CLINICAL SCIENCE

Correlation of *MGMT* promoter methylation status with gene and protein expression levels in glioblastoma

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OBJECTIVES: 1) To correlate the methylation status of the O^6-methylguanine-DNA-methyltransferase (*MGMT*) promoter to its gene and protein expression levels in glioblastoma and 2) to determine the most reliable method for using *MGMT* to predict the response to adjuvant therapy in patients with glioblastoma.

BACKGROUND: The *MGMT* gene is epigenetically silenced by promoter hypermethylation in gliomas, and this modification has emerged as a relevant predictor of therapeutic response.

METHODS: Fifty-one cases of glioblastoma were analyzed for *MGMT* promoter methylation by methylation-specific PCR and pyrosequencing, gene expression by real time polymerase chain reaction, and protein expression by immunohistochemistry.

RESULTS: *MGMT* promoter methylation was found in 43.1% of glioblastoma by methylation-specific PCR and 38.8% by pyrosequencing. A low level of *MGMT* gene expression was correlated with positive *MGMT* promoter methylation ($p = 0.001$). However, no correlation was found between promoter methylation and MGMT protein expression ($p = 0.297$). The mean survival time of glioblastoma patients submitted to adjuvant therapy was significantly higher among patients with *MGMT* promoter methylation (log rank = 0.025 by methylation-specific PCR and 0.004 by pyrosequencing), and methylation was an independent predictive factor that was associated with improved prognosis by multivariate analysis.

DISCUSSION AND CONCLUSION: *MGMT* promoter methylation status was a more reliable predictor of susceptibility to adjuvant therapy and prognosis of glioblastoma than were MGMT protein or gene expression levels. Methylation-specific polymerase chain reaction and pyrosequencing methods were both sensitive methods for determining *MGMT* promoter methylation status using DNA extracted from frozen tissue.

KEYWORDS: Glioblastoma; *MGMT* promoter methylation; *MGMT* gene; MGMT protein; Prognosis.

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INTRODUCTION

Gliomas are the most common primary brain tumors in adults.[1] Glioblastomas (GBMs, World Health Organization Grade IV) are the most frequent and malignant of these gliomas, with tumorigenicity demonstrated even in xenograft models.[2] The median survival of GBM patients rarely exceeds 12 months.[3,4] GBMs are divided into two subgroups: primary GBMs that emerge *de novo* and secondary GBMs that are formed from lower-grade astrocytomas.[5-7] Radiotherapy, either alone or in association with chemotherapy, is a frequent complementary treatment to surgical resection in GBM. Recent clinical trials have demonstrated that the combined use of radiotherapy and alkylating agents, particularly temozolamide, improves overall survival.[7,8] Nonetheless, only one third of GBM patients seem to benefit from these therapies. The epigenetic silencing of the O^6-methylguanine-DNA-methyltransferase (*MGMT*) gene by promoter hypermethylation is emerging as a clinically relevant predictor of response to treatment in glioma patients; this predictive value may be limited to GBM.[9]

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No potential conflict of interest was reported.

MGMT promoter hypermethylation can be detected in approximately half of gliomas and is associated with longer overall survival (OS) in patients who receive alkylating chemotherapy in association to radiotherapy.[10,11] Alkylating agents, most commonly chloroethylnitrosoureas (carmustine [BCNU], lomustine, and fotemustine), procarbazine, and temozolomide, induce cell death by forming crosslinks between adjacent DNA strands through alkylation of the O^6 position of guanine. Transcriptionally active MGMT rapidly removes the alkyl adducts, preventing the formation of crosslinks and thereby causing resistance to alkylating drugs.[11,12] Hypermethylation of the *MGMT* promoter with consequent loss of MGMT protein expression reduces the DNA repair activity of glioma cells, overcoming their resistance to alkylating agents.[11]

To translate this finding into a molecular diagnosis, *MGMT* promoter methylation assessment must be reliable and applicable to clinical practice. Several different methodologies are available for assessing the methylation status: 1) direct study of *MGMT* promoter methylation or 2) indirect assessment of its mRNA or protein expression levels.

Various assays have been reported for determining the *MGMT* promoter methylation status,[13] but the most widely used technique is methylation-specific polymerase chain reaction (MSP) analysis after bisulfite treatment.[14] MSP detects CpG island methylation with high sensitivity and specificity, particularly when high-quality DNA extracted from frozen tissue is analyzed. Significant risks of false-positive or false-negative results have been reported, especially when the DNA quality and/or quantity is low as in cases of DNA extracted from paraffin-embedded material.[15] Although MSP is a non-quantitative method, the methylated *MGMT* allele is attributed solely to neoplastic cells[16] by bisulfite treatment; MSP is, therefore, considered a cost-effective method for determining the *MGMT* promoter methylation status in tumor samples. Pyrosequencing (PyroS) was recently introduced as an alternative method based on sequencing by the synthesis principle to yield quantitative results for each individual CpG position,[17-19] including an internal control to check the efficacy of the bisulfite treatment. Because of these characteristics, PyroS has been reported to be the most accurate, robust, and high-throughput method for determining *MGMT* methylation status.[20,21]

Alternatively, *MGMT* methylation status may also be inferred indirectly from *MGMT* gene expression levels determined by real-time PCR or from MGMT protein expression level detected by immunohistochemistry (IHC), a well-established method that is available in the majority of histopathology laboratories.[22] Recently, the *MGMT* mRNA expression level has been associated with malignant glioma outcome independently of *MGMT* methylation status.[23]

With the availability of these various methodologies for exploring *MGMT* as a predictor of outcome or response to therapy, it is important to determine which method presents the best combination of sensitivity, specificity, and favorable cost-benefit ratio using the same set of samples.

