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# Prognostic significance of *MGMT* methylation and expression of MGMT, P53, EGFR, MDM2 and PTEN in glioblastoma multiforme

## *Signification pronostique de la méthylation de MGMT et l'expression de protéines MGMT, P53, EGFR, MDM2 et PTEN dans le glioblastome multiforme*

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**Abstract.** The study investigated the pattern of *MGMT* promoter methylation and the expression of MGMT, P53, EGFR, MDM2 and PTEN proteins in glioblastomas multiforme (GBM) and evaluated their prognostic significance. We carried out a retrospective study of 80 GBM. Expression of MGMT as well as of P53, EGFR, MDM2 and PTEN was investigated by immunohistochemistry. *MGMT* promoter methylation was investigated by methylation specific-PCR of bisulfite-treated DNA. Twenty-five GBM exhibited MGMT expression. Methylation of *MGMT* promoter was detected in 35.1% of cases. No significant concordance was reported between *MGMT* promoter methylation and protein expression (κ=-0.047, $p$=0.11). *MGMT* promoter methylation was significantly associated only with PTEN expression ($p$=0.001): no other significant association was identified with clinical parameters as well as with expression of P53, EGFR and MDM2 ($p$ >0.05). Tumor recurrence was significantly associated with unmethylated *MGMT* promoter ($p$=0.01) but not with MGMT expression ($p$=0.51). Recurrence-free survival (RFS) was significantly better among patients with methylated *MGMT* promoter (log rank, $p$ <0.0001) and PTEN expression (log rank, $p$=0.025) but not with MGMT expression (log rank, $p$=0.308). As well, using univariate analysis, *MGMT* promoter methylation ($p$=0.001) and PTEN expression ($p$=0.044) were significantly associated with RFS. In multivariate analysis, only *MGMT* promoter methylation was significantly associated with RFS ($p$=0.003). Together, our findings support that MGMT protein expression doesn't reflect the *MGMT* promoter methylation status. Furthermore, *MGMT* promoter methylation remains a useful prognostic marker in Tunisian patients with GBM. PTEN expression could be a potential prognostic marker of this tumor.

***Key words:*** *glioblastomas multiforme, MGMT promoter methylation, PTEN expression, prognosis, immunohistochemistry*

**Résumé.** L'étude a examiné le pattern méthylation du promoteur *MGMT* et l'expression des protéines MGMT, P53, EGFR, MDM2 et PTEN dans les glioblastomes multiformes (GBM) et a évalué leur signification pronostique. Nous avons réalisé une étude rétrospective de 80 GBM. L'expression de MGMT ainsi que de P53, EGFR, MDM2 et PTEN a été étudiée par immunohistochimie. La méthylation du promoteur *MGMT* a été étudiée par PCR spécifique de méthylation de l'ADN modifié au bisulfite. Vingt-cinq GBM ont montré une expression de MGMT. La méthylation du promoteur *MGMT* a été détectée dans 35,1 % des cas. Aucune concordance significative n'a été identifiée entre

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la méthylation du promoteur *MGMT* et l'expression de MGMT (κ = -0,047, $p$ = 0,11). La méthylation du promoteur *MGMT* était associée significativement à l'expression de PTEN ($p$ = 0,001) : aucune autre association significative n'a été identifiée avec tous les paramètres cliniques ni avec l'expression de P53, EGFR et MDM2 ($p > 0,05$). La récidive tumorale était significativement associée au promoteur *MGMT* non méthylé ($p$ = 0,01) mais pas à l'expression de MGMT ($p$ = 0,51). La survie sans récidive était significativement meilleure chez les patients présentant un promoteur *MGMT* méthylé (log rank, $p < 0,0001$) et une expression de PTEN (log rank, $p$ = 0,025) mais pas avec une expression de MGMT (log rank, $p$ = 0,308). De plus, à l'aide d'une analyse univariée, la méthylation du promoteur *MGMT* ($p$ = 0,001) et l'expression de PTEN ($p$ = 0,044) étaient associées de manière significative à la RFS. En analyse multivariée, seule la méthylation du promoteur *MGMT* était associée de manière significative à la RFS ($p$ = 0,003). Ensemble, nos résultats confirment que l'expression de MGMT ne reflète pas l'état de méthylation du promoteur *MGMT*. En outre, la méthylation du *MGMT* reste un marqueur pronostique utile des GBM chez les patients tunisiens. L'expression de PTEN pourrait être un marqueur pronostique potentiel de cette tumeur.

***Mots clés :*** glioblastomes multiformes, méthylation du promoteur MGMT, expression de PTEN, pronostic, immunohistochimie

Glioblastomas multiforme (GBM), or high-grade glioblastomas, are high-grade glial tumors and represent the most common glioma subtype in adults, accounting for 50% of all gliomas and 20% of all central nervous system (CNS) tumors [1]. GBM were associated with an extremely worse prognosis. The median overall survival (OS) is only 14.5 months in clinical trials and the 3-year survival reaches rarely 5% [2]. Current management of GBM includes surgical resection, radiation and alkylating agent-based therapy such as temozolomide, carmustine and procarbazine [3].

