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MGMT Promoter Hypermethylation in a Series of 104 Glioblastomas

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Abstract. *Aim: To evaluate MGMT promoter hypermethylation as prognostic factor in a retrospective study of 104 cases of glioblastoma multiforme (GBM). Materials and Methods: The O^6-methylguanine-DNA methyltransferase (MGMT) status was evaluated by methylation-specific PCR (MSP), immunohistochemistry and Western blotting analysis in formalin-fixed paraffin-embedded surgical samples. Results: The MGMT gene was found to be methylated in 29 of 101 tumors (28.7%) by MSP, according to the evaluation methods employed. By immunohistochemistry, different categories were identified on the basis of reaction intensity, percentage of positive cells and homogeneous or heterogeneous distribution. MSP did not correlate with immunohistochemistry, with the exception of the category with the highest percentage of positive cells and homogeneity of immunostaining. Western blotting analysis correlated with immunohistochemical findings (Pearson's correlation coefficient r=0.268, p=0.0211), but not with MSP. By Kaplan-Meier survival analysis, radiotherapy was a significant prognostic factor (p=0.0001). When uncensored patients alone were considered, MGMT methylation status showed a significant correlation with survival (p=0.026). Temozolomide therapy correlated with survival (p=0.022), but not with MGMT methylation. After multivariate Cox regression analysis, only radiotherapy remained as an independent prognostic factor (p=0.0001). Conclusion: Correlation was inconclusive among MSP, immunohistochemistry and Western blotting analysis, despite the sophisticated score system for the immunohistochemical quantitative evaluation. MGMT expression is a complex event in which many factors beside epigenetic silencing are implicated.*

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Alkylating agents are known to be carcinogenic because of the formation of O^6-alkylguanine from alkylation of the O^6 position of DNA and they are responsible for malignant transformation and mutations (1). At the same time, alkylating agents are also active antineoplastic drugs that induce cell death by apoptosis (2, 3). O^6-methylguanine-DNA methyltransferase (MGMT) plays a role in the mechanism of resistance to alkylating agents by repairing O^6-alkylguanine by removing the alkyl group and restoring guanine. In this regard, the amount of MGMT in a tumor and its rate of re-synthesis are of paramount importance because they condition the resistance to alkylating agents such as temozolomide (TMZ) that usually produce cell death *via* DNA interstrand crosslinking and apoptosis (4). MGMT repair of O^6-alkylation by TMZ reverses the formation of adducts before they provoke apoptosis (5).

MGMT is variably expressed in tumors and its function is frequently lost after CpG island hypermethylation in the promoter region. This occurs in many tumors, including 40% of gliomas (6), and it acts as an epigenetic mechanism of tumor progression (7, 8). Some studies demonstrated that inactivation of the *MGMT* gene by promoter hypermethylation was associated with longer survival in patients with glioblastoma. At 18 months, survival was 62% for patients with methylated *MGMT* and 8% for those without (7). Patients with *MGMT* methylation treated with radiotherapy and TMZ had prolonged survival rates (21.7 months with both treatments compared to 15.3 months with radiotherapy alone) (9).

There are demonstrations of a better outcome of patients with *MGMT* hypermethylation (7, 10-12), but other studies did not confirm these findings (13-16). In this discrepancy, the non-homogeneous composition of the tumor series could play a role (17). In other experiences, the correlation between *MGMT* methylation and survival was only found if patients had received chemotherapy (17). In fact, *MGMT* silencing correlated with increased survival in patients who

receive radiotherapy plus TMZ (7). The evaluation of the effects of TMZ treatment depends also on the modalities of its administration (18).

The methods of evaluation of *MGMT* hypermethylation are very important in order to verify *MGMT* epigenetic silencing. *MGMT* hypermethylation has been demonstrated by methylation-specific polymerase chain reaction (MSP) and inferred by analysis of the enzymatic activity (19) and by immunohistochemistry (IHC) of the protein; the latter method has been considered to have the advantage of being simpler and less expensive, but neither of the latter two methods are suitable. Several studies reported significant associations of IHC with patient outcome in glioma (17, 20-23). In a recent study, however, MGMT expression investigated by tissue microarray procedures, showed a poor immunohistochemical correlation with *MGMT* promoter hypermethylation and no correlation with patient outcome (24). Results of MGMT immunohistochemistry do not have the same prognostic significance as those from MSP (24).