Therefore, the objective of the present study was to compare these various methods using the same set of GBM samples and to correlate the results with the clinical endpoint of overall survival of the GBM patients.

MATERIALS AND METHODS

Tissue samples

GBM specimens were obtained during therapeutic surgical management of patients by the neurosurgery group at Hospital das Clínicas, Department of Neurology, School of Medicine, University of São Paulo, São Paulo, Brazil. The specimens were examined by a neuropathologist at the Department of Pathology of the same institution. GBM cases were all primary and were diagnosed within three months of the initial appearance of symptoms. This study was approved by the local research ethics committee (#691/05), and informed consent was obtained from each patient. Fresh GBM samples and non-neoplastic brain tissue from temporal lobectomy for epilepsy[23,24] were macrodissected and immediately snap-frozen in liquid nitrogen upon surgical removal. A 4 µm-thick cryosection of each sample was analyzed under a light microscope after hematoxylin-eosin staining for assessing necrosis and the presence of cellular debris and non-neoplastic areas; following removal from the frozen block, samples were microdissected prior to DNA and RNA extractions. Fifty-one GBM samples from 17 female and 34 male patients with a mean age of 50.2 (SD $\pm$ 14.6) years, and 19 non-neoplastic tissue samples (mean age: 37 years) were included in the present study. Twenty-nine out of 51 GBM patients were submitted to adjuvant radiotherapy (fractionated focal irradiation in daily fractions of 2 Gy given five days per week for six weeks for a total of 60 Gy) and/or chemotherapy (carmustine). The degree of tumor resection was classified as gross total resection (GTR) when more than 90% of the tumor was resected or partial resection (PR) when less than 90% of the tumor was resected. Demographic and clinical findings are presented in Table 1.

DNA extraction and bisulfite treatment

DNA was extracted from frozen tissue using standard phenol/chloroform methods. To evaluate the DNA concentration and purity, we measured the absorbances at 260 and 280 nm. A260/A280 ratios in the range of 1.8–2.0 were considered satisfactory for purity standards. Bisulfite treatment of up to 800 ng of DNA was performed using EpiTect Bisulfite Kits (Qiagen, Hilden, Germany).

Total RNA extraction and cDNA synthesis

Total RNA was isolated from tissues using RNeasy Mini Kits (Qiagen). A conventional reverse transcription reaction was performed to yield single-stranded cDNA. The first strand of cDNA was synthesized from 1 µg of total RNA previously treated with 1 unit of DNase I (FPLC-pure, GE Healthcare, Uppsala, Sweden) using random and oligo (dT) primers, RNase inhibitor, and SuperScript III reverse transcriptase according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). The resulting cDNA was subsequently treated with 1 unit of RNase H (GE Healthcare), diluted with TE buffer, and stored at -20℃ until later use.

Quantitative real time PCR

The relative expression level of *MGMT* was analyzed by quantitative real-time PCR (qRT-PCR) using the SYBR Green approach in duplicate. Quantitative data were normalized relative to the following internal housekeeping genes: hypoxanthine guanine phosphoribosyltransferase (*HPRT*), beta-glucuronidase (*GUSB*), and TATA-box binding protein (*TBP*). The geometric mean of the three genes

Table 1 - Demographic characteristics of and clinical data from the GBM patients analyzed in this study.

Case no.	Age at dx (years)[1]	Gender[2]	Surgical resection[3]	Treatment[4]
1	71	M	GTR	RT&CT
2	65	F	GTR	SR
3	47	F	GTR	RT
4	78	F	GTR	RT
5	45	M	PR	RT&CT
6	54	F	PR	RT
7	68	M	PR	SR
8	67	M	GTR	RT&CT
9	57	F	PR	SR
10	17	F	PR	CT
11	59	F	PR	RT
12	41	F	PR	RT&CT
13	55	M	GTR	SR
14	42	M	PR	RT
15	56	F	GTR	SR
16	45	M	GTR	RT&CT
17	62	F	PR	SR
18	52	M	PR	SR
19	51	M	GTR	SR
20	35	M	PR	SR
21	39	M	PR	RT&CT
22	60	M	PR	SR
23	46	M	PR	RT&CT
24	35	M	PR	SR
25	49	M	PR	SR
26	52	F	GTR	RT&CT
27	57	M	PR	RT
28	16	M	PR	SR
29	55	M	GTR	RT
30	40	M	GTR	SR
31	26	M	GTR	RT
32	40	M	PR	RT&CT
33	68	F	PR	SR
34	28	F	PR	SR
35	38	F	PR	RT
36	32	M	GTR	RT&CT
37	55	M	PR	RT&CT
38	54	M	PR	RT&CT
39	61	F	PR	SR
40	52	M	PR	RT&CT
41	63	M	PR	SR
42	52	M	PR	SR
43	76	M	PR	SR
44	39	M	PR	RT&CT
45	68	F	PR	RT
46	58	F	PR	RT
47	26	M	PR	SR
48	69	M	PR	RT
49	31	M	PR	SR
50	47	M	PR	RT&CT
51	63	M	PR	RT&CT