The *O6-methylguanine-DNA methyltransferase (MGMT)* gene (OMIM156569), located in the 10q26 locus, encodes a DNA repair enzyme that removes alkyl groups from the O-6 position of guanine DNA nucleotide to a cysteine residue and repairs promutagenic DNA damage [4]. *MGMT* gene is silenced in various cancers, including colorectal, gastric, lung and oral cavity cancers [5, 6]. In GBM, the *MGMT* counteracts with chemotherapy efficiency, mainly with alkylating agents such as temozolomide [7, 8]. Temozolomide induces the binding of an alkyl group to the O6-position of guanine, causing DNA mismatching and DNA-double-strand breakage, leading to apoptosis in proliferating cells [7-9]. However, by removing the alkyl group from O6-guanine, MGMT activity inhibits the apoptosis and counteracts the cytotoxic effects of temozolomide-based therapy [7-9]. Several reports described a significant association between *MGMT* promoter methylation and a positive clinical outcome for GBM patients treated with alkylating agents with or without radiotherapy [10-12]. Despite the extensive characterization of the molecular bases of the GBM aggressiveness, the pattern of *MGMT* promoter methylation remains the only prognostic biomarker of affected patients as well as a useful molecular predictive factor of chemosensitivity in clinical practice [10-15]. Nevertheless, over the last decades, other potential molecular predictive factors have been largely investigated, including P53, epidermal growth factor receptor (EGFR), murine double minute (MDM2) protein and phosphatase and tensin homolog (PTEN). However, their prognostic role in GBM is still debated [16-18].

In the current study, we investigated the methylation status of the *MGMT* promoter and the expression of MGMT protein as well as P53, EGFR, MDM2 and PTEN proteins. Then, we evaluated their prognostic significance in GBM in Tunisian patients.

## Material and methods

### *Tissue samples*

We carried out a retrospective study of 80 GBM diagnosed in the Pathology Department, Farhet Hached University Hospital, Sousse (Tunisia) and registered in the Cancer registry of central Tunisia during 2009-2015. This study was approved by the local Human ethics committee at the Farhet Hached University Hospital of Sousse (Tunisia) and it is conform to the provisions of the declaration of Helsinki.

**Table 1.** Immunohistochemistry conditions and evaluation.

| Expression | Clone | Provenance | Dilution | Retrieval solution | Positive immunostaining |
|---|---|---|---|---|---|
| P53 | DO-7 | NovoCastra | 1:800 | Citrate 0.01M, pH 6.0 | Nuclear staining |
| EGFR | DAK-H1-WT | DAKO | 1:100 | Citrate 0.01M, pH 9.0 | Membranous staining |
| PTEN | 6H2.1 | DAKO | 1:100 | Citrate 0.01M, pH 9.0 | Cytoplasmic staining |
| MDM2 | 1B10 | NovoCastra | 1:50 | Citrate 0.01M, pH 6.0 | Nuclear staining |
| MGMT | MT23.2 | Zymed laboratories | 1:100 | Citrate 0.01M, pH 6.0 | Nuclear staining |

Patient's clinicopathological data, including age at diagnosis, gender, tumor localization, type of surgery and the follow up were collected. Patient overall survival (OS) and tumor recurrence were recorded. Histological diagnosis review of all cases was performed by two pathologists on hematoxylin and eosin (HE) stained sections (MM and MTY). All tissues had been routinely fixed in 10% buffered formalin and paraffin embedded.

### *Immunohistochemistry*

Immunohistochemistry analysis of P53, EGFR, MDM2, PTEN and MGMT proteins expression was conducted using Envision kit (DakoCytomation, Denmark) as previously described (*table 1*) [19]. Appropriate positive controls were performed according manufacture's instruction. Negative controls were obtained by substitution of the primary antibody by phosphate buffered saline. Nuclear MGMT immunolabeling detected within the endothelial cells served as an internal positive control. Immunostaining findings were evaluated semi-quantitatively by estimating the fraction of positive cells as described elsewhere [20].

### *DNA extraction*

DNA extractions from paraffin-embedded tissues were performed from 5-µm thick tissue sections using QIAamp DNA FFPE Tissue kit according to the manufacturer's protocol (QIAGEN, France). The integrity of extracted DNA was tested by amplifying a fragment of a 268-bp fragment of the human β-*globin* gene using a set of primers as described elsewhere [21].

### *DNA bisulfite treatment*

The bisulfite treatment method is used to determine the promoter methylation pattern. Sodium bisulfite deaminates selectively unmethylated cytosines (but not 5-methylcytosines) to uracils. The bisulfite reaction was performed as described previously [21]. The presence of bisulfite-treated DNA in each sample was determined by amplification of a 133-bp DNA fragment of the β-*actin* gene, using a specific primer set for the amplification of bisulfite-modified DNA (but not wild-type DNA), irrespective of the methylation status of the sample.

### *Methylation-specific PCR*

The *MGMT* promoter methylation pattern was carried out using methylation-specific PCR (MS-PCR) as described previously [21]. The bisulfite-modified DNA was used as a template for methylation specific amplification with primers specific for the methylated (F: 5′-TTTCGACGTTCGTAGGTTTTCGC-3'; R: 5′-GCACTCTTCCGAAAACG AAACG-3′) and unmethylated (F: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′; R: 5′-AACTCCACACTCTTCCAAAAACAAAACA-3') *MGMT* promoter sequences [22]. The annealing temperature was 59°C [22]. In our study, MS-PCR is designed to detect the methylation of CpGs located in exon 1 of the *MGMT* gene. This part of the gene has 97 CpGs sites, of which four sites showed strong association with gene expression [23].