The positive or negative correlation largely depends not only on a careful immunohistochemical analysis, but mainly on the molecular procedures used for the demonstration of *MGMT* methylation status. Since in the literature there are still controversies regarding any correlation, in the present study we sought to compare *MGMT* hypermethylation as detected by MSP after capillary electrophoresis (CE)-fragment analysis with that determined by immunohistochemistry and Western blotting in order to evaluate it as a prognostic factor in a series of 104 patients with glioblastoma.

Materials and Methods

Patients. The study included 104 patients with glioblastoma operated on at the Department of Neuroscience, University of Turin in the period between 1995 and 2007. Surgical samples from all patients were fixed in buffered 4% formalin and embedded in paraffin within 48 h. Histological diagnosis was performed with WHO guidelines (25) after surgery, mostly of partial resection. No biopsy was included in this series. The mean age of patients at surgery was 60.3±10.43 years, with a range of 27-81 years; 63 males and 43 females were included in the study.

All patients provided informed consent allowing the use of tumor specimens for molecular studies. The study was approved by Ethics Committee of the University Hospital.

Patient stratification. Survival analysis could be performed in 80 patients only, and 55 out of these received postoperative standard fractionated radiotherapy (60 Gy total dose; 2 Gy × 5 days/week for 6 weeks). Twenty-eight out of 55 irradiated patients received concomitant chemotherapy with temozolomide 75 mg/m^2/daily for 6 weeks, followed by adjuvant temozolomide 200 mg/m^2 from day 1 to day 5 every 4 weeks for 6-12 cycles. Irradiation is standard therapy for glioblastoma, when feasible. Eleven patients did not undergo radiotherapy or received <20 Gy because of their low Karnofsky status (<60), or death within 2-8 months after surgery. In 14 patients surviving <8 months, radiotherapy was <20Gy. Twenty-four patients were excluded from survival analysis because their survival data were not available, but not from the correlations between MGMT MSP and immunohistochemistry and Western blotting.

MGMT hypermethylation analysis by MSP. The DNA methylation status in the CpG islands of the MGMT gene was determined after chemical modification of unmethylated but not methylated cytosines to uracil. Subsequently, MSP with primers specific for either modified methylated or unmethylated DNA sequences was carried out (6).

Genomic DNA was extracted according to a standard phenol-chlorophorm protocol. Only those tumor areas previously identified as proliferating by haematoxylin and eosin (H&E) staining were selected and drawn from paraffin blocks by a lancet. One microgram of genomic DNA was denatured with sodium hydroxide (3 M) and modified by sodium bisulfite with a MethylEasy™ DNA Bisulphite Modification Kit (Human Genetic Signatures Pty Ltd, Macquarie Park, Sydney, Australia) according to the manufacturer's instructions. CpGenome™ Universal Methylated DNA (Chemicon International Inc., Temecula, CA, USA) and normal lymphocyte DNA were used as methylated and unmethylated controls, respectively. They were subjected to bisulphite treatment and PCR amplification in parallel with tissue from the patients. For each sample, two distinct PCR reactions were performed to confirm the result. The amplified products of MSP were analyzed by CE on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Figure 1A, B). Data were collected by the GeneMapper Software v4.0 for fragment analysis (Applied Biosystems). The MSP products for the methylated and unmethylated allele corresponded to a 81 and 93-base pair peak, respectively. The peak height ratio between peaks for the methylated and unmethylated allele was considered and values >0.1 were scored as evidence of the methylated status of the *MGMT* gene. In addition, a second evaluation was carried out by considering the methylated peak only with three different intensity cut-offs.

Immunohistochemistry. The analysis of MGMT was performed on 5 μm-thick sections by a labeled streptavidin-biotin protocol after heat-induced antigen retrieval in 0.01 M sodium citrate buffer (pH 7.4) (3×5 minutes at 650 W). Slides were then incubated with the anti-human MGMT mouse monoclonal antibody (MAB16200, clone MT3.1, 1:100 dilution; Chemicon International Inc.) overnight at 37˚C. After incubation with biotinylated anti-mouse secondary antibody and ABC complex (StreptABC Complex/HPR; Dako Carpinteria, CA, USA), the reaction was developed by diaminobenzidine substrate (DAB; Roche Diagnostics GmbH, Penzeberg, Germany). Nuclei were counterstained with Mayer's haematoxylin. A negative control was performed by the omission of the primary antibody. Nuclear expression in endothelial cells and lymphocytes provided positive internal controls for binding of the primary antibody.