[1]Age at diagnosis was calculated from date of birth to date of surgery.
[2]M, male; F, female.
[3]GTR, gross total resection; PR, partial resection.
[4]SR = surgical resection; RT = radiotherapy; CT = chemotherapy; RT & CT = radiotherapy and chemotherapy.

was used for relative expression analysis. The primer sequences were as follows (5′ to 3′): *MGMT* F: GCTGAATGCCTATTTCCACCA, MGMT R: CACAACCTTCAGCAGCTTCCA, *HPRT* F: TGAGGATTTGGAAAGGGTGT, HPRT R: GAGCACACAGAGGGCTACAA; *GUSB* F: AAAATACGTGGTTGGAGAGCTCATT, GUSB R: CCGAGTGAAGATCCCCTTTTTA; *TBP* F: AGGATAAGAGAGCCACGAACCA and TBP R: CTTGCTGCCAGTCTGGACTGT.

SYBR Green I amplification mixtures (12 µl) contained 3 µl of cDNA, 6 µl of 2X Power SYBR Green I Master Mix (Applied Biosystems, Foster City, CA) and forward and reverse primers. PCR reactions were run on an ABI Prism 7500 sequence detector (Applied Biosystems) as follows: 2 min at 50℃, 10 min of polymerase activation at 95℃, and 40 cycles of 15 s at 95℃ and 1 min at 60℃. All of the primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). The minimum primer concentrations necessary were determined to be those concentrations that gave the lowest threshold cycle (Ct) and maximum amplification efficiency while minimizing non-specific amplification; primer concentrations used were 200 nM for *MGMT*, *HPRT* and *TBP* and 400 nM for *GUSB*. Analysis of the DNA melting curves demonstrated a single peak for all primers. Standard curves were analyzed for all genes to check the efficiency of amplification of each gene. Additionally, agarose gel electrophoresis was employed to check the size of the PCR product amplified.

The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative *MGMT* expression in tumor samples compared to the mean of the non-neoplastic tissues where $\Delta Ct = Ct$ (*MGMT* gene) – Ct (geometric mean of housekeeping genes) and $\Delta\Delta Ct = \Delta Ct$ (tumor) – mean ΔCt (non-neoplastic tissues).[25] For statistical analysis, the *MGMT* expression status was scored as high- or low-expression according to the median of the GBM relative expression values.

Methylation-specific PCR

MSP analyzed positions 118-137 and 174-195 with the following specific primers designed to distinguish methylated (MetMGMT) from unmethylated DNA (UnmetMGMT)[26] (5′-3′): UnmetMGMT F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT, UnmetMGMT R: AACTCCACACTCTTCCAAAAACAAAACA, MetMGMT F: TTTCGACGTTCGTAGGTTTTCGC-3′ and MetMGMT R:GCACTCTTCCGAAAACGAAACG.

MSP using SYBR Green I was performed using PCR Core Reagents (Applied Biosystems) with 20 ng of bisulfite-treated DNA. Final concentrations in a volume of 10 µl were 1X SYBR Green PCR Buffer, 1 mM of each dNTP, 200 nM of primers and 0.3 units of AmpliTaq DNA polymerase. PCR was carried out on an ABI Prism 7500 sequence detector (Applied Biosystems) with the following amplification program: 10 min at 95℃ followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. Universal unmethylated and universal poly-methylated DNA (EpiTect Control DNA Set, Qiagen) were included as controls in each set of reactions in addition to a negative control sample without DNA. Methylated and unmethylated *MGMT* promoter sequences were analyzed by comparing the melting curves of control DNAs. Additionally, the reactions were checked by 3% agarose gel electrophoresis to verify the presence of methylated and unmethylated *MGMT* promoter PCR products of lengths 81 bp and 93 bp, respectively.

