

MGMT-Methylated Alleles Are Distributed Heterogeneously Within Glioma Samples Irrespective of *IDH* Status and Chromosome 10q Deletion

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Abstract

Several molecular markers drive diagnostic classification, prognostic stratification, and/or prediction of response to therapy in patients with gliomas. Among them, *IDH* gene mutations are valuable markers for defining subtypes and are strongly associated with epigenetic silencing of the methylguanine DNA methyltransferase (*MGMT*) gene. However, little is known about the percentage of *MGMT*-methylated alleles in *IDH*-mutated cells or the potential association between *MGMT* methylation and deletion of chromosome 10q, which encompasses the *MGMT* locus. Here, we quantitatively assessed *MGMT* methylation and *IDH1* mutation in 208 primary glioma samples to explore possible differences associated with the *IDH* genotype. We also explored a potential association between *MGMT* methylation and loss of chromosome 10q. We observed that *MGMT* methylation was heterogeneously distributed within glioma samples irrespective of *IDH* status suggesting an incomplete overlap between *IDH1*-mutated and *MGMT*-methylated alleles and indicating a partial association between these 2 events. Moreover, loss of one *MGMT* allele did not affect the methylation level of the remaining allele. *MGMT* was methylated in about half of gliomas harboring a

10q deletion; in those cases, loss of heterozygosity might be considered a second hit leading to complete inactivation of *MGMT* and further contributing to tumor progression.

Key Words: 10q LOH, Glioma, *IDH* mutation, *MGMT* methylation.

INTRODUCTION

Gliomas are the most common primary brain tumors in adults, accounting for approximately 70% of all CNS neoplasms (1). They are classified on the basis of clinicopathological and histological assessment as either low-grade gliomas (LGGs), including World Health Organization (WHO) grade I and grade II tumors, or high-grade gliomas (HGGs), designated grade III and grade IV tumors (2, 3).

The optimal treatment regimen of patients with LGGs is currently disputed but the therapeutic approach for primary HGGs includes maximal safe resection of the tumor followed by a 6-week course of radiotherapy, with concomitant systemic therapy using the alkylating agent temozolomide (TMZ) (4–6). Use of this combination therapy (known as the Stupp protocol) has increased the survival of patients with HGGs, although median survival remains only approximately 15 months (7).

The conventional classification of glioma subtypes can be enhanced by their molecular characterization, which is aimed at identifying clinically important biomarkers. A number of genetic and epigenetic features useful for diagnostic classification, prognostic stratification, and/or prediction of response to therapy have indeed been identified (8–12). Among them, mutations in the *IDH1* and *IDH2* genes, which encode isocitrate dehydrogenases, are considered valuable diagnostic and prognostic markers. *IDH1* is mutated in 50%–80% of astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, with R132H the most common mutation (90%). *IDH2* is mutated in approximately 3% of gliomas (13). It is likely that mutations in the *IDHs* represent an early event in tumor development, and it has been suggested that they may occur in the neoplastic cell of origin (14). Indeed, *IDH1* mutations are strongly associated with other driver alter-

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This study was supported by Ministero della Salute Regione Lombardia (Ricerca Finalizzata 2011-2012, RF-2011-02347106).

The authors have no duality or conflicts of interest to declare.

Supplementary Data can be found at <http://www.jnen.oxfordjournals.org>.

ations, namely, *TP53* mutations and the 1p/19q co-deletion, in diffuse gliomas (15).

Mutations of the *IDH* genes confer an enzymatic gain-of-function phenotype, associated with production of the alternative metabolite, 2-hydroxyglutarate (16). Accumulation of 2-hydroxyglutarate impairs DNA demethylation, leading to methylome and transcriptome remodeling (17, 18), thereby triggering the glioma-CpG island methylator phenotype (G-CIMP) (19, 20). Among methylated genes, the O6-methylguanine DNA methyltransferase (*MGMT*) promoter frequently undergoes methylation in the presence of *IDH* mutations, suggesting a possible molecular link between the two events (21, 22). *MGMT* acts as a tumor suppressor gene that functions in DNA repair (23) and plays a fundamental role in maintaining genome integrity by removing O6-alkylguanine DNA adducts induced by radiotherapy or alkylating agents (i.e. TMZ or nitrosourea derivatives), which are used as adjuvant therapy in patients with HGGs. Because the best understood mechanism of *MGMT* inactivation is its epigenetic silencing via promoter methylation, *MGMT* methylation status predicts the response to alkylating therapy and is, therefore, an indicator of patient survival (24). Overall, 80% of LGGs and 35%–45% of HGGs show *MGMT* methylation (25).

Although *MGMT* methylation has been extensively studied in a number of tumors, many key points remain unclear. Indeed, while the association between *MGMT* methylation and *IDH* mutations is well established (26), little is known about the timing of *MGMT* silencing during gliomagenesis, the percentage of *MGMT*-methylated alleles in *IDH*-mutated cells, or the possible association of *MGMT* loss with *MGMT* methylation.

MGMT maps to chromosome 10q26.3. Loss of heterozygosity (LOH) of 10q is frequent in gliomas and is classically associated with HGGs, where it is a negative prognostic marker (27). In theory, 10q LOH spanning the *MGMT* locus should lead to its haploinsufficiency and, therefore, have an analogous effect on gene expression to that of the methylation of one allele.

To date, *IDH* mutation analysis has been performed by direct sequencing or immunohistochemistry, which allow the detection but not the quantification of mutations; comparable results are found using both techniques (28). *MGMT* methylation is routinely assessed by methylation-specific PCR, despite the proclivity of this technique to generate false-positive/negative results and the fact that it is not quantitative (29).

In this study, we used a quantitative approach to evaluate *IDH1* mutations and *MGMT* promoter methylation in 208 primitive gliomas. We also analyzed *MGMT* deletion in the same cases to explore the distribution of these biomarkers and possible associations between them, considering potential additive effects and their clinical significance.