The evaluation was carried out considering staining intensity (+, ++, +++), percentage of positive cells (< or >20% and >50%), and homogeneous or heterogeneous distribution. The score system is described in Table I. Only nuclear staining was considered for evaluation. Infiltrating lymphocytes, microglial cells and endothelial cells were not considered in the counts, being excluded on the basis of their morphology and by CD68 staining. On parallel sections, immunohistochemistry by anti-human CD68 mouse monoclonal antibody (M0814, 1:100 dilution; Dako) was performed after heat-induced antigen retrieval as described.

Western blotting analysis. Half of the sample drawn from paraffin

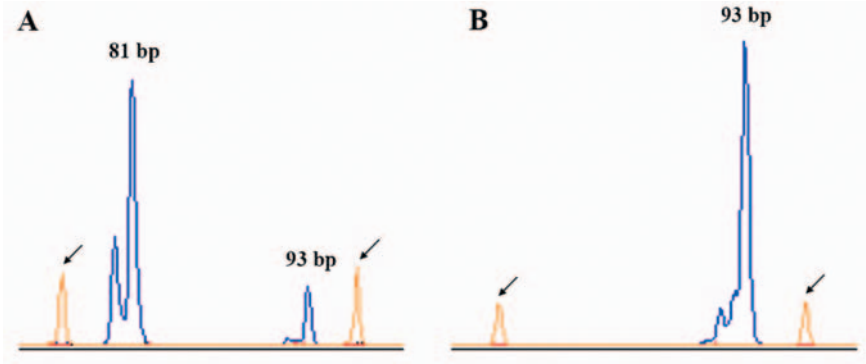

Figure 1. *Capillary electrophoresis based fragment analysis of the MGMT gene. A, Electropherogram showing a case with MGMT promoter hypermethylation; B, Electropherogram showing a case without MGMT promoter hypermethylation. The 81-base pair peak refers to the methylated allele and the 93-base pair to the unmethylated allele. Arrows indicate the GeneScan™ -500 LIZ™ Size Standard (Applied Biosystems).*