Pyrosequencing analysis

PyroS analysis was carried out for 5 CpG sites in exon 1 (positions 17 to 39, Ensembl ID: OTTHUMT00000051009) of the *MGMT* promoter using a PyroMark Q24 System (Biotage, Sweden). Primers (PyroMark Assay Database, Biotage) were designed to hybridize with CpG-free regions to secure methylation-independent amplification as an internal control. PCR was performed with 20 ng bisulfite-treated DNA,

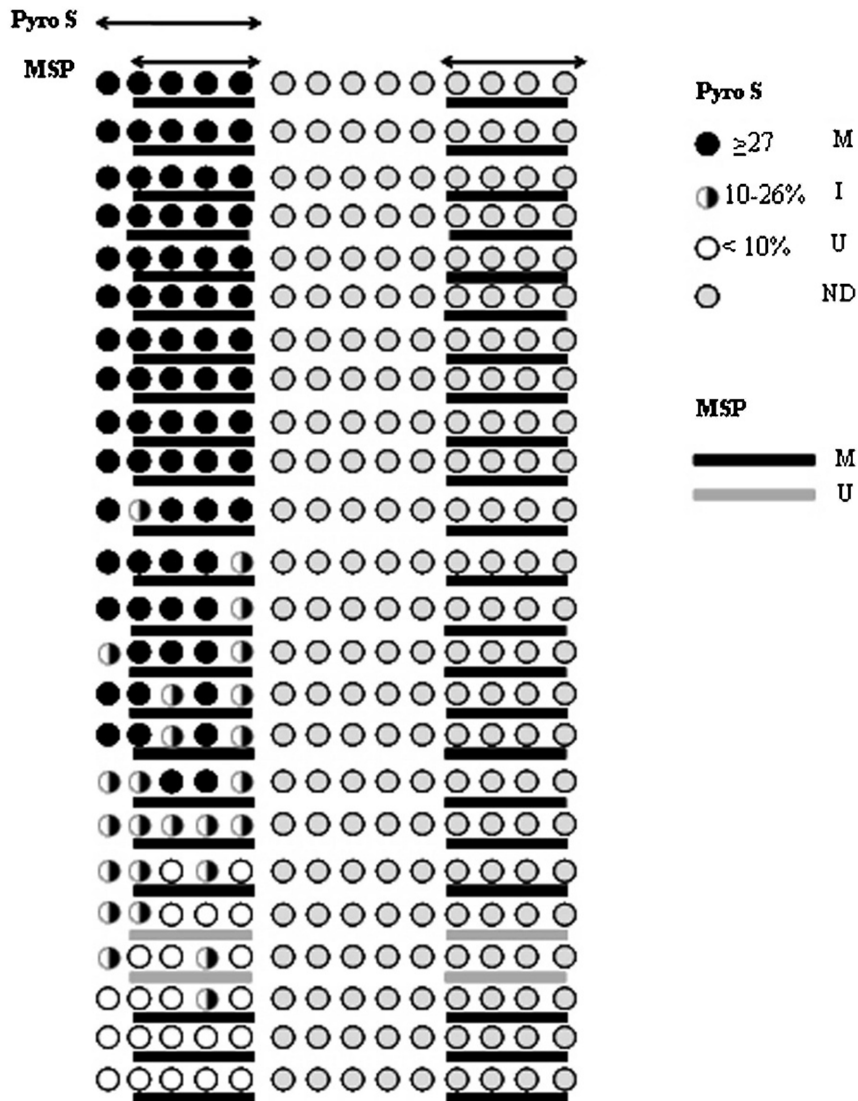

Figure 1 - Comparison of the *MGMT* promoter methylation status of GBM cases analyzed using MSP and PyroS. Five CpG sites were analyzed using PyroS, and 8 CpG sites were analyzed using MSP. For PyroS, the *MGMT* methylation status was scored according to the average percentage of specifically methylated CpG sites as unmethylated (U) when <10% CpG sites were methylated, intermediate (I) when 10 to 26% CpG sites were methylated, or methylated (M) when ≥27% of CpG sites were methylated. For MSP, the *MGMT* methylation status was scored as methylated (M) or unmethylated (U) according to the presence or absence of the specific PCR amplification.

200 mM of each primer, 12.5 µl PyroMark 2X PCR master mix, 2.5 µl CoralLoad Concentrate 10X (provided in the PyroMark PCR Master Mix, Qiagen), and HotStar Taq polymerase. PCR conditions were 95℃ for 15 min; 45 cycles of 94℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s; and 72℃ for 10 min. PCR products of 104 bp were checked by 2% agarose gel electrophoresis. Subsequent quantification of the methylation density of five CpG sites was performed using the PyroMark Q24 software. Subsets of GBM cases were reanalyzed with repeated PCR and PyroS reactions to test the reproducibility of the findings. Two subgroups were defined for statistical analysis according to an average of CpG residue methylation: 1) an unmethylated group, for which the mean range was <10%, and 2) a methylated group, including those samples with an intermediate status (mean range of 10-26%) or a methylated status (mean range ≥27%).

Immunohistochemistry

Four-micrometer sections were prepared from paraffin-embedded tumor blocks for IHC analysis. Briefly, antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) at 122℃ for 3 min using an electric pressure cooker (BioCare Medical, Walnut Creek, CA). Specimens were subsequently blocked and incubated further with a mouse monoclonal antibody raised against human MGMT (Clone MT 3.1, Neomarkers Inc., Fremont, CA) at a final dilution of 1:500 at 4℃ overnight. Antigen-antibody reactions were revealed using a commercial kit (Novolink, Novocastra, Newcastle, UK) at room temperature; diaminobenzidine was used to detect the signal, and Harris hematoxylin was used as a nuclear stain. A positive control tissue (colon adenocarcinoma) was used to confirm the consistency of the immunostaining, and all samples were stained in a single batch.