MATERIALS AND METHODS

Patients and Samples

This study included 208 primitive gliomas collected consecutively between 2011 and 2014. The series included 29 LGGs (12 diffuse astrocytomas, 13 oligodendrogliomas, and 4

oligoastrocytomas) and 179 HGGs (37 grade III, including 24 anaplastic astrocytomas, 12 anaplastic oligoastrocytomas, and 1 anaplastic ganglioglioma, and 142 grade IV glioblastomas) (Supplementary Data Table S1). Eighty-one patients were female and 127 were male. The median age at surgery for patients with grade II, grade III, and grade IV tumors was 48.7 years (range: 19.0–77.0 years), 51.7 years (range: 18.0–78.0 years), and 59.1 years (range: 23.0–91.0 years), respectively. All surgical treatments were performed at Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy.

Karnofski Performance Status was assessed on the day before surgery. The patients underwent surgical gross total resection of the lesion. All HGG patients received 6 weeks of radiotherapy, with concomitant systemic TMZ. Standard treatment involved the administration of a total of 60 Gy in 30–35 fractions of 1.8–2.0 Gy, 5 days per week. Concomitant TMZ was administered at a dose of 75 mg/m²/day on days 1–42, 1–1.5 hours before radiotherapy, followed by subsequent TMZ adjuvant therapy of 150–200 mg/m²/day on days 1–5 every 28 days for 12 cycles (6).

After surgery, histological diagnosis was performed using hematoxylin and eosin-stained, formalin-fixed paraffin-embedded (FFPE) tissue samples. Gliomas were staged according to the WHO classification (3). Following histological diagnosis, all patients with HGGs (grades III and IV) underwent concomitant chemo-radiation therapy according to the Stupp protocol (7). Overall survival (OS) analysis was performed in the patients with available follow-up (172 patients). At the end of the study, 16% of patients were alive.

The median OS times for patients with grade II, grade III, and grade IV gliomas were 29 months (range: 11–53 months), 26 months (range: 5–62 months), and 18 months (range: 1–90 months), respectively.

Tumor DNA was retrieved from FFPE sections consisting of at least 80% cancer cells, estimated by histological evaluation, using the Biostic FFPE tissue DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA), following the manufacturer's instructions.

For *MGMT* methylation analysis, we also analyzed FFPE sections from 16 non-tumor brain samples (controls), obtained from autopsies of age-matched individuals (range: 18–90 years), who died of causes other than brain malignancies. DNA was also isolated from peripheral blood lymphocytes (PBLs) using the QiAMP DNA Mini Kit, according to the manufacturer's instructions.

Genotyping of *IDH* Genes

IDH1 (codon 132) and *IDH2* (codon 172) in 172 of 208 gliomas were screened by pyrosequencing. For the remaining cases, there was insufficient DNA to perform the analysis.

PCR and pyrosequencing were carried out with modifications as described by Cykowski et al. (30). The primers used for PCR and pyrosequencing and the amplification, and sequencing conditions are shown in Supplementary Data Table S2A. Results were analyzed using PyroMark ID 1.0 software (Biotage AB, Uppsala, Sweden). The pyrosequencing approach allows accurate detection of the proportion of mutated alleles in the sample (31). Considering that mutations of *IDH*

genes are usually heterozygous, a value of 50% for the mutated allele indicates that the alteration is present in virtually 100% of cells.

Evaluation of MGMT Methylation

MGMT methylation was evaluated in all glioma samples and normal brain tissue controls. DNA was modified with sodium bisulfite, using the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Irvine, CA). PCR was performed on 20–100 ng of bisulfite-treated DNA, using 10 pmol each of forward and reverse primers. We analyzed a region covering 10 CpG sites located in the promoter region (Supplementary Data Table S2B). We focused on these CpGs because it was previously reported that the methylation of this region shows a reliable correlation with MGMT gene expression and includes CpG sites also considered in survival analyses of large clinical trials (32, 33). Quantitative DNA methylation analysis was carried out on the Pyro Mark ID instrument using Pyro Gold Reagents (Qiagen) and 1 pmol of sequencing primer. PCR and pyrosequencing primers, and amplification and sequencing conditions are detailed in Supplementary Data Table S2B. Methylation data were analyzed with Q-CpG software v1.09 (Qiagen) (34, 35). The percentage of methylation was defined as the mean of the methylation percentages at each CpG site of the investigated region.

Chromosome 10q LOH

We investigated chromosome 10q deletions in 84 of 208 glioma samples, depending on DNA availability. We used 2 different approaches as follows: when PBLs were available (76 cases), we compared the genotypes of short tandem repeats (STRs) in PBLs and tumor DNAs, whereas in the absence of normal tissue for comparison, array-comparative genomic hybridization (CGH) was performed (8 cases).

For STR analysis, 100 ng of DNA from both tumor and PBL samples was PCR amplified and analyzed by capillary gel electrophoresis on the GeneMapper ABI 3130XL system (Applied Biosystems, Foster City, CA). Seven STRs spanning 11 Mb (D10S1483, D10S587, D10S1727, D10S1676, D10S169, D10S1770, and D10S212) were used to investigate the region neighboring MGMT (10q26.3). The genomic locations and primer sequences of STRs are provided in Supplementary Data Table S2C. MGMT LOH was defined as LOH of at least 2 informative STRs encompassing the MGMT locus and was calculated according to the peak-height ratio, as previously reported (36).