The PCR amplifications were carried out in a final volume of 25 µL containing 3 µL bisulfite-modified DNA template, 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 2.5 mM MgCl2, 0.25 mM of each dNTP, 0.2 µM of each primer, and 1 U Taq DNA polymerase (Promega). The amplification was performed in a PTC 200$^{TM}$ DNA engine thermal cycler. The cycling conditions were as follows: denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72°C. The reactions were finished by a 10 min extension at 72°C. The PCR products were run on 2% agarose gels containing ethidium bromide and visualized under ultraviolet illumination using a GelDoc2000 System (Bio-Rad, Marnes-la-Coquette, France). A sample was considered as methylation-negative when the resulting PCR product was obtained only with the U set and was regarded as methylation-positive when the resulting PCR product was obtained with the M or with both the U and the M set. In each experiment CpG universal methylated DNA (Qbiogene, Carlsbad, CA, USA) was used as a positive control for methylated alleles and DNA from normal lymphocytes was used as a negative control for unmethylated alleles. In

addition, negative controls without DNA were included in each experiment. All experiments were performed at least twice [21].

### *Statistical analysis*

Statistical analysis was performed using SPSS software, version 22.0. The concordance between the *MGMT* promoter methylation and protein expression were calculated using Cohen's kappa. The relationships between *MGMT* promoter methylation and proteins expression and GBM features were evaluated by Chi-square test. Recurrence-free survival curves were generated using the Kaplan-Meier estimates, with the Log-rank test being applied for the comparison of survival curves. Furthermore, hazard ratios and 95% confidence intervals (CI) computed from univariate and multivariable Cox regression models were used to investigate the relationship between studied features and survival. The probability value ($p$) $< 0.05$ was considered as statistically significant.

## Results

Patient age ranged from 5 to 75 years with a mean age of 54 years. There were 44 male and 36 female. The tumors were diagnosed in the frontal lobe (21.2%), temporal lobe (20%), parietal lobe (15%) occipital lobe (1.2%) and mixed localizations (18.5%). In the remaining GBM cases (23.7%), the tumor localization was unspecified (*figure 1*).

MGMT protein expression was detected in 26 tumors (32.5%). Twenty-five tumors showed strong nuclear expression (score 3) and only one case with low expression (score 1). MGMT immunostaining was considered positive only in tumors of score 3. Hence, 31.2% of GBM cases exhibited MGMT protein expression (*figure 2A*).

Expression of P53, EGFR, PTEN and MDM2 proteins was described in 43.2%, 79.7%, 73% and 32.4% of GBM cases, respectively (*figure 2B-E*). The remaining cases were considered negative (*table 2, figure 2F-H*).

Among the 80 cases included in our study, 74 cases (92.5%) showed a gene amplification of β-globin, justifying the presence of DNA of good quality. Methylation of the *MGMT* promoter was detected in 26 GBM (35.1%); in the 48 remaining cases, *MGMT* promoter was unmethylated (64.9%) (*figure 3*).

The MGMT protein expression was detected in 17 cases with unmethylated *MGMT* promoter and 8 cases with methylated *MGMT* promoter. Meanwhile, the loss of MGMT protein expression was observed in 18 cases with methylated *MGMT* promoter as well as in 31 GBM cases