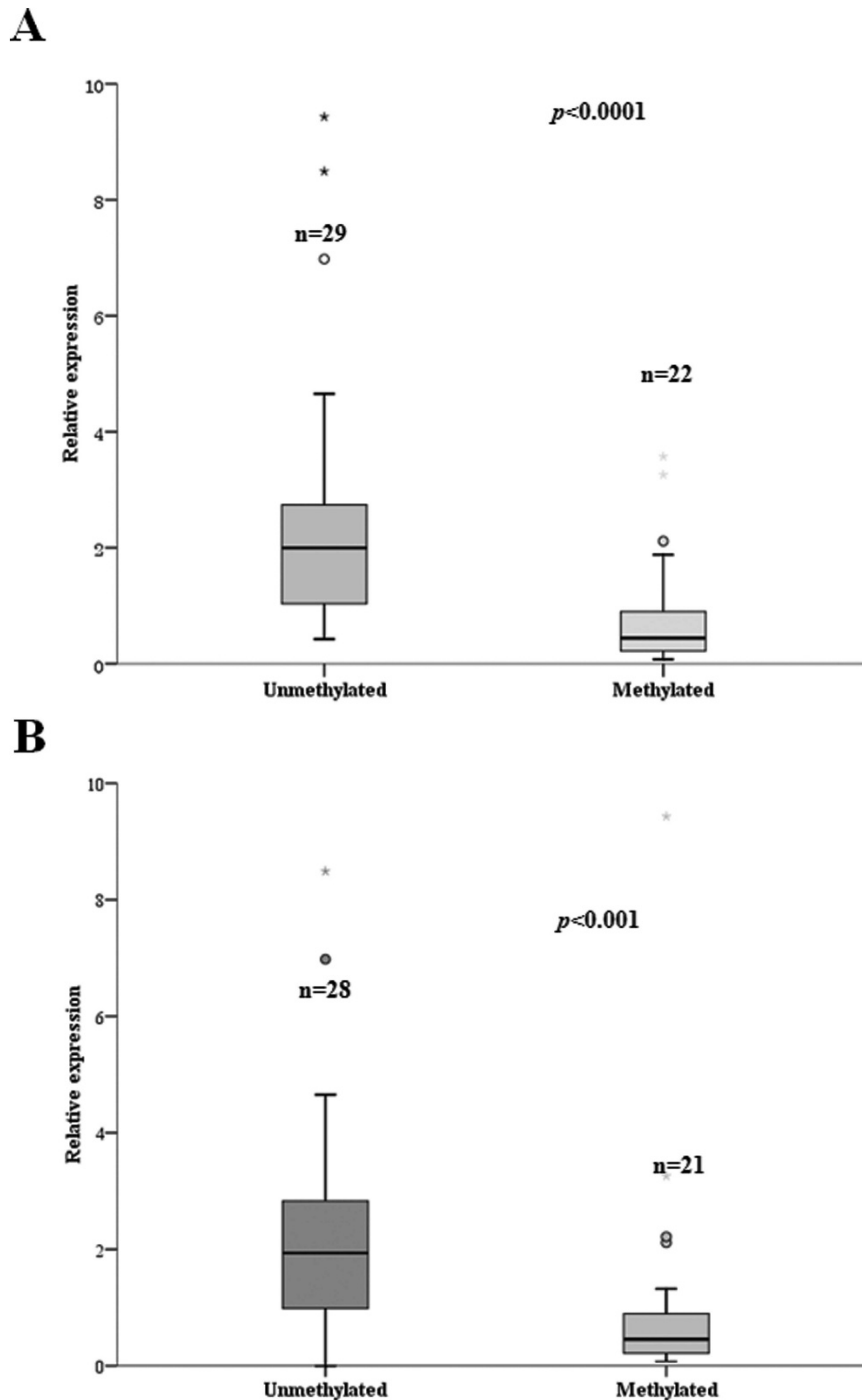

Figure 2 - Correlation between relative gene expression level of *MGMT* and promoter methylation status determined using MSP (A) and PyroS (B). *MGMT* expression levels in the GBM samples were determined relative to non-neoplastic tissues. The horizontal bars show the median values for the relative expression of *MGMT* in each group. The Mann-Whitney test was used to evaluate the differences in the relative expression level between the methylated and unmethylated groups.

MGMT expression was assessed and scored in tumor cells by two independent observers who were blinded to tumor methylation status and clinical data according to the following semi-quantitative classification method based on percent of nuclei that were positive: 0 (no staining), 1 (10–25%), 2 (26–50%), or 3 (>50%). Endothelial staining was

used as an internal control for MGMT immunostaining. In accordance with previous reports, MGMT staining was considered positive when uniform MGMT staining was detected in the cell nuclei.[27] Reactivity that was restricted to the cytosol and granular nuclear reactivity were considered negative. [27] Endothelial cells and perivascular lymphocytes were excluded from positive cell counts. For statistical analysis, scores of 0 were defined as the absence of protein expression, and scores of 1 to 3 were defined as positive for protein expression. The χ^2 test was used to evaluate the association between the *MGMT* promoter methylation status (negative *versus* positive) and protein expression levels (absence *versus* positive).