Array-CGH analysis was performed using the Agilent Technologies Platform (Santa Clara, CA) and samples were screened with the Sure Print G3 Human CGH Microarray containing 60,000 oligonucleotide probes. Labeling, purification, and hybridization of DNA samples were carried out according to the manufacturer's protocols (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, version 7.3). Slides were evaluated by the DNA Microarray Scanner (Agilent Technologies) and TIFF images were obtained using Agilent Scan Control software. Raw data were generated using Agilent Feature extraction and analyzed by Agilent Cytoge-

nomics 2.7. Copy number variation analysis was performed using the ADAM2 algorithm. To improve the accuracy of the results, the Diploid Peak Centralization algorithm was applied. The aberration filter was set to detect a minimum of 3 consecutive probes/region and the minimum absolute average log ratio (MAALR) was ± 0.25 . A second analysis was performed using a MAALR of ± 0.15 with a minimum number of 5 probes/region to detect low level of mosaicism.

Statistical Analyses

The Fisher exact, Chi-square, and Mann-Whitney tests were used to identify possible correlations between molecular markers and clinical variables. Kaplan-Meier survival curves and the Log-rank test allowed us to investigate survival differences among groups. Correlations between MGMT methylation levels and OS were identified by Cox regression and Kruskal-Wallis tests.

RESULTS

The Significance of Molecular Markers in Glioma Grading

IDH1 R132H was the only mutation present in the samples. It was found in 30 of 172 tumors (17%) and 84%, 26%, and 5.5% of grade II, grade III, and grade IV, respectively, confirming an inverse correlation with tumor grade ($p < 0.00001$, Chi-square test) (Fig. 1A) (13).

For MGMT methylation analysis, we first defined the normal methylation range in 16 control brain samples as 0%–4% (Supplementary Data Table S3). Taking into account the analytical sensitivity of pyrosequencing ($\sim 5\%$), we considered cases with MGMT methylation levels $\geq 9\%$ to be methylated. Moreover, MGMT methylation at all the analyzed CpG sites was stable, in both controls and glioma samples (Supplementary Data Tables S3 and S4). Overall, MGMT methylation was present in 121/208 samples (58%). LGGs were more frequently methylated (83% of cases) compared with HGGs (65% of grade III and 53% of grade IV cases) ($p = 0.008$, Chi-square test) (Fig. 1A). In addition, considering all cases, MGMT methylation percentages inversely correlated with tumor grade ($p = 0.04$, Kruskal-Wallis test). The median methylation levels (interquartile range) in grade II, grade III, and grade IV gliomas were 29% (9%–44%), 24% (3%–61%), and 9% (3%–47%), respectively (Fig. 1B).

Chromosome 10q LOH spanning the MGMT locus was observed in 9%, 56%, and 75% of grade II, grade III, and grade IV gliomas, respectively, confirming a positive correlation with tumor grade ($p = 0.0002$, Chi-square test) (36) (Fig. 1A).

In grade II and grade III gliomas, the IDH1 mutation was predominantly observed in MGMT-methylated tumors: 16 of 19 (84%) grade II tumors ($p = 0.0012$, Fisher exact test) and 7 of 27 (26%) grade III tumors ($p = 0.0216$, Fisher exact test). Conversely, grade IV tumors lacked this association. Specifically, of the 126 grade IV gliomas, 4 were positive for IDH1 mutation and MGMT methylation, 3 were IDH1-mutated and MGMT-unmethylated, 55 were IDH wild-type and MGMT-unmethylated, and 66 were IDH wild-type and MGMT

