGMT* promoter methylation and the role of TMZ in tumor management. The relevant genes on 1p and 19q are still unidentified, and it is unclear whether alterations in one or more genes on these chromosome arms, or rather in completely different chromosomal locations, may account for the clinically less aggressive behavior of 1p/19q deleted tumors. The observation that *MGMT* promoter hypermethylation is common in oligodendrogliomas with losses on 1p and 19q may point to at least one possible mechanism contributing to the chemosensitivity of these tumors.[19] In our study, seven PAs were all unmethylated. PAs can be considered a grade I glial tumor, and PAs are frequently unmethylated; however, sufficient references to PA methylation status, compared with our results are not available yet. Our three grade II ependymomas were unmethylated. In contrast, Koos *et al.*[20] analyzed 142 ependymal tumors and showed that 27% of ependymal tumors were methylated by MSP, and that methylation of the *MGMT* promoter region was significantly less frequent when compared with malignant astrocytic gliomas. Nicholson *et al.*[21] reported that the majority of ependymomas respond poorly to TMZ treatment. It might be too early to determine the usefulness of *MGMT* promoter methylation status for low grade gliomas, so further comprehensive study will be needed.

Several methods to determine promoter methylation have been described, including the use of restriction enzymes[22] or genomic bisulfite sequencing.[23] The overwhelming majority of published data uses MSP following bisulfite treatment.[24] MSP is highly sensitive and has been used widely in this context.[24,25] However, as there are no inbuilt measures of the adequacy of bisulfite treatment, the possibility of false positives due to inadequate conversion of non-methylated cytosine to uracil exists. Another potential source of false positives is mis-priming, and this may be a greater problem when high numbers of PCR cycles or nested primers are used. Previously described methods for controlling mis-priming include re-analysis by methylation-sensitive restriction enzymes[26] or subsequent bisulfite DNA sequencing.[27] An accurate quantitative analysis is very important for a DNA methylation analysis, and MSP has many weaknesses in this regard. One of the major issues with MSP is the use of relative quantification by means of an external control using a different PCR for the control gene. The accuracy and reproducibility of this approach has still not been adequately addressed for the large diversity of genes. Development of DNA methylation risk prediction panels based on diverse genes will be required to develop and optimize an accurate and reproducible method.

Cut-off values for the percentage methylation might be one of the critical issues to determine methylation status using PSQ analysis. Mikeska *et al.*[7] reported that unmethylated tumor samples and control samples show ratios of $<10\%$ at all positions with a small standard deviation and suggested a $Score_{py15}$ formula to separate unmethylated and methylated cases by employing the percentage values of four specified CpGs. Dunn *et al.*[14] stated that GBMs should be considered methylated if they have $\geq 9\%$ average methylation and unmethylated cases should have an average methylation $<9\%$ in all samples. Karayan-Tapon *et al.*[9] analyzed methylation status in patients with GBM by five different methods. They used the PyroMark MGMT™ kit, containing five CpGs, which overlapped with the sequence of Mikeska *et al.*[7] for analysis. Patients with a CpG4 percentage methylation greater than the median value (7.9%) had highly significant longer overall survival than patients with mean methylation for the five CpGs $>8\%$ (median value). Three researchers have used different cut-off values of $>10\%$, 9%, and 8%, which look very similar, but cause a problem if a case has a borderline value such as 8.5% or 9.5% which could represent methylated or unmethylated status. Our study adopted a cut-off value $>9\%$, and four case values were very close to 9%. Two were AAs, with cut-offs of 8.2% and 8.5%, respectively, and considered unmethylated status and the remaining were GBMs, with

9.7% and 9.9% cut-offs, were categorized as methylated. Dunn *et al.*[14] reported a 3.2±2.89% MP for control non-neoplastic brain samples and 51.2% for GBM and average methylation across all CpGs in at least one clinical sample greater than that of non-neoplastic brain (≥9%) were classified as methylated. Mikeska *et al.*[7] estimated methylation status based on logistic regression of a full validation dataset to calculate optimal separation between unmethylated and methylated cases. These are current limitations to the PSQ method, including a lack of consensus on how the data should be interpreted. In clinical practice, an optimal threshold to convert percentage methylation into a qualitative score must be established as a binary classification for clinical testing.

Another issue for *MGMT* promoter methylation analysis is the relevant CpG sites for methylation status. The *MGMT* promoter methylation pattern is very heterogeneous from tumor to tumor, and the CpG sites that need to be methylated to silence transcription and provide a favorable outcome have not been established. Several studies have investigated the methylation status at individual CpGs within *MGMT* CGI and compared them with gene expression. Watts *et al.*[28] studied the methylation status of 108 CpGs across *MGMT* CGI using bisulfite sequencing, and they identified three distinct regions within CGI where high methylation levels were found but only in the *MGMT* non-expressing cell line. Recently, Malley *et al.*[29] reviewed a distinct region of the *MGMT* CGI for transcriptional regulation. Watt's and Malley's regions roughly overlapped with CpG1-25, differentially methylated region (DMR) 1 and 2.[28,29] They[29] found that DMR2 is always methylated when DMR1 is methylated. These results indicated that while both DMR1 and DMR2 may be important for promoter activity, DMR2 alone is practically sufficient for assessing *MGMT* CGI methylation during clinical testing. They concluded that at least several specific CpGs within DMR2 play an important role in *MGMT* CGI promoter activity; therefore, DMR2 appears to be the optimal target for clinical *MGMT* promoter methylation testing. Karayan-Tapon *et al.*[9] assessed *MGMT* promoter methylation status by five different methods including PSQ and his PSQ primer sets included 5 CpGs in DMR2 region, which were used in this study. They concluded that PSQ predicts overall survival well in patients initially treated with radiotherapy plus TMZ using FFPE samples.