Statistical analysis

The χ^2 test was used to evaluate the associations between the *MGMT* promoter methylation status (negative *versus* positive) and the gene and protein expression levels. The Mann-Whitney test was used to analyze the differences in the relative expression levels between the two groups (methylated *versus* unmethylated) as determined by MSP and PyroS. Overall survival (OS) was calculated from the day of surgery to the day of death and was expressed in months. The Kaplan-Meier survival curve was analyzed using the *log rank* (Mantel Cox) test and multivariate analysis using the Cox proportional hazards model. The logistic regression model included the following parameters: age at diagnosis, gender (female *versus* male), degree of tumor surgical resection (gross total resection, GTR *versus* partial resection, PR) and MGMT promoter methylation status (methylated *versus* unmethylated) assessed using PyroS and MSP. Calculations were performed using STATA version 7 (STATA Corp., College Station, TX) and SPSS 15.0 (SPSS, Chicago, IL). Also, *p*-values lower than 0.05 were considered statistically significant.

RESULTS

MGMT promoter methylation status

MGMT promoter methylation was detected in 43.1% (22 out of 51) of the GBM samples by MSP and in 38.8% (4 intermediate and 15 methylated out of 49) of the samples by PyroS. Two of the 51 GBM cases were excluded from the PyroS method analysis, due to unsuccessful PCR amplification. MGMT methylation status was determined with 91% concordance for the two methods. Although PyroS revealed two cases of intermediate methylation that went undetected by MSP, the former method failed to detect three methylated cases that were detected by MSP. These three cases were methylated at a CpG site revealed by the primer set for PyroS, as shown in Figure 1.

MGMT gene expression

MGMT gene expression was determined by qRT-PCR. The median expression levels in GBM did not differ significantly from those of non-neoplastic brain tissues (1.16 *versus* 0.91, respectively, $p = 0.597$). However, when *MGMT* expression levels were analyzed in the two subgroups (methylated *versus* unmethylated), a significant difference was observed with higher expression levels found in the methylated subgroup using either MSP ($p < 0.0001$, Figure 2A) or PyroS ($p < 0.001$, Figure 2B). Furthermore, as revealed by qualitative analysis, the correlation between the methylation status of the *MGMT* promoter and the relative *MGMT* expression level (either positive promoter methylation and low expression or negative promoter methylation and high expression) was statistically significant ($p = 0.001$, 72.5% concordance for MSP; $p = 0.002$, 71.4% concordance for PyroS).

MGMT protein expression

Positive expression of MGMT protein in tumor cell nuclei (as analyzed by IHC) was observed in 38 out of 51 (74.5%) GBM cases; there were 33 cases with a positivity score of 1+ positivity and only five cases with a score of 2+ (Figure 3). Among the 38 positive cases, 47.4% (by MSP) and 57.1% (by PyroS) were positive for *MGMT* promoter methylation. In contrast, only 31% (4 out of 13) of cases showing no staining on IHC were positive for *MGMT* promoter methylation. Consequently, no significant correlation between MGMT protein expression and *MGMT* promoter methylation status was found. The concordances between IHC and either MSP or PyroS were only 47% and 57.1%, respectively. Other comparative analyses of the IHC protein expression scores and methylation status (considering 0 and 1+ staining as negative protein expression or 2+ as positive protein expression) demonstrated a similarly low concordance between the two methods.

An overview of the results of the present study is shown in Figure 3 as a heatmap and includes *MGMT* promoter methylation status and gene and protein expression findings.

Influence of *MGMT* promoter methylation status and gene and protein expression on prognosis

The mean OS of the GBM cases was 13 ± 14.0 months. *MGMT* gene and protein expression levels were not correlated with OS ($p > 0.05$, data not shown). Similarly, *MGMT* promoter methylation status (as determined by MSP) did not appear to affect OS, which was 17.2 months for the methylated group and 9.5 months for the unmethylated group ($p = 0.297$). However, when only the 29 GBM cases submitted to adjuvant chemotherapy with radiotherapy and/or chemotherapy were considered, the mean OS times differed significantly between the two groups (27.4 months for the methylated group and 12 months for the unmethylated group, $p = 0.025$), indicating a stronger therapeutic response in the methylated group (Figure 4A). The mean OS was also significantly longer in the methylated group (31.7 months) than in the unmethylated group (11.8 months) ($p = 0.004$) determined by PyroS (Figure 4B). A

Figure 3 - Heatmap of the MGMT analyses showing methylation status as assessed by MSP and PyroS, quantitative real-time PCR (qRT-PCR) for *MGMT* relative gene expression and immunohistochemistry (IHC) for MGMT protein expression. MSP and PyroS: green, methylated; yellow, intermediate; red, unmethylated; and white, not determined. qRT-PCR: green, low expression; red, high expression. IHC: green, negative staining; red, positive staining.