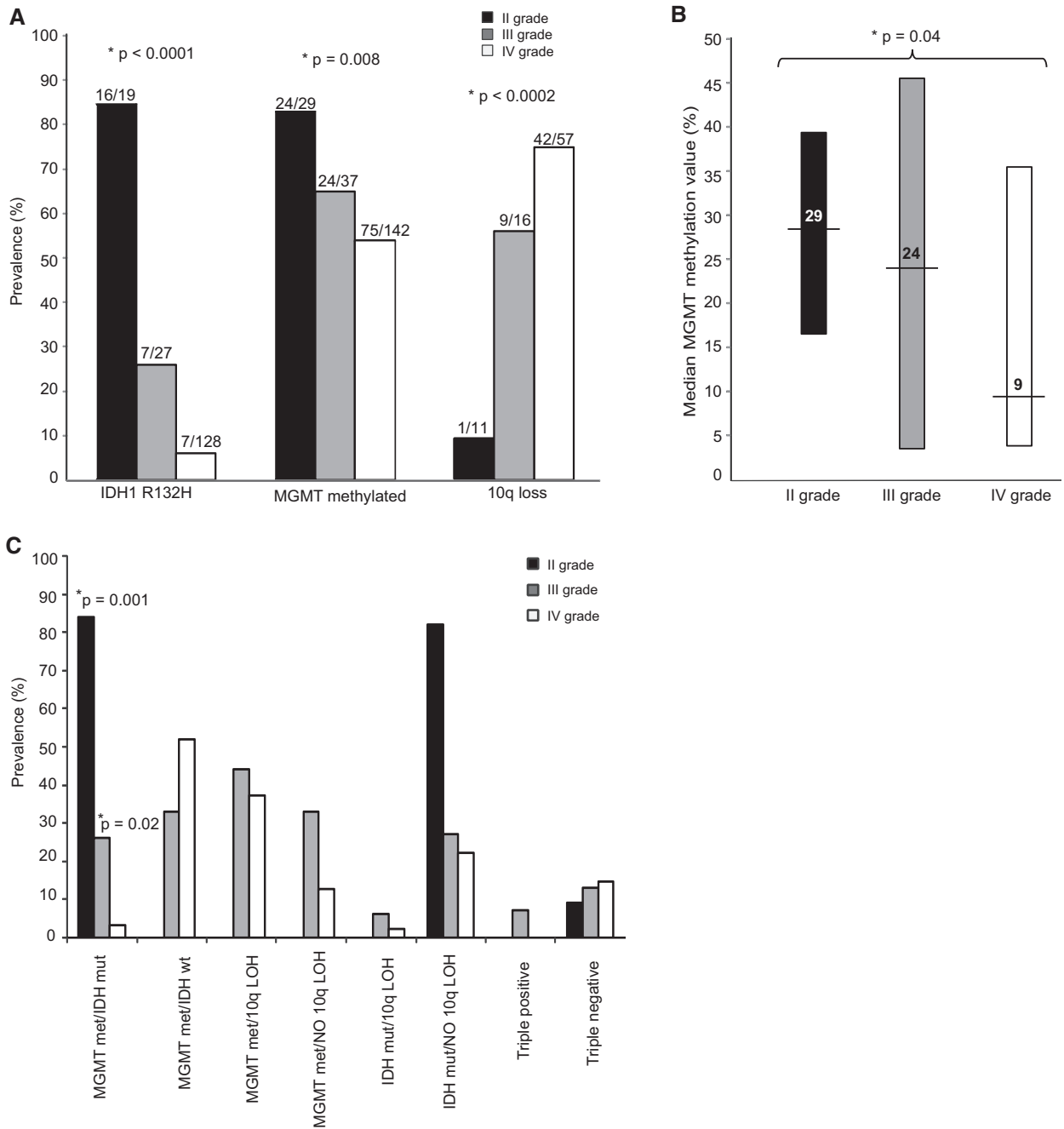


FIGURE 1. Prevalence of the *IDH1* mutation, methylguanine DNA methyltransferase (*MGMT*) methylation, and 10q loss of heterozygosity (LOH) and association of these molecular markers in histologically classified gliomas. **(A)** The *IDH1* R132H mutation and *MGMT* methylation are inversely correlated with tumor grade, whereas 10q loss correlates positively with tumor grade. *p values were calculated using the Chi-square test. The number of cases is indicated at the top of each vertical bar. **(B)** *MGMT* methylation values (%) correlate inversely with tumor grade. Vertical bars indicate the interquartile range (IQR). *p value was calculated using the Kruskal-Wallis test. **(C)** Significant associations (*) were found between *IDH1* mutation and *MGMT* methylation in grade II and III gliomas. No associations were observed between 10q LOH and the other analyzed markers.

methylated (Fig. 1C). This suggests that in grade IV gliomas, *MGMT* methylation occurs independently of *IDH1* genotype. When examining the possible association between 10q LOH and *MGMT* methylation, we noticed that among the 10q deleted cases, 28 (54%) were *MGMT* methylated, suggesting that,

in these cases, *MGMT* was completely inactivated by the 2 events. In addition, the majority of 10q deleted tumors (92%) were *IDH1* wild-type (Supplementary Data Table S1; Fig. 1C).

Evaluation of the distribution of *MGMT* methylation levels in methylated cases (methylation $\geq 9\%$) according to

TABLE. Median MGMT Methylation Values in Cases Showing Methylation levels $\geq 9\%$, According to Tumor Grade, IDH1 Status and 10q Loss

		MGMT methylation values (%)					
		Grade II		Grade III		Grade IV	
		No.	Median (range)	No.	Median (range)	No.	Median (range)
IDH1 status	Mut (R132H)	7	36 (16–70)	16	32 (15–67)	4	32 (22–45)
	WT	9	40 (10–74)	0		63	34 (10–87)
10q	LOH	0		7	40 (10–70)	21	47 (9–86)
	NO LOH	9	31 (22–67)	5	36 (16–52)	7	41 (20–56)

LOH, loss of heterozygosity.

IDH1 mutation status did not provide any evidence of differences in median methylation levels (median range in all groups, 32–47%) (Table), suggesting that the IDH1 mutation does not influence MGMT methylation levels. Similarly, no association was observed between MGMT methylation levels and 10q LOH (Table).

Distribution of MGMT-Methylated Alleles in Gliomas

Quantitative IDH1 genotyping showed that the R132H mutation was present in 21–53% of alleles (mean: 46%) (Fig. 2A; Table; Supplementary Data Table S1). Considering that tumor samples contained at least 80% tumor cells, as determined by histological evaluation, and that IDH mutations are commonly heterozygous, we can assume that the majority of tumor cells carried one mutated allele.