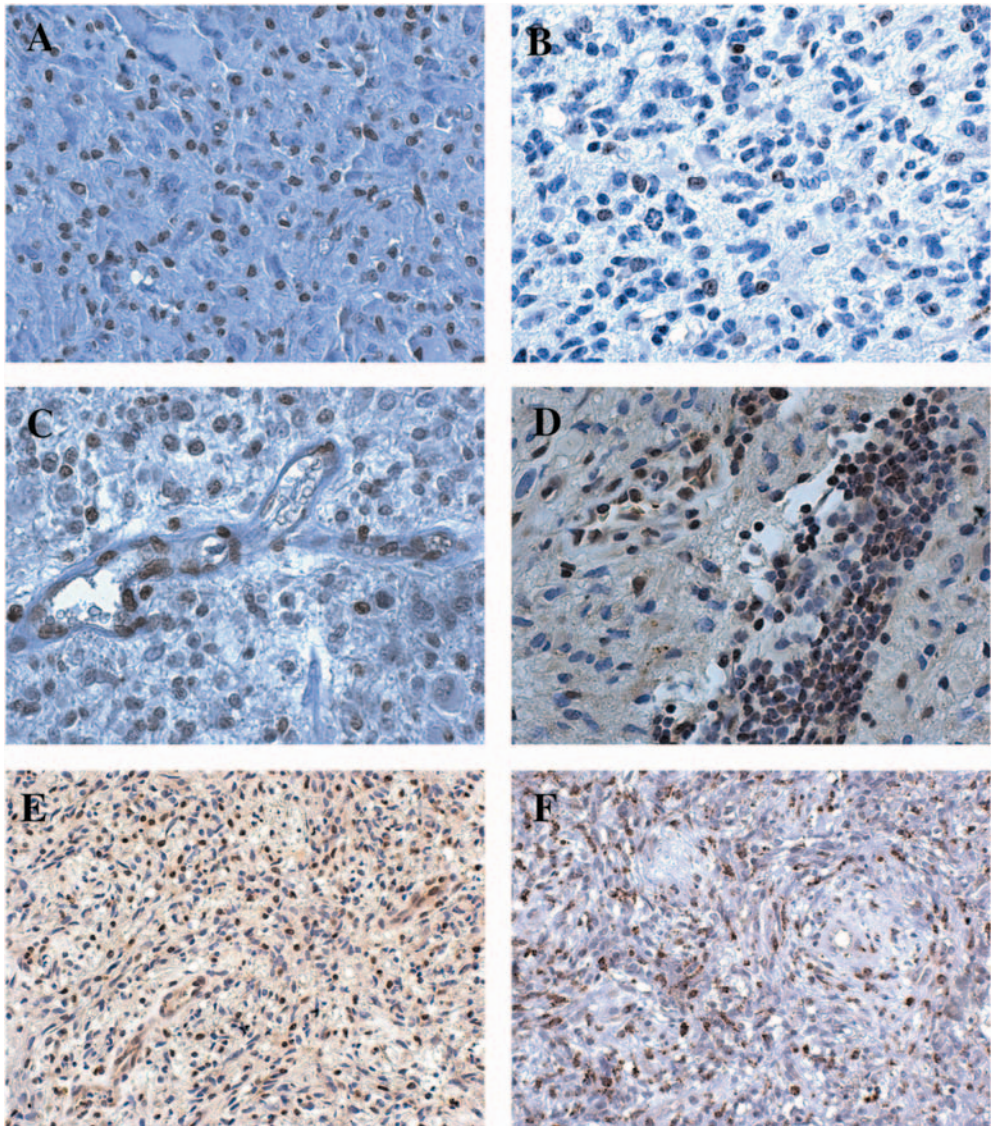

Figure 2. *MGMT Immunohistochemistry. A, High frequency of positive nuclei, ×200; B, low frequency of positive nuclei, ×200; C, positive endothelial cells, ×200; D, positive lymphocytes, ×200; E, area with a high frequency of MGMT-positive cells, ×100; F, the same area with CD68-positive cells, ×100, DAB.*

blocks in each case was deparaffinized and homogenized in a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal, 2% sodium dodecyl sulfate (SDS), 0.5% Na deoxycholate, 10 mM EDTA and a Protease Inhibitor Cocktail (Sigma Aldrich Co., St. Louis, MO, USA)) for protein extraction.

Protein concentration was measured by the BCA method (Pierce Biotechnology, Rockford, IL, USA), and 80 μg of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in 0.2% Tween 20-phosphate-buffered saline (T-PBS) 1X overnight at 4˚C. Blots were incubated for 2 h at room temperature with a mouse monoclonal MGMT antibody (# MS-470-P0, 1:500 dilution; Neomarkers, Fremont, CA, USA) and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) in blocking buffer for 1 hour at room temperature. Protein signal detection was performed using the ECL Detection System (GE Healthcare, Buckinghamshire, UK). A specific anti-α tubulin antibody (LF-PA0146; LabFrontier, Seoul, Korea) was used for normalizing sample loading and transfer. Band intensities were quantified by densitometry using NIH Image J (RSB, NIMH, Bethesda, MD, USA).

Statistical methods. Associations between categorical variables were evaluated using 2×2 contingency tables *via* the χ^2 test. When required due to the small number of cases, two-tailed Fisher's exact test was used. Correlation analysis was performed using Pearson's correlation coefficient. Survival curves were estimated by the Kaplan-Meier method, and the log-rank test (Mantel-Cox) was performed to compare two survival curves for different groups of individuals. A multivariate analysis with the Cox proportional-hazards regression model was performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL). The following variables were investigated: age (≤50 or >50 years), sex, *MGMT* methylation status, radiotherapy, and chemotherapy with TMZ.

Results

MGMT methylation status and clinical variables. MSP was performed on samples from 104 glioblastoma patients and the *MGMT* methylation status was successfully determined in 101 of these (97.1%). The *MGMT* gene was considered methylated when the ratio methylated/unmethylated peaks was >0.1. Methylation was found in 29 out of 101 tumors (28.7%). This value is lower than those of the literature. However, if also cases with a weak methylation peak (*i.e.* a ratio between 0.01 and 0.09, n=7) was considered, the percentage of *MGMT* methylation increased to 35.6% .

Calculating the occurrence of the methylated allele only, independently of its ratio to the unmethylated allele, the same percentage of 35.6% of methylation was found, even when a different intensity cut-off was considered. *MGMT* methylation was not associated with sex (χ^2 test, p=0.403), patient age (≤50 or >50 years, χ^2 test, p=0.990), or tumor location (χ^2 test, p=0.520).