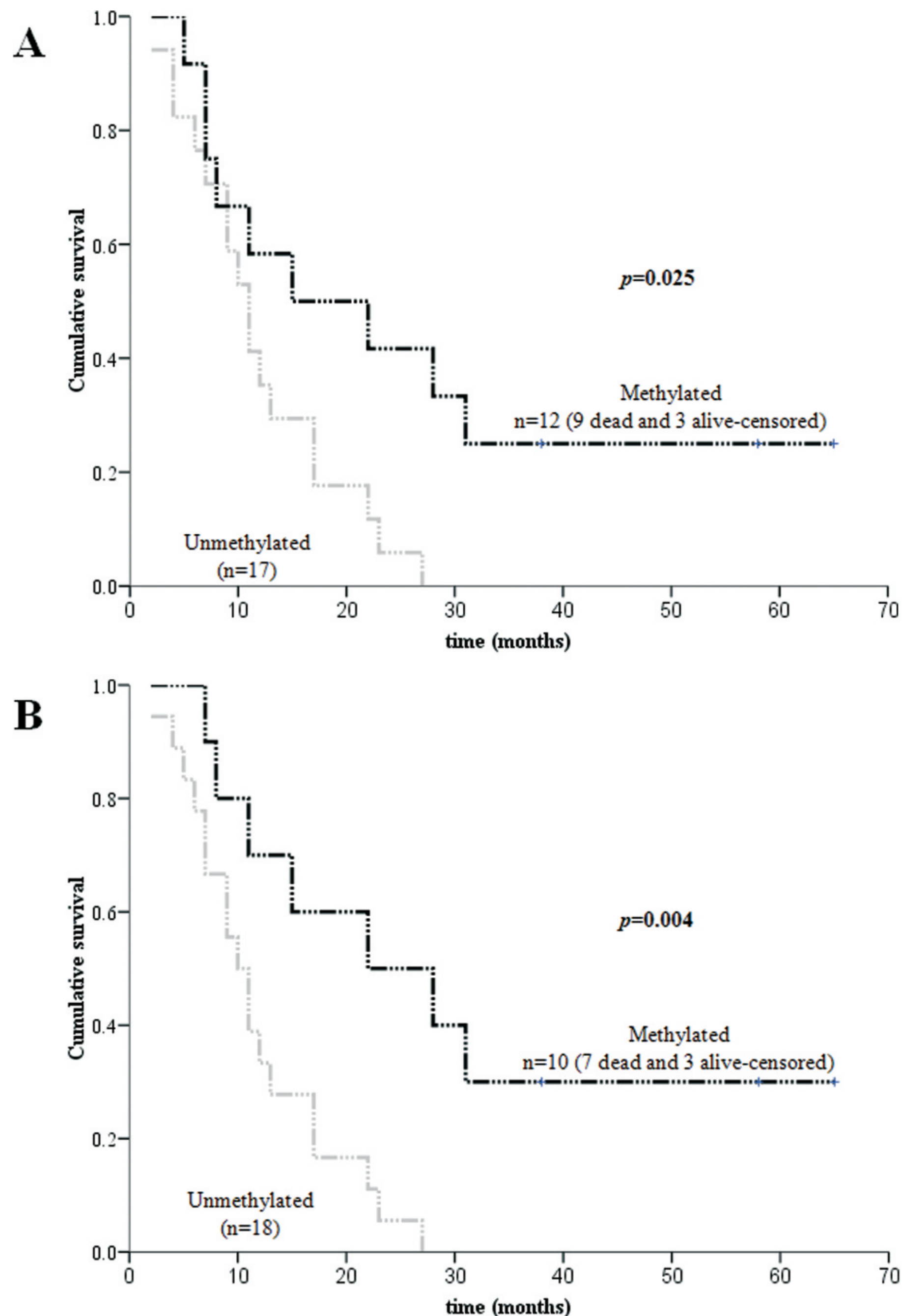

Figure 4 - Kaplan-Meier curves showing the overall survival of GBM patients submitted to adjuvant therapy (radiotherapy and/or chemotherapy) and grouped according to *MGMT* promoter methylation status as determined by MSP (A) and PyroS (B). The difference in overall survival times between the methylated and unmethylated groups was statistically significant for both methods (*log-rank* test: $p = 0.025$ for MSP and $p = 0.004$ for PyroS).

multivariate Cox regression model (which considered age at diagnosis, gender, degree of tumor surgical resection, and *MGMT* promoter methylation status) showed that only *MGMT* promoter methylation status assessed either by MSP or PyroS was an independent prognostic factor (hazard ratio = 0.342, $p = 0.023$ and hazard ratio = 0.218, $p = 0.005$, respectively) as indicated in Table 2. Comparison of the methylation levels of the five CpG sites individually (above *versus* below the median level) yielded similar results ($p = 0.004$ for CpG1, CpG2 and CpG5 and $p = 0.002$ for CpG3 and CpG4, data not shown).