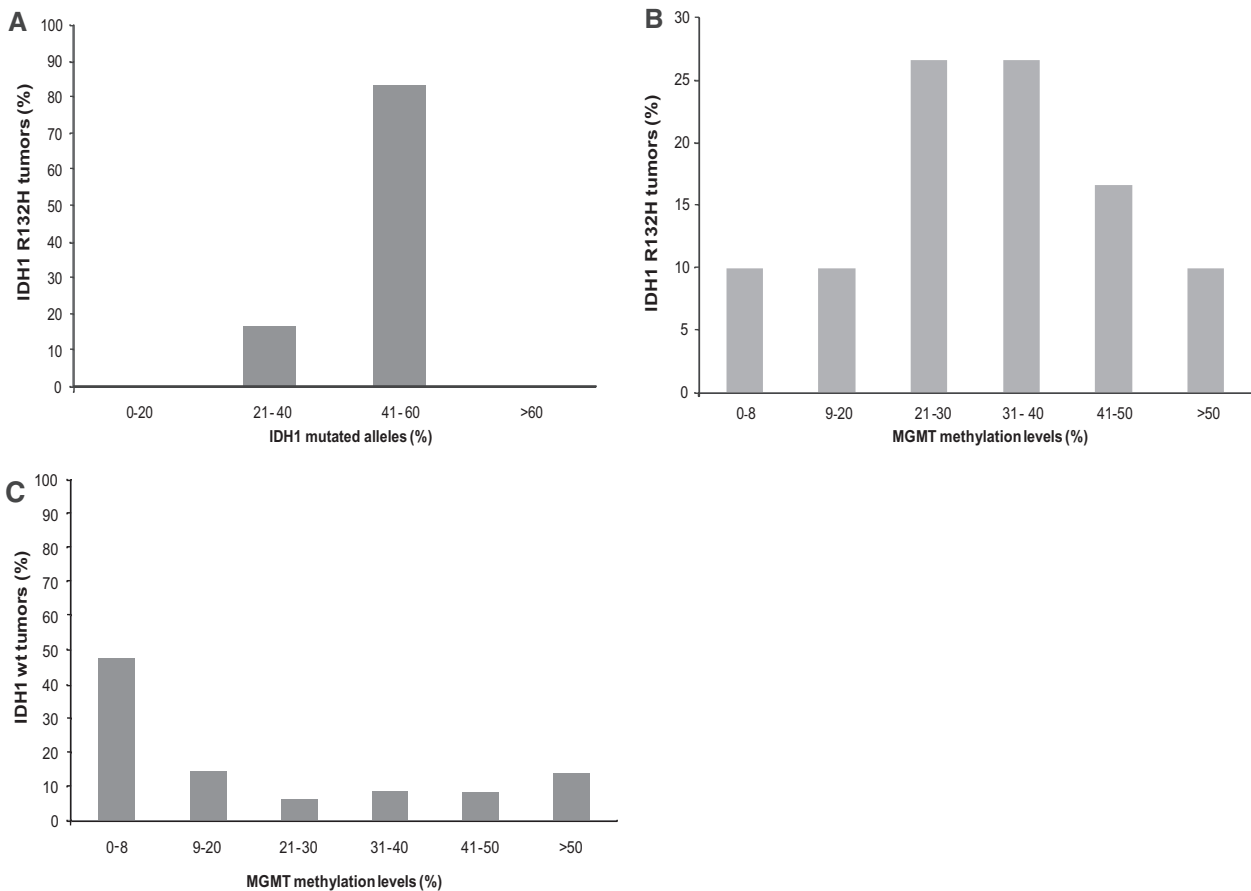


FIGURE 2. Distribution of IDH1 R132H-mutated and MGMT-methylated alleles in IDH1-mutated and wild-type gliomas. **(A)** Distribution of IDH1-mutated alleles in glioma samples. The majority of cases carrying mutations (25 of 30) harbored approximately 50% (41%–60%) of mutated alleles, indicating that all tumor cells in the analyzed samples carry the heterozygous mutation. **(B)** Distribution of MGMT-methylated alleles in IDH1-mutated tumors. Three of 30 (10%) IDH1-mutated tumors were MGMT unmethylated (mean methylation: 0%–8%). Twenty-seven of 30 mutated tumors showed a heterogeneous distribution of the methylated alleles, ranging from 15% to 70%, suggesting that not all cells in the tumor carry MGMT-methylated alleles. **(C)** Distribution of MGMT-methylated alleles in IDH1 wild-type tumors. Sixty-nine out of 144 (48%) IDH1 wild-type gliomas were MGMT-unmethylated (mean methylation level 0%–8%); the remaining 75 cases showed variable levels of MGMT methylation (9%–87%).

In samples with *IDH1* mutations, the percentage of *MGMT* methylation ranged from 5% to 70% (mean 32%; Fig. 2B). In principle, in a homogenous cell population in which *MGMT* is either methylated or not, the methylation percentage of CpG stretches directly involved in gene expression should approximate the following values: 0% (both alleles unmethylated); 50% (1 allele methylated and 1 unmethylated; i.e. imprinted loci); and 100% (both alleles methylated). Intermediate percentages of methylation (as we found in the majority of the samples) suggest that the tumor contains a mixture of cells carrying fully methylated alleles and cells with

unmethylated alleles. The nonoverlapping distribution of *IDH1*-mutated and *MGMT*-methylated alleles in a tumor sample indicates that while the *IDH1* mutation is present in virtually all cancer cells, *MGMT* is methylated in a variable subgroup (Figs. 2B and 3A–C). Analogous results can be extrapolated for *IDH* wild-type cases, in which histological evaluation confirmed that samples consisted of at least 80% cancer cells (Figs. 2C and 3D–F).

Finally, *MGMT* methylation was present in 28 of 52 cases, with 10q LOH spanning the *MGMT* locus. These tumors were mostly (21 of 28) grade IV, suggesting that, in more