To date, *MGMT* methylation analysis is one of the most recommended molecular assays in clinical neuro-oncology. Due to lack of therapeutic alternatives, *MGMT* evaluation is only currently essential for clinical trials, although that is likely to change once effective alternative therapies are identified. A range of new methodologies are available for *MGMT* testing that potentially allow higher levels of sensitivity, specificity, robustness, and reproducibility, and PSQ is one of the promising techniques for *MGMT* evaluation in daily practice.

REFERENCES

1. Lee CH, Jung KW, Yoo H, Park S, Lee SH. Epidemiology of primary brain and central nervous system tumors in Korea. J Korean Neurosurg Soc 2010; 48: 145-52.
2. Hegi ME, Diserens AC, Gorlia T, *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005; 352: 997-1003.
3. Stupp R, Mason WP, van den Bent MJ, *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352: 987-96.
4. Verbeek B, Southgate TD, Gilham DE, Margison GP. O6-Methylguanine-DNA methyltransferase inactivation and chemotherapy. Br Med Bull 2008; 85: 17-33.
5. Esteller M, Garcia-Foncillas J, Andion E, *et al.* Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000; 343: 1350-4.
6. Marsh S. Pyrosequencing applications. Methods Mol Biol 2007; 373: 15-24.
7. Mikeska T, Bock C, El-Maarri O, *et al.* Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. J Mol Diagn 2007; 9: 368-81.
8. Etcheverry A, Aubry M, de Tayrac M, *et al.* DNA methylation in glioblastoma: impact on gene expression and clinical outcome. BMC Genomics 2010; 11: 701.
9. Karayan-Tapon L, Quillien V, Guilhot J, *et al.* Prognostic value of O6-methylguanine-DNA methyltransferase status in glioblastoma patients, assessed by five different methods. J Neurooncol 2010; 97: 311-22.
10. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc 2007; 2: 2265-75.
11. Brakensiek K, Wingen LU, Länger F, Kreipe H, Lehmann U. Quantitative high-resolution CpG island mapping with Pyrosequencing reveals disease-specific methylation patterns of the CDKN2B gene in myelodysplastic syndrome and myeloid leukemia. Clin Chem 2007; 53: 17-23.
12. Jones AV, Kreil S, Zoi K, *et al.* Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 2005; 106: 2162-8.

13. Shaw RJ, Liloglou T, Rogers SN, *et al.* Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. Br J Cancer 2006; 94: 561-8.
14. Dunn J, Baborie A, Alam F, *et al.* Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. Br J Cancer 2009; 101: 124-31.
15. Costello JF, Berger MS, Huang HS, Cavenee WK. Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. Cancer Res 1996; 56: 2405-10.
16. Lee SH, Hwang TS, Koh YC, *et al.* A consideration of MGMT gene promotor methylation analysis for glioblastoma using methylation-specific polymerase chain reaction and pyrosequencing. Korean J Pathol 2011; 45: 21-9.
17. Alonso ME, Bello MJ, Gonzalez-Gomez P, *et al.* Aberrant promoter methylation of multiple genes in oligodendrogliomas and ependymomas. Cancer Genet Cytogenet 2003; 144: 134-42.
18. Everhard S, Kaloshi G, Crinière E, *et al.* MGMT methylation: a marker of response to temozolomide in low-grade gliomas. Ann Neurol 2006; 60: 740-3.
19. Möllemann M, Wolter M, Felsberg J, Collins VP, Reifenberger G. Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. Int J Cancer 2005; 113: 379-85.
20. Koos B, Peetz-Dienhart S, Riesmeier B, Frühwald MC, Hasselblatt M. O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation is significantly less frequent in ependymal tumours as compared to malignant astrocytic gliomas. Neuropathol Appl Neurobiol 2010; 36: 356-8.
21. Nicholson HS, Kretschmar CS, Krailo M, *et al.* Phase 2 study of temozolomide in children and adolescents with recurrent central nervous system tumors: a report from the Children's Oncology Group. Cancer 2007; 110: 1542-50.
22. Singer-Sam J, Grant M, LeBon JM, *et al.* Use of a HpaII-polymerase chain reaction assay to study DNA methylation in the Pgk-1 CpG island of mouse embryos at the time of X-chromosome inactivation. Mol Cell Biol 1990; 10: 4987-9.
23. Frommer M, McDonald LE, Millar DS, *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992; 89: 1827-31.
24. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996; 93: 9821-6.
25. Derks S, Lentjes MH, Hellebrekers DM, de Bruïne AP, Herman JG, van Engeland M. Methylation-specific PCR unraveled. Cell Oncol 2004; 26: 291-9.
26. Belinsky SA, Klinge DM, Dekker JD, *et al.* Gene promoter methylation in plasma and sputum increases with lung cancer risk. Clin Cancer Res 2005; 11: 6505-11.
27. Kawamoto K, Enokida H, Gotanda T, *et al.* p16INK4a and p14ARF methylation as a potential biomarker for human bladder cancer. Biochem Biophys Res Commun 2006; 339: 790-6.
28. Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS, Futscher BW. Methylation of discrete regions of the O6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. Mol Cell Biol 1997; 17: 5612-9.
29. Malley DS, Hamoudi RA, Kocialkowski S, Pearson DM, Collins VP, Ichimura K. A distinct region of the MGMT CpG island critical for transcriptional regulation is preferentially methylated in glioblastoma cells and xenografts. Acta Neuropathol 2011; 121: 651-61.