DISCUSSION

In the present study, *MGMT* promoter methylation as determined by two distinct methods had a significant impact on overall survival among patients treated with radiotherapy and/or chemotherapy as reported by other researchers in clinical trials and meta-analyses.[10,11,28-31] The frequency of *MGMT* promoter methylation in our study was in agreement with previously described results in primary GBM, which range from 36-45%.[10,15,32-33] Using one set of GBM samples, we were able to predict the therapeutic

Table 2 - Multivariate proportional hazards analysis (Cox model) of age, gender, degree of surgical tumor resection, and *MGMT* methylation status (by MSP and PyroS) of patients with GBM who underwent adjuvant therapy.

Variable	HR (95% CI)	*p*-value
MSP		
MGMT promoter methylation status (by MSP)[1]	0.342 (0.13-0.86)	**0.023**
Age at diagnosis	1.00 (0.97-1.04)	0.783
Gender[2]	1.54 (0.67-3.55)	0.311
GTR[3]	0.54 (0.21-1.40)	0.205
PyroS		
MGMT promoter methylation status (by PyroS)[1]	0.218 (0.08-0.63)	**0.005**
Age at diagnosis	1.02 (0.98-1.06)	0.230
Gender[2]	1.30 (0.54-3.13)	0.553
GTR[3]	0.55 (0.21-1.42)	0.216

HR, hazard ratio; CI, confidence interval; GTR: gross total resection, PR: partial resection.
Age at diagnosis (from date of birth to date of surgery).
[1]MGMT methylated compared to MGMT unmethylated.
[2]Compared to male.
[3]Compared to PR.

response using either a qualitative (MSP) or a quantitative (PyroS) method to assess the methylation status of the *MGMT* promoter. A high level of concordance (91%) between the two methods was observed. MSP provided slightly higher sensitivity while covering more CpG sites in the analysis than PyroS (8 *versus* 5 CpG sites). However, PyroS detected an intermediate methylation state that was not revealed by MSP. Additionally, the analysis of the five CpG sites together or separately predicted response to therapy in our set of GBM cases. In fact, the status of only a few CpG sites may be an adequate predictor of OS if high quality DNA is extracted from frozen tissue, which improves the sensitivity of methylation assessment,[32] and adequate internal controls to detect incomplete bisulfite conversion and false priming are used. The accurate and robust results for *MGMT* promoter methylation status achieved by assessment of only four CpG sites[20] located immediately downstream of those investigated in the present study corroborate this notion. Moreover, the methylation status of CpG4 alone has also proved to be a good predictor of OS.[31]

Another important issue in bringing a result from the laboratory bench to bedside practice is the cost-benefit ratio. To this end, we analyzed the reliability of two low cost methods for indirectly assessing MGMT methylation status: 1) *MGMT* gene expression level by straightforward qRT-PCR method and 2) MGMT protein expression level by IHC, a routine method widely available in histopathology laboratories. We compared the results of both methodologies to the *MGMT* methylation status. The positive correlation observed between the presence of *MGMT* promoter methylation and low expression of this gene may be explained by the use of the same microdissected frozen tumor fragment for DNA and RNA extractions and also by normalization of qRT-PCR results using three housekeeping genes instead of one.[34,35] Nevertheless, the concordance between the *MGMT* gene expression level and methylation status determined by MSP was 72.5%, and this discrepancy may be due to the difficulty of determining the cut-off level for dividing the cases into groups with high or low *MGMT* gene expression. In the present study, the cut-off was arbitrarily determined as the median average value of the gene expression level in all GBM cases (1.16), although other criteria may also be acceptable. Recently, *MGMT* mRNA expression has been shown to play a direct role in mediating tumor sensitivity to alkylating agents independently of *MGMT* promoter methylation.[23] Some methodological differences, such as the housekeeping genes used in that study compared to the present one, may explain the discrepancy, as may the fact that some CpG sites better reflect the *MGMT* gene expression level than others.[36] Therefore, the reliability of assessing *MGMT* methylation status indirectly by *MGMT* qRT-PCR remains an open question.

No significant correlation between MGMT protein expression (as determined by IHC) and methylation status was found, and concordances of only 47.4% (MSP) and 57.1% (PyroS) were observed. The presence of sampling bias in the methods with the smaller frozen fragments needed for the methylation study compared to the typically larger paraffin-embedded sections used for IHC, and the inclusion of endothelial cells, tumor-infiltrating lymphocytes or a variety of normal resident cells preserved within tumors may represent confounding factors for evaluation by IHC.[21,37,38] Heterogeneity of the cell subpopulation comprising GBM tumors is an additional factor that could explain the discrepancy between the results of these two methods. The concomitant detection of both methylated and unmethylated status in the majority of GBM specimens analyzed in the present study corroborates the existence of this heterogeneity, as described previously.[39,40] Furthermore, other factors, such as the p53 status, may also influence the final level of MGMT protein expression. Tumors with normal p53 status are more likely to have low or absent MGMT expression independently of the *MGMT* promoter methylation status.[41] Therefore, although IHC is a more accessible method than MSP or PyroS, MGMT protein expression is not a reliable method for inferring MGMT methylation status.

CONCLUSION

In summary, in one set of samples, the *MGMT* promoter methylation status but not the MGMT mRNA or protein expression levels was confirmed as a factor predicting the response to adjuvant therapy in GBM patients.

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