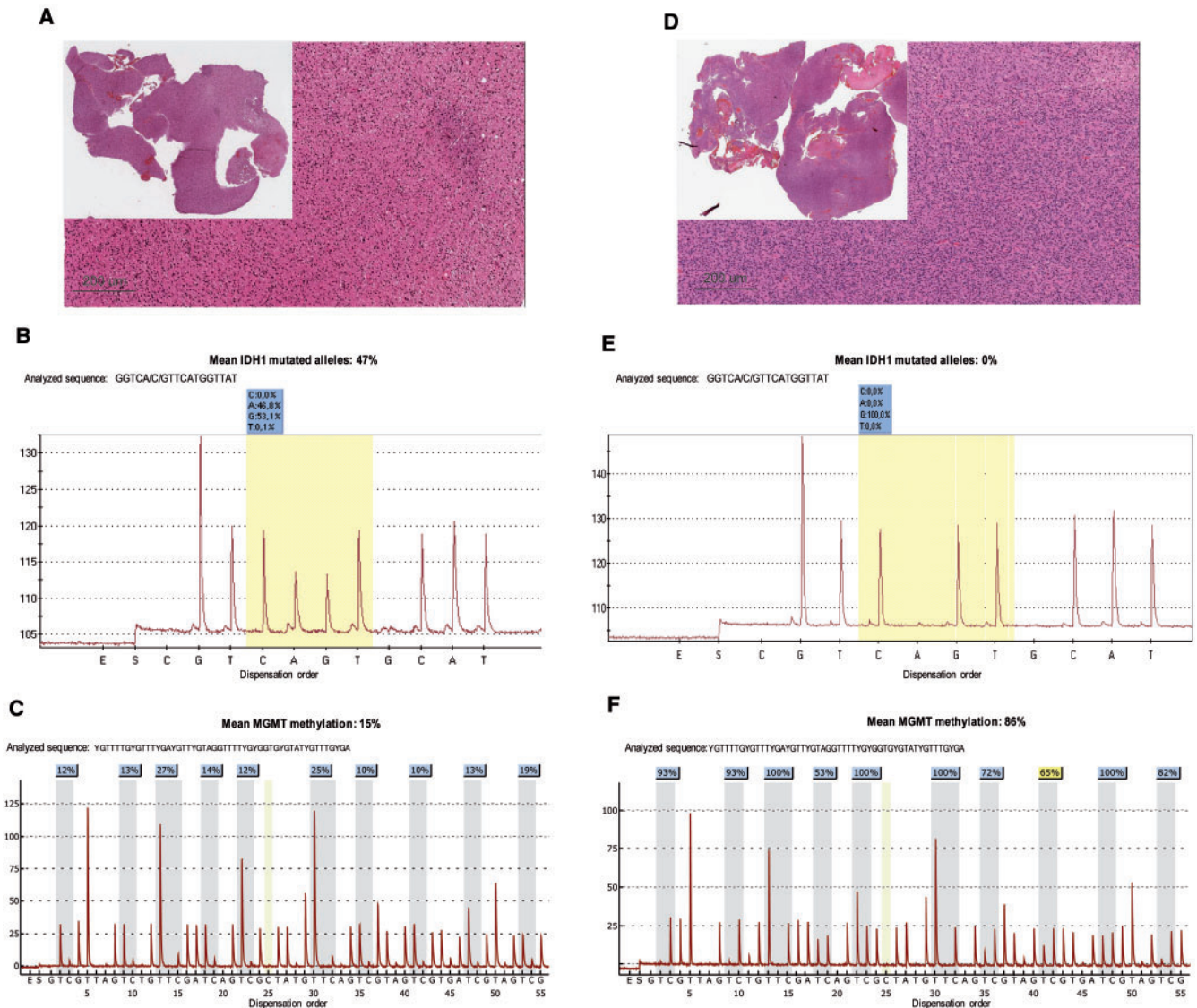


FIGURE 3. *IDH1* R132H-mutated and *MGMT*-methylated alleles within glioma samples. Hematoxylin and eosin (H&E) staining (**A**, **D**), *IDH1* R132H (**B**, **E**), and *MGMT* methylation (**C**, **F**) in 2 representative cases (DA8 and GBM90) (Table; Supplementary Data Table S1). (**A**–**C**) H&E staining of a representative case of diffuse astrocytoma showing approximately 80% of tumor cells (**A**). *IDH1* pyrograms indicating that 47% of alleles carry the R132H mutation (**B**) and the percentage of *MGMT* methylation in the sample is 15% (**C**). (**D**–**F**) H&E staining of a representative case of glioblastoma, showing approximately 80% of tumor cells (**D**). *IDH1* pyrograms indicating that the tumor is wild-type (**E**) and that the percentage of *MGMT* methylation in the sample is 86% (**F**).

advanced tumors, *MGMT* can be silenced by a two-hit mechanism (i.e. 1 allele deleted and 1 methylated).

MGMT Methylation Affects OS Independently of IDH1 Mutation

Correlations between molecular markers and OS were evaluated in all patients with available follow-up (172 cases in total). Because all patients followed the same therapeutic protocol, we assume that the observed differences in OS between experimental groups were independent of treatment.

Univariate analysis of molecular markers highlighted that *IDH1* mutation correlated positively with OS in both LGGs and HGGs ($p < 0.0001$ and $p = 0.0116$, respectively, Log-rank test) (Fig. 4A, C). The median OS for patients with *IDH1* mutations was 31 months for LGGs and 34 months for HGGs. Conversely, the median OS of *IDH1* wild-type patients was 14 months for LGGs and 18 months for HGGs.

MGMT methylation conferred a survival advantage on both LGGs and HGGs ($p < 0.0001$, Log-rank test). The median OS for LGGs patients was 32 months in methylated versus 14 months in non-methylated cases (Fig. 4B). Similarly, the median OS for HGGs patients was 24 months in methylated versus 14 months in nonmethylated cases (Fig. 4D). Importantly, a significant positive correlation was found between methylation levels and OS ($p < 0.0001$ and $e = -0.019$, Cox regression) (Fig. 5A, B). The distribution of OS (determined at 6-month intervals) for unmethylated compared with methylated cases revealed that 75% of *MGMT*-unmethylated patients died within 18 months from surgery, whereas more than half of *MGMT*-methylated patients survived for longer than 18 months. In particular, 24 of 98 (24%) *MGMT*-methylated cases can be considered long-term survivors (OS >36 months) (Fig. 5B). We also found that the methylation levels in glioma samples grouped by OS intervals (every 6 months) increased with increasing OS ($p = 0.001$, Kruskal-Wallis test) (Fig. 5C). Moreover, patients with *MGMT*-methylated HGGs