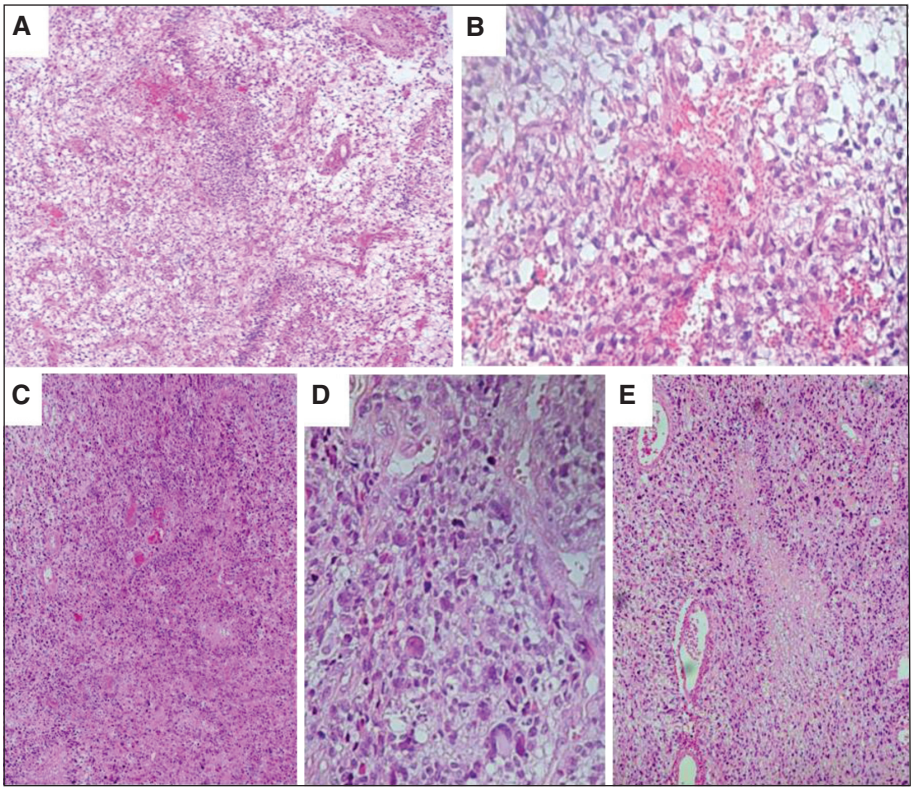


**Figure 1.** Histopathological features of GBM. (**A-C**) Malignant proliferation of glial cells: pleomorphic and atypical cells (A-C, Mx200; B, Mx400), stromal proliferation of endothelial-capillary (**D**, Mx400), tumor necrosis surrounded by tumoral cells (**E**, Mx200).

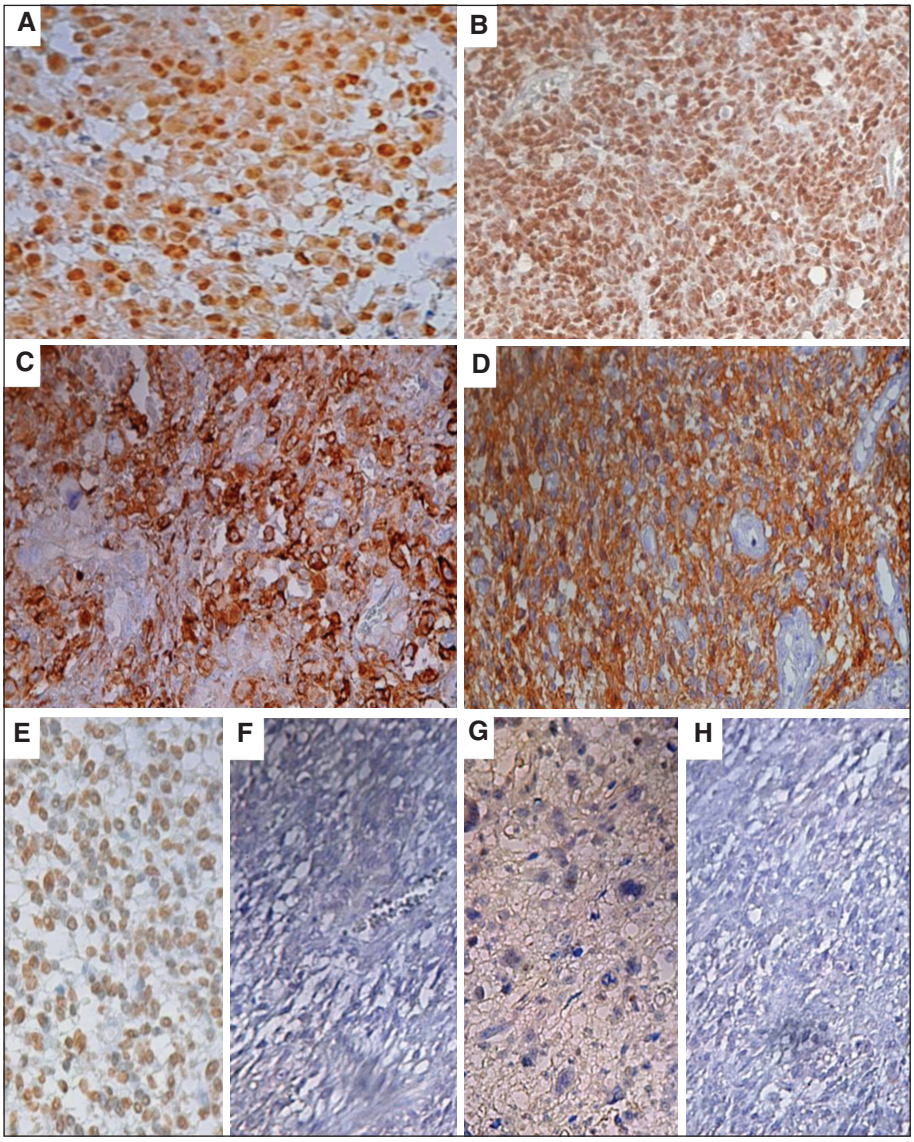


**Figure 2.** Immunohistochemical results of GBM (Mx400). Nuclear MGMT expression (**A**). Nuclear P53 expression (**B**). Membranous expression of EGFR (**C**). Cytoplasmic expression of PTEN (**D**). Nuclear expression of MDM2 (**E**). No specific expression of PTEN (**F**), P53 (**G**) and EGFR (**H**).

with unmethylated *MGMT* promoter (*table 2*). No significant concordance between *MGMT* promoter methylation and MGMT protein expression was reported (κ=-0.047, $p$=0.11).

Overall, there was no significant correlation between *MGMT* promoter methylation and the clinicopathological parameters of GBM cases, including patient age, gender, tumor localization and surgery type ($p$ >0.05 for all, *table 2*). However, a significant correlation was found between *MGMT* promoter methylation and PTEN expression ($p$=0.001). No relationship was found between the *MGMT* methylation pattern and the immunoexpression of P53, EGFR and MDM2 proteins ($p$ >0.05 for all, *table 2*).