MGMT immunohistochemistry. MGMT: Nuclear staining only was considered, and 96 cases were evaluated (Figure 2A, B). The distribution of positive cells according to the scoring system used is reported in Table II. The cases were subdivided into five categories according to criteria of homogeneity/ heterogeneity, percentage of positive cells and staining intensity (Table II). Positive staining for MGMT was also observed for endothelial cells, lymphocytes, microglia and macrophages (Figure 2C, D). Neurons did not show any immunopositivity. Heterogeneity occurred in 37.3% of unmethylated cases and 48.2% of methylated cases. No correlation was found between MGMT expression as assessed by immunohistochemistry and overall survival (OS). *CD68:* Abundant microglial cells and macrophages were present in all cases, especially in the tumor periphery or around necroses. The CD68 distribution was variable in different cases, and exhibited no correlation with MGMT. In some cases with a high methylation peak upon MSP and a high score of MGMT expression, however, a high percentage of CD68-positive cells was recorded (Figure 2E, F).

MGMT methylation status, protein expression and Western blotting analysis. A significant inverse correlation between MGMT methylation status and immunohistochemistry score was found (χ^2 test, p=0.0175) only for cases (n=29) with the score 5 (Table III). The correlation with scores ranging from 0-4 was not significant.

A correlation between MSP and Western blotting was not found, whereas a positive correlation between MGMT immunohistochemistry (percentage of positive cells) and Western blotting analysis was identified (Pearson's correlation coefficient r=0.268, p=0.0211) (Figure 3A, B). Additionally, Western blotting did not correlate with OS.

MGMT methylation status and survival. OS was evaluated in 80 patients, because 24 patients were excluded due to unavailable data or death within 1 month after surgery.

Radiotherapy evaluated in the whole series emerged as a prognostic factor (log-rank test, p=0.0001) (Figure 4A). The main bias was that almost all patients surviving less than 8 months were not irradiated.

A statistically non-significant difference in median OS was found between patients with methylated (13 months) and those with unmethylated (11 months) *MGMT* by MSP (long-rank test, p=0.303). Excluding censored (still alive) patients because of their asymmetrical distribution, due to the recent introduction of TMZ therapy, (1 methylated *versus* 12 unmethylated), a statistically significant difference (log-rank test, p=0.026) of 12 months *versus* 9 months was found. This finding suggested that the *MGMT* methylation status has a prognostic value (Figure 4B). The same effect was observed after stratification of patients for radiotherapy (log-rank test, p=0.44 *versus* p=0.065, respectively). Considering the methylated peak only, we found that the correlation with

Table I. *Scoring system for evaluation of MGMT immunostaining.*

Score	Category		
	Distribution	Intensity	Percentage of positive cells
0	No staining	–	–
1	Heterogeneous	+	<20%
2	Heterogeneous	++/+++	>20%
3	Homogeneous	+	<20%
4	Homogeneous	++/+++	>20%
5	Homogeneous	++/+++	>50%

Table II. *MGMT immunostaining according to score system.*

Score	Distribution (N=96)	Percentage of positive cells (%)
0	10	10.4
1	24	25.0
2	16	16.7
3	10	10.4
4	36	37.5
5	22	22.9

Cases with score 5 are included in those with the score 4.

Table III. *Correlation between methylation status of the MGMT promoter by MSP and protein expression by IHC.*

	Unmethylated (N=66)		Methylated (N=29)		
Score	No.	%	No.	%	*p*-Value
0	6	9.1	3	10.4	ns
1	16	24.2	8	27.6	ns
2	9	13.6	7	24.1	ns
3	6	9.1	4	13.8	ns
4	29	44.0	7	24.1	ns
5	19	28.8	2	6.9	0.0179

Cases with score 5 are included in those with the score 4; ns: not statistically significant.

OS increased with the peak's height; however, this increase was not statistically significant.

Therapy with TMZ was a prognostic factor for the whole group of 80 cases (log-rank test, p=0.022) but not for irradiated cases, or for those with *MGMT* methylation. It is likely that the stratification of treated cases provided too asymmetrical a distribution (21 unmethylated *versus* 59 methylated). Patients were treated by TMZ independently of the *MGMT* methylation status. Patients untreated by TMZ showed a trend towards a correlation of OS with methylation (log-rank test, p=0.058), taking into account that they have been mostly irradiated. Excluding from the evaluation all censored patients mostly under treatment by TMZ, *MGMT* methylation correlated significantly with OS (log-rank test, p=0.013) as it was for irradiated patients.