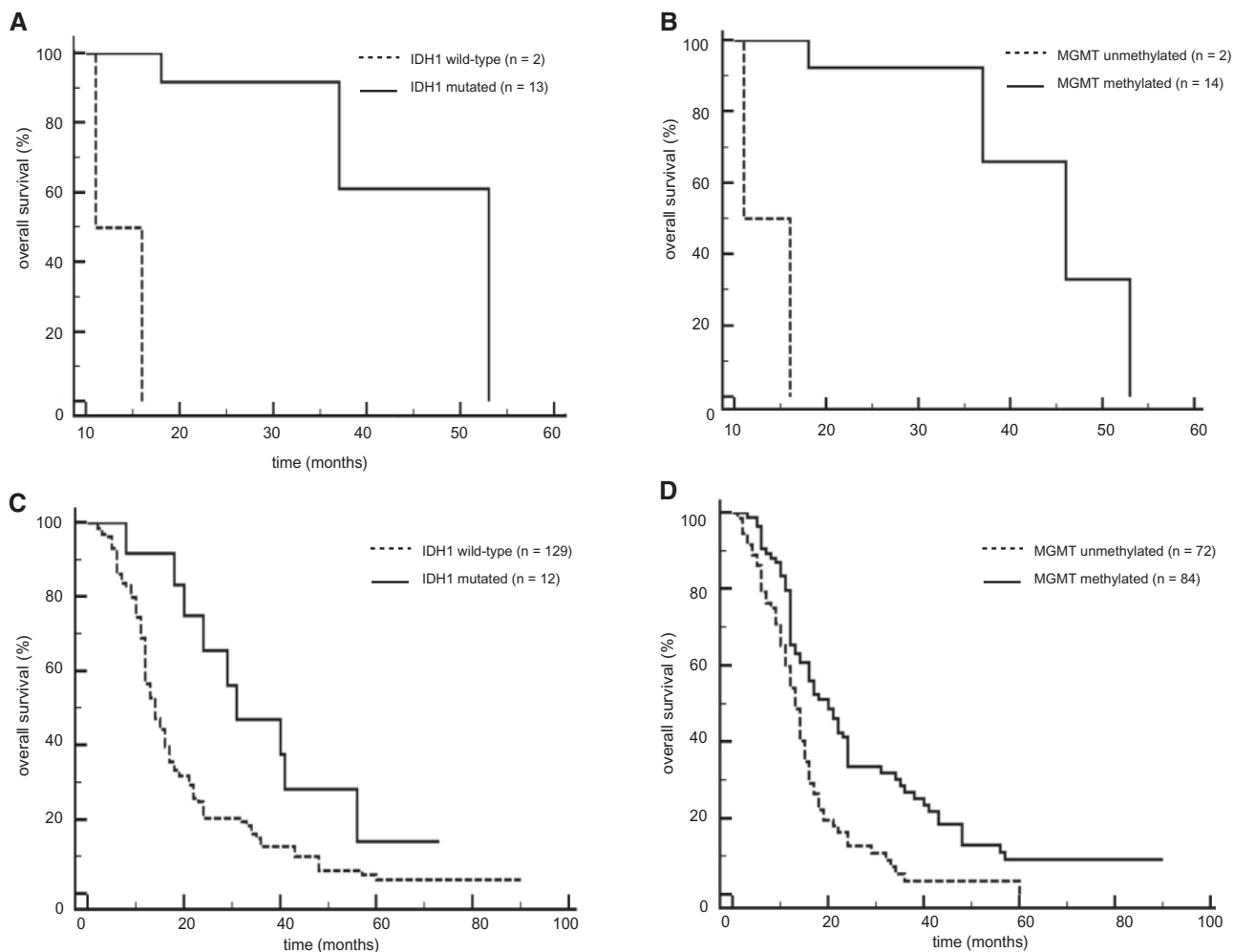


FIGURE 4. Prognostic significance of *IDH1* mutation and *MGMT* methylation in low-grade gliomas (LGGs) and high-grade gliomas (HGGs). (A–D) Kaplan-Meier survival analysis of LGGs (A, B) and HGGs (C, D) according to *IDH1* genotype (A, C) and *MGMT* methylation status (B, D). Both molecular markers are associated with improved OS.