Tumor recurrence occurred in 18 GBM patients. The majority of tumor recurrence was reported in patients exhibiting unmethylated *MGMT* promoter (88.9%). However, tumor recurrences occurred in only two patients with methylated *MGMT* promoter ($p$=0.01, *table 2*). In addition, only seven recurrent GBM cases expressed MGMT protein. Overall, no significant association was identified between tumor recurrence and expression of MGMT ($p$=0.51), P53 ($p$=0.61), EGFR ($p$=0.26), PTEN ($p$=0.07) and MDM2 ($p$=0.22).

The OS of GBM patients ranged between two to 61 months with a median of 12.5 months. Among patients with methylated *MGMT* promoter, the median OS was 22

**Table 2.** *MGMT* promoter methylation status and features of GBM.

| | | ***MGMT* promoter methylation status** | | |
|---|---|---|---|---|
| **Features** | **Total (%)** | **Unmethylated (n=48)** | **Methylated (n=26)** | ***p*-values** |
| **Age (years)** | | | | |
| <50 | 25 (33.8%) | 16 (64%) | 9 (36%) | *p*=0.54 |
| ≥50 | 49 (66.2%) | 32 (65.3%) | 17 (34.7%) | |
| **Gender** | | | | |
| Male | 40 (54%) | 26 (65%) | 14 (35%) | *p*=0.58 |
| Female | 34 (46%) | 22 (64.7%) | 12 (35.3%) | |
| **Tumor localization** | | | | |
| Temporal | 14 (18.1%) | 8 (57.1%) | 6 (42.8%) | |
| Parietal | 10 (13.5%) | 6 (60%) | 4 (40%) | |
| Frontal | 16 (21.6%) | 12 (75%) | 4 (25%) | *p*=0.57 |
| Occipital | 1 (1.3%) | 1 (100%) | 0 | |
| Mixed | 15 (20.3%) | 12 (80%) | 3 (20%) | |
| Unspecified | 18 (24.3%) | 9 (50%) | 9 (50%) | |
| **P53 expression** | | | | |
| Positive | 32 (43.2%) | 21 (65.6%) | 11 (34.4%) | *p*=0.60 |
| Negative | 42 (56.8%) | 27 (64.3%) | 15 (35.7%) | |
| **EGFR expression** | | | | |
| Positive | 59 (79.7%) | 36 (61%) | 23 (39%) | *p*=0.14 |
| Negative | 15 (20.3%) | 12 (80%) | 3 (20%) | |
| **MDM2 expression** | | | | |
| Positive | 24 (32.4%) | 16 (66.7%) | 8 (33.3%) | *p*=0.51 |
| Negative | 50 (67.6%) | 32 (64%) | 18 (36%) | |
| **PTEN expression** | | | | |
| Positive | 54 (73%) | 42 (77.8%) | 12 (22.2%) | *p*=0.001* |
| Negative | 20 (27%) | 6 (30%) | 14 (70%) | |
| **MGMT expression** | | | | |
| Positive | 25 (33.8%) | 17 (68%) | 8 (32%) | *p*=0.11 |
| Negative | 49 (63.2%) | 31 (63.3%) | 18 (36.7%) | |
| **Type of surgery** | | | | |
| Gross total resection | 55 (74.3%) | 38 (69.1%) | 17 (30.9%) | *p*=0.26 |
| Partial resection | 19 (25.3%) | 10 (52.6%) | 9 (47.4%) | |
| **Tumor recurrence** | | | | |
| Presence | 18 (24.3%) | 16 (88.9%) | 2 (11.1%) | *p*=0.01* |
| Absence | 56 (75.7%) | 32 (57.1%) | 24 (42.9%) | |
| **Median overall survival** | 12.5 months | 9 months | 22 months | *p* <0.0001* |

* Significant *p*-values.

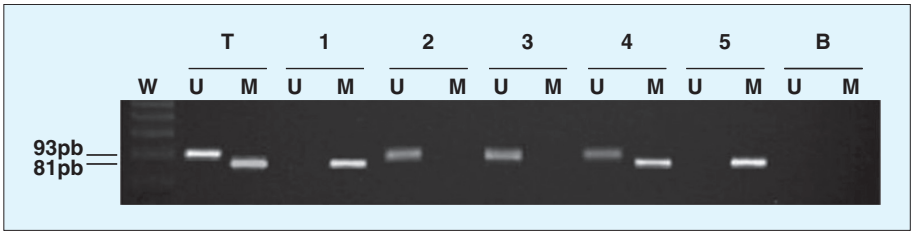


**Figure 3.** *MGMT* promoter methylation pattern in GBM. Representative experiments of methylation specific PCR of bisulfite-modified DNA. U: PCR products obtained using primers designed to amplify the unmethylated sequences (81 bp). M: PCR products obtained using primers designed to amplify the methylated sequences (93 bp). 1-5: GBM samples. W: Molecular weight marker at 50 bp (Promega). T: Positive control. B: Negative control.

months. However, it was only nine months within patients with unmethylated *MGMT* promoter ($p$ <0.0001). Using the Kaplan-Meier method, RFS was significantly associated with *MGMT* promoter methylation ($p$ <0.0001, *figure 4A*) and PTEN expression ($p$=0.025, *figure 4B*). The correspondent medians for survival time were 17.00 (95%CI, 1.265-32.735) and 39.00 (95%CI, 20.471-57.529), respectively. Nevertheless, no significant relationship was