Multivariate analysis with a Cox proportional-hazards model, revealed that radiotherapy was the only independent prognostic factor (p=0.0001).

Discussion

The frequency of *MGMT* promoter hypermethylation observed in the 101 cases with a methylated/unmethylated peak height ratio >0.1 was 29% in this study. This percentage is lower than those observed in previous studies, which ranged between 30 and 45% (8, 10, 26, 27). Lowering the cut-off between 0.01 and 0.09, our percentage increased to 35.6%, roughly in line with previous percentages. The significance of *MGMT* methylation in relation to survival can explain why the percentage in long-term survivors is much higher (28, 29).

In some studies, the percentage of methylated tumors is higher in secondary than in primary glioblastoma (8, 30-32). The explanation for this prevalence may reside in the secondary glioblastomas arising from previous astrocytomas, and in the finding that the methylation percentage of the latter is remarkable, even though lower than in glioblastomas. This problem must be discussed in the light of the relationship found between *MGMT* methylation and *TP53* mutations (30, 31, 33). It could be inherent to tumor progression and its clonal evolution. Our studies being confined to the comparison of immunohistochemistry and MSP in regard to the prognostic meaning of *MGMT* methylation, no comparison has been made between MGMT methylation, *EGFR* amplification and *TP53* mutation. The method used to evaluate methylation *i.e.* electrophoretic analysis on agarose or polyacrylamide gels, or CE on a genetic analyzer after MSP, did not influence the methylation percentage, because the same values were obtained by the two methods. *MGMT* methylation has been also evaluated by an activity assay which demonstrated a very low percentage of methylated cases, even considering that the glioblastomas examined were entirely primary tumors (19). Very importantly, as previous studies have also shown, a proportion of unmethylated *MGMT* by MSP is always present, as in our cases (6, 27). Of course, contamination by normal non-tumor cells must be considered in this regard. This could be the source of error and must be avoided by using parallel histological section analysis to exclude the presence of normal nervous tissue or necrosis as much as possible (27). Our technique of drawing from paraffin blocks