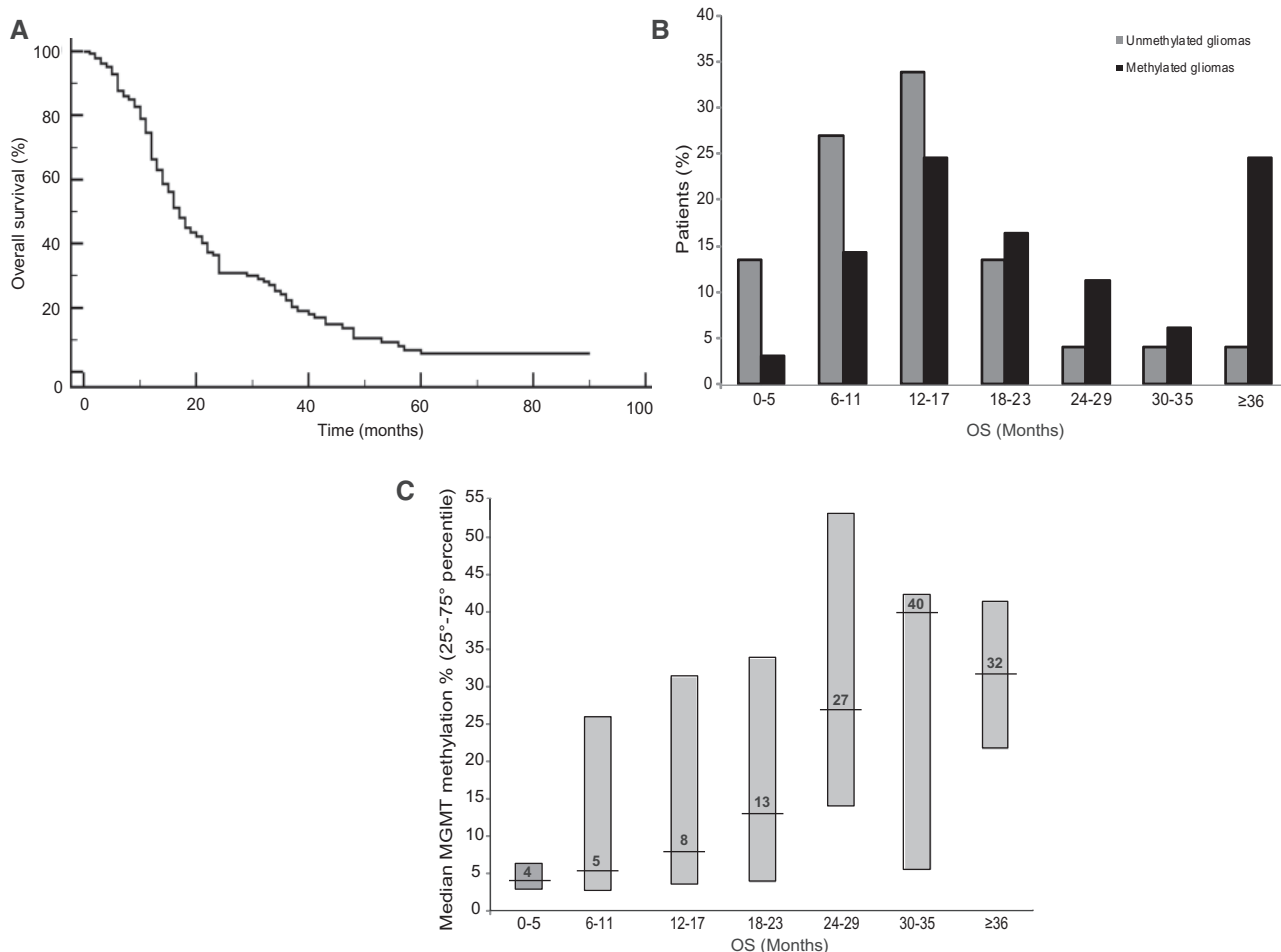


FIGURE 5. Correlation between *MGMT* methylation level and overall survival (OS). **(A)** Kaplan-Meier survival curve of combined LGGs and HGGs. **(B)** Correlation between *MGMT* methylation levels and OS in both LGGs and HGGs. The overall data highlight a positive correlation between the level of *MGMT* methylation and increased OS. Stratification of patients into OS intervals (every 6 months), according to *MGMT* methylation ($\geq 9\%$), highlights the positive correlation between *MGMT* methylation status and OS. **(C)** *MGMT* methylation levels correlate positively with OS. Vertical bars indicate the IQR and the horizontal line indicates the median value.

had longer OS, independent of *IDH1* mutation status ($p = 0.0337$ and $p = 0.0020$ for *IDH1* mutated and wild-type, respectively, Log-rank test) (Supplementary Data Fig. S1).

Multivariate analysis suggested that both *IDH1* mutation and *MGMT* methylation significantly affected OS ($p = 0.0162$ and $p = 0.0037$, respectively), but *MGMT* methylation shows a more positive correlation with OS ($e = -0.01476$, hazard ratio = 0.9853) compared to *IDH1* mutation ($e = -0.8057$, hazard ratio = 0.4468), emphasizing its role as a strong marker of positive prognosis.

Finally, in our population, 10q LOH did not influence OS independently of *MGMT* methylation status.

DISCUSSION

This study demonstrates that, whatever the underlying mechanism, *MGMT* promoter methylation is not homogeneously distributed in glioma samples, suggesting that only

a fraction of cells in the tumor bulk is sensitive to *MGMT* epigenetic silencing. Overall, our data on grade II and grade III gliomas indicate that *IDH1* mutation and *MGMT* methylation are often found concomitantly, as previously reported (26). However, the non-homogeneously overlapping distribution of *IDH1*-mutated and *MGMT*-methylated alleles is indicative of an incomplete association between these 2 molecular events. In particular, the distribution of *IDH1*-mutated alleles confirmed, as expected for a heterozygous mutation, that the gene was mutated in virtually all cancer cells and that the *IDH1* mutation is an early event in gliomagenesis. This result supports the hypothesis that *IDH1* mutation triggers G-CIMP (20) and confers only an increased likelihood of *MGMT* methylation in tumor cells (37).

In addition, in grade IV gliomas, *IDH* mutation and *MGMT* methylation seem to be independent events, as indicated by the presence of a consistent subgroup of *MGMT*-methylated/*IDH1* wild-type tumors. Similar to *IDH1*-mutated

gliomas, wild-type tumors showed a heterogeneous distribution of *MGMT*-methylated alleles, suggesting that, whatever the underlying mechanism, *MGMT* epigenetic silencing occurs in only a subset of tumor cells.

Our data suggest that *IDH1* mutation (or other unknown mechanisms) may predispose to *MGMT* methylation in a variable percentage of tumor cells. Whether methylated cells are randomly distributed or occur in a precise niche within the tumor is unknown. It is conceivable that, given the crucial role of *MGMT* in the maintenance of genome integrity, its epigenetic silencing may lead to mutation acquisition and thus contribute to promotion or maintenance of neoplastic transformation.

We confirmed that *MGMT* methylation is inversely correlated with tumor grade (13); however, no differences were found in *MGMT* methylation levels of glioma samples grouped according to 10q LOH, implying that the lack of one *MGMT* allele does not affect the methylation of the remaining allele. We also observed that in approximately 50% of HGGs with LOH at the *MGMT* locus, the second allele was methylated, suggesting that in advanced stages of gliomagenesis, *MGMT* could be completely inactivated by methylation of one allele and deletion of the other.

Our data also confirm *MGMT* methylation as a positive prognostic factor in both *IDH1*-mutated and wild-type gliomas (25), with the percentage of *MGMT* methylation showing a direct correlation with increasing OS (38). Increased OS in patients with *MGMT* methylation is attributable to a positive response to TMZ, rather than a biological effect on tumor development. Because we did not observe any improvement in the OS of patients with both *MGMT* methylation and 10q LOH, we hypothesize that the positive effect of complete *MGMT* silencing on therapy response can be counterbalanced by the loss of other tumor suppressor genes (e.g. *PTEN*, *ERCC6* and *DMBT1*) mapping to the same region of chromosome 10q and associated with reduced OS (39, 40).

These findings confirm the importance of quantitative evaluation of *MGMT* methylation in the clinical assessment of patients with glioma, taking into account the proportion of tumor cells in the samples, to facilitate accurate evaluation of the percentage of *MGMT* methylation in the tumor. To enable a better understanding of the role of *MGMT* methylation phenomenon in gliomagenesis, the mechanism underlying *MGMT* methylation should be examined in detail along with the identification of tumor cells that are more susceptible to the acquisition of DNA methylation.

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