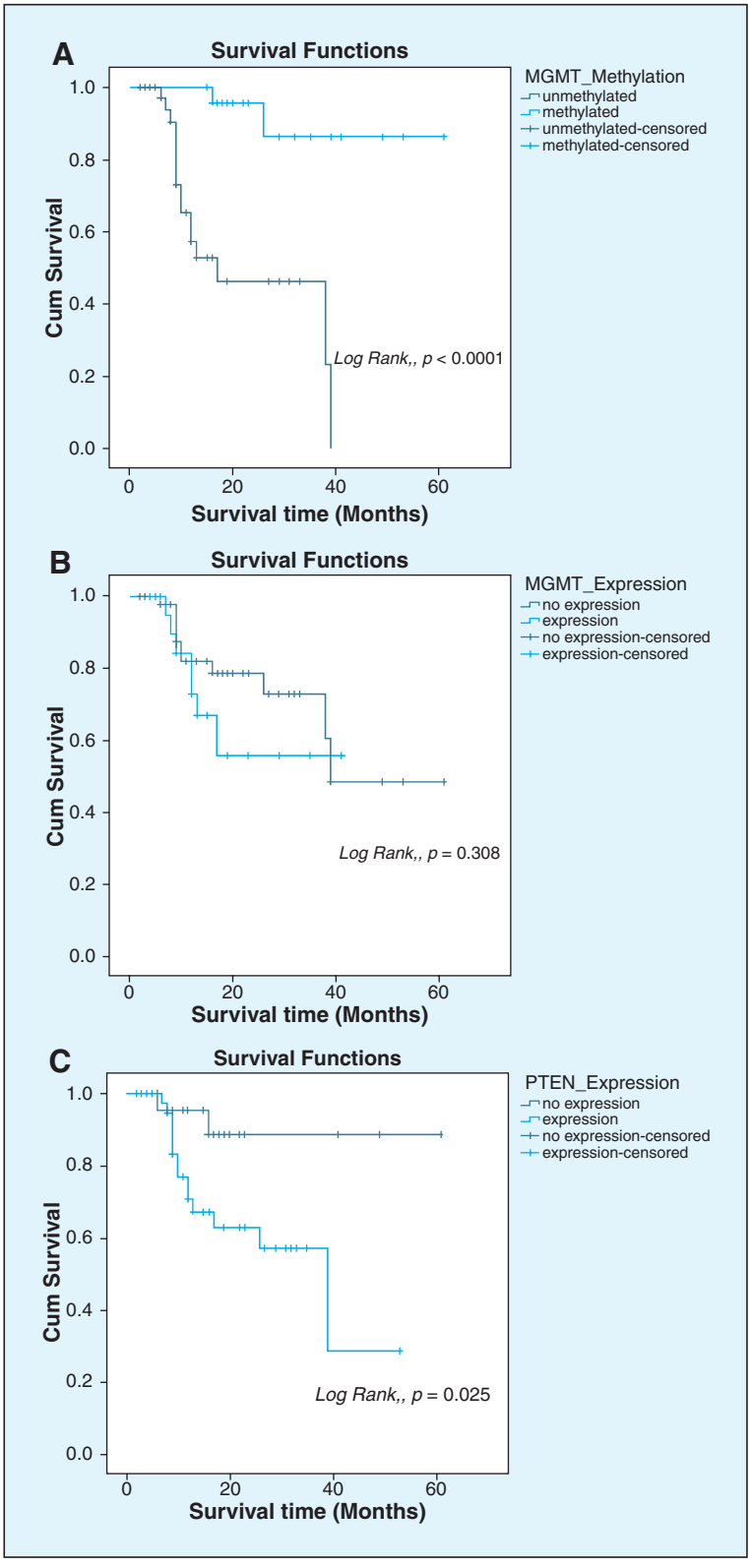


**Figure 4.** Kaplan-Meier survival curves for patients with GBM according to the *MGMT* promoter methylation pattern (**A**), MGMT protein expression (**B**) and PTEN expression (**C**). Using the Kaplan-Meier, *MGMT* promoter methylation and PTEN expression were significantly associated with RFS ($p < 0.0001$ and $p=0.025$, respectively; Log Rank test); whereas, no significant relationship was indentified with MGMT expression ($p=0.308$, log rank test).

**Table 3.** Univariate and multivariable analysis of recurrence-free survival for patients with GBM.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | *p*-values | Hazard ratio | 95% Confidence interval | *p*-values | Hazard ratio | 95% Confidence interval |
| Age | 0.917 | 0.950 | (0.366-2.440) | 0.287 | 2.965 | (0.401-21.902) |
| Gender | 0.432 | 1.451 | (0.574-3.666) | 0.220 | 2.699 | (0.553-13.177) |
| Surgery type | 0.522 | 0.666 | (0.192-2.308) | 0.322 | 2.911 | (0.351-24.124) |
| P53 expression | 0.627 | 0.768 | (0.266-2.233) | 0.983 | 0.983 | (0.200-4.837) |
| MDM2 expression | 0.243 | 0.515 | (0.169-1.568) | 0.194 | 0.427 | (0.118-1.541) |
| PTEN expression | 0.044* | 4.645 | (1.046-20.632) | 0.719 | 1.336 | (0.277-6.446) |
| EGFR expression | 0.359 | 0.614 | (0.217-1.739) | 0.834 | 0.792 | (0.090-6.955) |
| MGMT expression | 0.320 | 1.627 | (0.624-4.245) | 0.188 | 2.040 | (0.706-5.897) |
| MGMT promoter methylation | 0.001* | 0.078 | (0.018-0.348) | 0.003* | 0.096 | (0.020-0.460) |