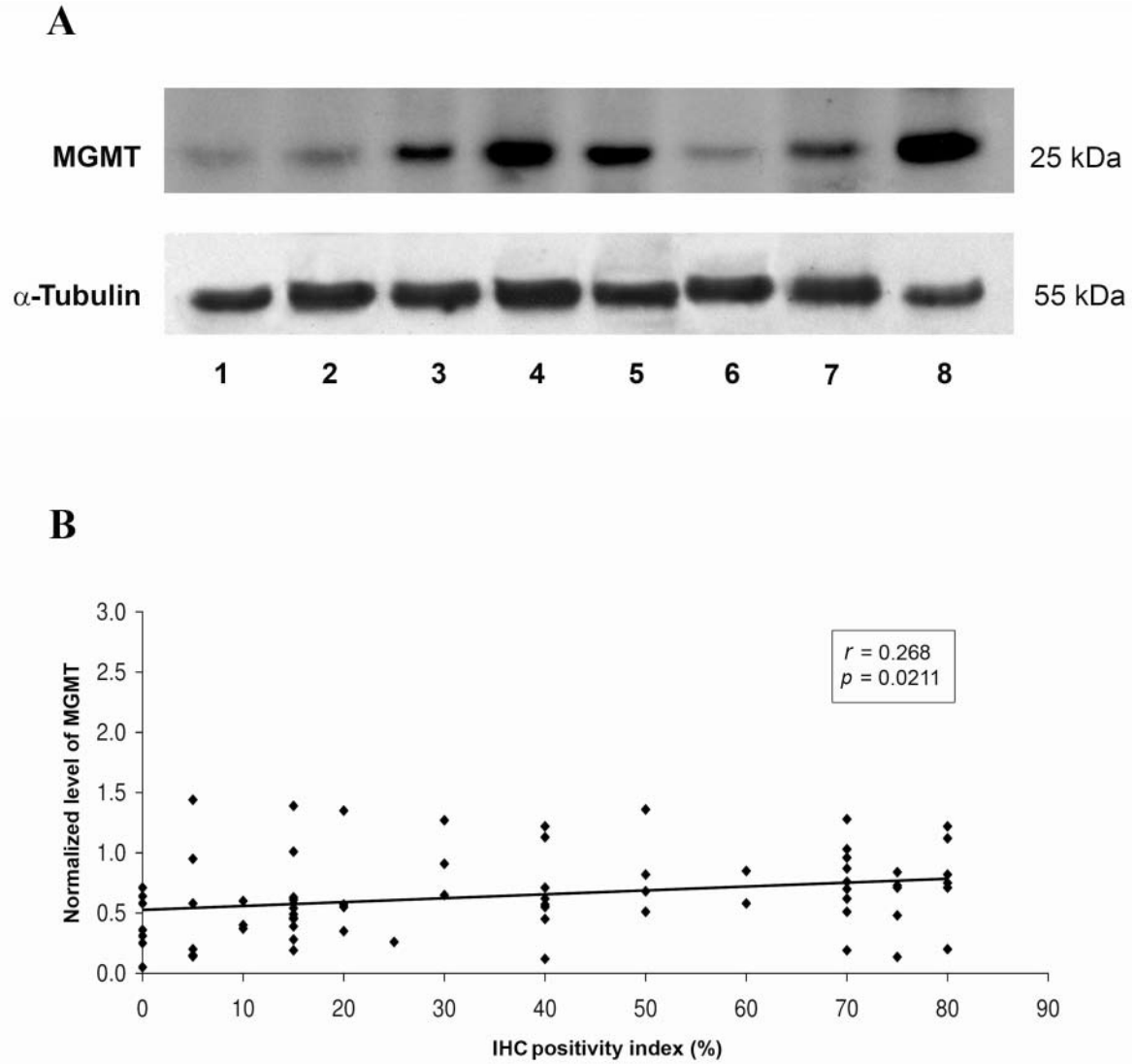

Figure 3. *A, Western blotting of MGMT: lanes 1-7 tumor samples with different levels of MGMT protein; lane 8 normal nervous tissue. B, Positive linear correlation of MGMT immunohistochemical index and Western blotting.*

areas histologically verified as containing only proliferating tumor cells prevents such contamination, but not that from microglial cells (see below). From the literature, it does not seem that the use of fresh *versus* deparaffinized tissue influences the results, even though a recent paper reports the opposite (34).

Radiotherapy appears in our cases to be a strongly significant prognostic factor. This finding could be overestimated, however, because radiotherapy for glioblastoma is systematically used when applicable. In fact, radiotherapy is regularly performed for glioblastoma unless the clinical conditions and the Karnofsky status of the patients are prohibitive, *i.e.* the lifespan of the patient is very short. For this reason, in our cases, the favourable effect of radiotherapy is overstated. On the contrary, *MGMT* methylation, regardless of its association with radiotherapy, in our cases showed only a trend toward a correlation with OS. It was clearly prognostic only when uncensored patients were included in the evaluation, in line with previous observations (8, 26), especially in relation to progression-free survival (PFS) (26).

Incomplete conclusions can be drawn regarding TMZ. In the whole series, TMZ was a prognostic factor (p=0.022). However, it was not statistically significant in relation to methylation status of the *MGMT* gene, even evaluated in association with radiotherapy. In other series (26), TMZ was identified as being prognostic in association with methylation and radiotherapy, both for OS and more significantly for PFS. The lack of correlation in our case may be a result of