*Significant *p*-values.

found between RFS and expression of MGMT ($p$=0.308, *figure 4C*), P53 ($p$=0.621), MDM2 ($p$=0.228) and EGFR ($p$=0.816) as well as with all clinicopathological features studied, including age ($p$=0.916), gender ($p$=0.423), tumor localization ($p$=0.778) and surgery type ($p$=0.513).

*Table 3* indicated the univariate and multivariable Cox regression analysis of RFS for patients with GBM. The univariate analysis showed that the significant factors associated with RFS included *MGMT* promoter methylation ($p$=0.001) and PTEN expression ($p$=0.044). In multivariate analysis, only *MGMT* promoter methylation was significantly associated with RFS ($p$=0.003). No other significant association was identified between the remaining features and RFS of GBM patients (*table 3*).

## Discussion

In Tunisia, gliomas are the most common primary tumors of the CNS and GBM are the most aggressive and frequent glioma subtype, accounting around 60% of all gliomas [24]. Until nowadays, most GBM remains an incurable cancer and major available treatments remain only palliative [3, 9, 25]. The understanding of the mechanisms of chemoresistance improves the strategies of management of these aggressive tumors [13]. The most alkylating agents used for GBM chemotherapy is temozolomide which methyls the O6 of guanine, provoking tumor cell apoptosis [3, 13]. MGMT activity repairs these cytotoxic effects by removing the O6-alkyl group from guanine, leading to tumor chemoresistance [7]. Several studies investigated the expression of the *MGMT* gene as well as its promoter methylation status as a potential tumor biomarker in GBM [9, 12, 14, 15].

In the present study, among 80 cases of GBM, a strong nuclear MGMT expression was identified in 31.2% of cases and only one case exhibited mild expression. Using different MGMT antibody clone, Trabelsi *et al.* [26] reported MGMT expression in 35% of cases similar to that reported previously by Brell *et al.* study [27]. Among Brazilian patients, Uno *et al.* [28] found that 38 GBM exhibited positive MGMT staining (74.5%) and only five cases expressed moderately MGMT protein. Using RT-PCR, Everhard *et al.* [29] described a strong MGMT expression in 30% of GBM and the remaining cases showed only a mild expression. The difference of results in the rate of MGMT-positive cases reported by these studies could be explained by the method used and technical discrepancies such as the choice of antibody, clone, immunostaining protocol and scoring [9, 20].

During the last decade, the methylation status of the *MGMT* promoter in GBM has been well-investigated [7, 12, 14, 26, 30]. Herein, the pattern of *MGMT* promoter methylation in Tunisian GBM patients was carried out by MS-PCR, since this method is the most frequent technique used for the diagnosis of promoter methylation for histopathological specimen [8]. Overall, 35.1% of GBM were methylated for *MGMT* promoter. Using different techniques, previous reports found *MGMT* promoter methylation in 4% to 88% of GBM cases [22, 28, 31]. Esteller *et al.* [22] detected 41% of GBM with methylated *MGMT* promoter. Among 110 GBM, Lechapt-Zalcman *et al.* [32] reported promoter methylation in 57.3% of samples. Using MS-PCR and pyrosequencing techniques, methylation of *MGMT* promoter was detected in 43.1% and 38.8%, respectively [28]. Using pyrosequencing on paraffin-embedded fine needle aspiration biopsies, Xie *et al.* [31] identified *MGMT* promoter methylation in 37% of GBM. More recent studies proposed specific cut-off levels of methylation [12, 14]. Gurrieri *et al.* [12] proposed an average level of methylation between all investigated CpGs of 9% to discriminate between methylated and unmethylated tumors. Nevertheless, compared to pyrosequencing and immunohistochemistry, Wang *et al.* [33] demonstrated that quantitative MSP is an effective and rapid detection method for routine use in pathol-

ogy laboratories for the identification of *MGMT* promoter methylation.

Previously, a significant correlation was reported between the *MGMT* promoter methylation status and its protein expression [22, 29]. In fact, *MGMT* promoter methylation has been found to be associated with the loss of MGMT protein expression, whereas, unmethylated promoter correlated with protein expression [22, 29]. Nevertheless, herein, no significant correlation was observed between MGMT protein expression and its promoter methylation pattern as previously reported in GBM [27, 28, 34] and in other tumors such as low-grade glioma, colorectal cancer and lymphoma [27]. This discordance could be explained by the tumor heterogeneity of GBM that hardens the discrimination between nuclear immunolabeling of tumor cells and non-tumor nuclear staining. In addition to immunostaining problems cited above, immunohistochemistry and MS-PCR techniques could be done in different tumor area of GBM sample. Furthermore, since the methylation mechanism is not bi-allelic in some tumors, one allele can express the active protein while the promoter is methylated.

Based on this discordance, our results supported the fact that immunohistochemistry could not be reliable in making clinical decisions for GBM patients. More sophisticated techniques have been proposed to improve these discrepancies, such as RT-PCR and CHIP for protein expression quantification and PCR-MLPA, methyl-BEAMing, pyrosequencing, quantitative MSP, methylation-sensitive high-resolution melting (MS-HRM), MassARRAY technique, and next generation sequencing for promoter methylation analyses [5, 12, 14, 26, 28, 35-37]. Nevertheless, controversy remains about the most appropriate method to use for analyzing MGMT status. Yamashita *et al.* [35] found that MS-HRM is better than MS-PCR in term of detection of promoter methylation. Compared with MS-PCR, Xie *et al.* [31] found that pyrosequencing is comparably sensitive, relatively specific and also provides quantitative information for each CpG methylation. However, Yoshioka *et al.* [36] considered that the extent of methylated CpGs would be better assessed with real-time semi-quantitative MSP than with the standard gel-based MSP and with pyrosequencing.

In the current study, no protein expression was reported in 31 GBM with unmethylated *MGMT* promoter. The MGMT expression loss could be explained by methylation of intragenic CpG islands that could interfere with the activity of transcription factors, leading to elongation interruption during gene transcription [38]. Moreover, depending on the affected region of *MGMT* gene, mutations, deletions and/or gene rearrangements could contribute to transcriptional repression since the *MGMT* gene is located on 10q26, a region that is often altered in GBM [39, 40]. In addition, recent studies have shown that miR-603, miR -221, miR -222, miR-181d, miR-767-3p, miR-125b and miR-648 can induce loss of *MGMT* expression [41, 42].

In our study, eight GBM with methylated *MGMT* promoter exhibited a strong protein expression. Some factors could influence the level of MGMT protein expression, regardless of the promoter methylation status, such as P53 and NF-κB expression [40]. The overexpression of NF-κB is correlated with the increase of MGMT expression, despite the promoter methylation status [43, 44]. Furthermore, MECP, a protein of MBD family, could recruit other proteins activating the transcription of the *MGMT* gene, although its promoter is hypermethylated [38].

The methylation of *MGMT* promoter was found to be associated with a better survival rate in GBM patients [1, 11, 12, 15, 25, 30, 45-47]. Herein, the OS of GBM patients with methylated promoter was significantly better compared to patients with unmethylated promoter. As well, tumor recurrence occurred more frequently in patients with unmethylated *MGMT* promoter than in those with methylated pattern. Interestingly, in a recent meta-analysis of clinical trials, GBM patients with methylated *MGMT* promoter were associated with longer OS [11]. Moreover, methylation of the *MGMT* promoter was found to be correlated with a better progression-free survival and OS after gamma knife radiosurgery for recurrent GBM [45]. In a prospective study, Franceschi *et al.* [46] considered that survival was consistently longer among *MGMT* methylated females compared to males. On univariate and multivariable analysis, Lee *et al.* [30] found a strong association of the methylated *MGMT* promoter with improved OS in GBM patients from United States hospitals. Using pyrosequencing-based quantitative methylation and specific cutoff levels of methylation, the independent prognostic value of *MGMT* methylation pattern was confirmed [12, 14].

In addition to the methylated MGMT promoter status, previous studies reported other prognostic markers of GBM such as the loss of PTEN expression with conflicting results [16, 20]. In our study, tumors exhibiting PTEN expression were significantly associated with a longer patient survival. Interestingly, in the Trabelsi *et al.* study [20], GBM with a poor survival were characterized by the combination of PTEN expression loss with PDGFRa expression and/or EGFR amplification. By contrast, Carico *et al.* [16] showed that the loss of PTEN expression does not confer poor OS in GBM patients.

In the present study, no significant prognostic value was found for the expression of MGMT protein as well as for P53, EGFR and MDM2 immunoexpression in contrast with some previous reports [17, 18, 47]. Ogura *et al.* [17] suggested that the immunohistochemical analyses of IDH1, MGMT and P53 may be useful for prognostication of GBM. More recently, Dahlrot *et al.* [47] proposed

to incorporate the MGMT expression in future studies evaluating MGMT status before the potential integration into clinical practice as it was found to be an independent prognostic factor. Furthermore, Tini *et al.* [18] revealed that the EGFR expression evaluation refines the prognostic value of MGMT methylation status in GBM. The high-EGFR group had a shorter median time to progression and a higher rate of marginal/distant tumor recurrences [48]. More recently, other proteins have been demonstrated as independent predictors of GBM patient survival, such as insulin like growth factor-binding proteins and interferon regulatory factor [49].

In summary, altogether our results support the fact that MGMT protein expression doesn't reflect the *MGMT* promoter methylation in GBM. Hence, MGM expression by immunohistochemistry seems to be not sufficient to make clinical decisions for GBM patients. Furthermore, *MGMT* promoter methylation is a useful prognostic marker of patients. Nevertheless, it is not the only molecular mechanism leading to *MGMT* silencing in these aggressive tumors. As it was significantly associated with a better survival, PTEN expression could be a helpful prognostic marker for Tunisian patients with GBM malignancies. However, no prognostic value was found for P53, EGFR and MDM2 expression. Further analyses of more large series of GBM are required in order to confirm these results.

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