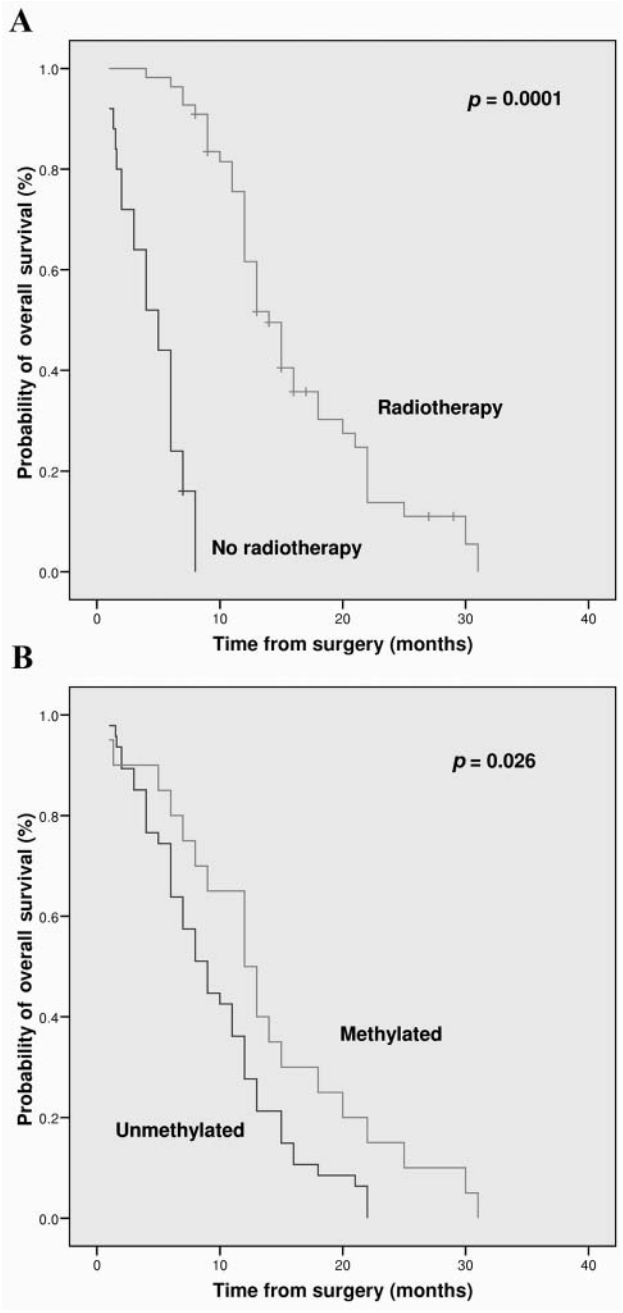

Figure 4. *Kaplan-Meier analysis of overall survival according to A, radiotherapy (all patients included) and B, the MGMT promoter methylation status (in all patients excluding the censored ones).*

number of patients, since if censored patients are not considered, very few cases treated by TMZ remain and this could be misleading in the statistical evaluation.

Based on immunohistochemical findings, MGMT expression was grouped into five categories according to staining intensity, percentage of positive cells, and homogeneity/heterogeneity. Only the fifth category inversely correlated with methylation by MSP. The second and the fourth scores together, which included cases with >20% of positive cells and high staining intensity, represent 54.2% of positive cases; this finding is consistent with the frequency observed in a previous study (17). Heterogeneity of protein expression occurs in 40% of cases, with no difference between methylated and unmethylated cases. The importance of heterogeneity was already stressed (17, 35) and a recent study emphasized that 82% of unmethylated and 27% of methylated cases express the protein (27). On the other hand, immunohistochemistry in our series did not correlate with MSP or OS. The use of computer-assisted fluorescence methods with evaluation do not ameliorate the quantitative protein expression evaluation.

Non-tumor-positive cells can be a source of contamination. This is documented by our observations with CD68, which accounts for proportion of MGMT-positive cells which could be misleading. On the other hand, other events can lead to the same results, for example, gene deletions or mutations which can be alternative mechanisms of gene silencing. MSP establishes whether methylation occurs, but it provides no information regarding heterogeneity, even though, after studying multiple samples from the same tumor by stereotactic procedures, heterogeneity was not found (32). That contamination can occur in both homogeneous and heterogeneous tumors is of paramount importance, to which up-regulation of protein expression by radio- and chemotherapy or steroids must be added (26, 36). Contamination affects all the three systems: MSP, immunohistochemistry and Western blotting.

The important question of whether immunohistochemistry, with its ease of performance and lower cost, can substitute for MSP in the assessment of *MGMT* methylation, seems to receive a negative answer. Our results may be more useful if directed toward identifying unmethylated cases than methylated ones, because it is difficult to establish a cut-off among the low scores. In our series, MGMT expression was shown to be poorly or moderately correlated with *MGMT* promoter methylation status. An inverse correlation between MGMT protein expression and MSP occurred only for the highest category of protein expression (*i.e.* when tissue is homogeneously stained and immunoreactivity is present in >50% of cells). The lack of any significant association between MGMT expression and patient outcome hampers the use of anti-MGMT immunohistochemistry as a clinical biomarker for routine diagnostic purposes (24).

MGMT promoter methylation is involved in the inactivation of the *MGMT* gene in numerous tumors and cancer cell lines, but the regulation of MGMT expression is a complex event in which many factors other than epigenetic silencing are implicated (17). It is likely that further control for MGMT expression at the transcriptional, post-transcriptional and translational levels occurs (8, 24).

Acknowledgements

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