

MGMT CpG island is invariably methylated in adult astrocytic and oligodendroglial tumors with *IDH1* or *IDH2* mutations

Shani Mulholland¹, Danita M. Pearson¹, Rifat A. Hamoudi¹, Deborah S. Malley¹, Caroline M. Smith¹, Jamie M.J. Weaver¹, David T.W. Jones¹, Sylvia Kocalkowski¹, L. Magnus Bäcklund², V. Peter Collins¹ and Koichi Ichimura¹

¹ Division of Molecular Histopathology, Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom

² Department of Oncology–Pathology, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden

We have previously identified a region containing 16 CpGs within the *MGMT* CpG islands which is critical for the transcriptional control of *MGMT* (Malley, *Acta Neuropathol* 2011). To investigate the patterns and incidence of *MGMT* methylation in astrocytic and oligodendroglial tumors, we quantitatively assessed methylation at these 16 CpGs using bisulfite modification followed by pyrosequencing of 362 gliomas not treated with temozolomide, and correlated the findings with previously identified patterns of genetic abnormalities, patients' age and survival. The *MGMT* gene was considered to be methylated when the mean methylation of the 16 CpGs was 10% or higher. This cut-off value distinguished diffuse astrocytomas with high and low *MGMT* expression. Within each tumor type, the patterns of methylation were highly variable and also highly heterogeneous across the 16 CpGs. A high incidence of *MGMT* methylation was observed in all subtypes of gliomas included in this study. Among a subset of 97 tumors where conventional methylation-specific PCR (MSP) was also applied, methylation was detected by both methods in 54 tumors, while the pyrosequencing results identified a further 17 tumors. No additional cases were found using MSP alone, indicating that pyrosequencing is a robust method for methylation analysis. All tumors with *IDH1/IDH2* mutations except two had *MGMT* methylation, while there were many tumors with *MGMT* methylation, particularly primary glioblastomas, which had no mutations of *IDH1/2*. We suggest that *MGMT* methylation may be one of the earliest events in the development of astrocytic and oligodendroglial tumors.

Gliomas are the most common group of primary brain tumors consisting of clinically and biologically distinct subtypes. The three main subgroups of glioma are astrocytic,

Key words: *IDH*, *MGMT*, pyrosequencing, astrocytoma, oligodendrogloma, MSP, methylation

Abbreviations: A, diffuse astrocytoma grade II; AA, anaplastic astrocytoma WHO grade III; AML, acute myeloid leukemia; AO, anaplastic oligodendrogloma WHO grade III; AOA, anaplastic oligoastrocytoma WHO grade III; CGI, CpG island; *IDH*, isocitrate dehydrogenase; *MGMT*, O⁶-methylguanine-DNA methyltransferase; MSP, methylation-specific PCR; O, oligodendrogloma WHO grade II; OA, oligoastrocytoma WHO grade II; pGB, primary glioblastoma WHO grade IV; sGB, secondary glioblastoma WHO grade IV; TMZ, temozolomide; TSS, transcription start site; WHO, world health organization

Additional Supporting Information may be found in the online version of this article.

Grant sponsors: Cancer Research, UK; Samantha Dickson Brain Tumour Trust; Cambridge Fund for the Prevention of Disease (CAMPOD)

DOI: 10.1002/ijc.26499

History: Received 4 Jun 2011; Revised 14 Aug 2011; Accepted 9 Sep 2011; Online 22 Oct 2011

Correspondence to: Koichi Ichimura, M.D., Ph.D., Molecular Histopathology, Level 3, Lab Block, Addenbrooke's Hospital Box 231, Cambridge CB2 0QQ, United Kingdom, Tel.: +44-1223-762084, Fax: +44-1223-586670, E-mail: ki212.ichimura@gmail.com

oligodendroglial and mixed gliomas, which are further malignancy graded into up to four grades according to the WHO classification.¹ Glioblastomas (WHO grade IV) are the most malignant and common astrocytic brain tumor in adults, the overall median survival being only just over 14 months despite modern combined therapies.² Most glioblastomas are primary glioblastoma (pGB), while secondary glioblastoma (sGB) generally develops by progression from astrocytomas WHO grade II or III. Patients with astrocytic or oligodendroglial tumors WHO grade II and III survive longer, however they always recur and are ultimately fatal.

Among the many genetic and epigenetic abnormalities commonly found in adult gliomas, three have been reported to predict response to treatment and/or prognosis: *IDH1/IDH2* mutations, concurrent deletion of the entire 1p and 19q chromosomal arms (total 1p/19q loss) and *MGMT* methylation.^{3,4} With the exception of pGB, mutations of either *IDH1* or *IDH2* occur in more than 50% of adult astrocytic, oligodendroglial and oligoastrocytic tumors.^{5–8} It has been shown that the presence of *IDH1/2* mutations is associated with longer patient survival.^{5–7,9,10} *IDH1/2* mutations have been proposed as the earliest genetic changes in a subset of WHO grade II/III astrocytic and oligodendroglial tumors.^{5,8,11} Mutant *IDH1/IDH2* are known to acquire a neomorphic enzymatic activity converting α -ketoglutarate to D-2-hydroxyglutarate (D-2-HG) using NADPH as a co-factor.¹² The cellular consequences of *IDH* mutations are currently unclear,^{12,13} although they have recently been

linked to a hypermethylation phenotype in glioma and acute myeloid leukemia (AML).^{14–17} Mutations of *IDH1/2* are also strongly associated with another prognostic marker, *i.e.*, total 1p/19q loss,^{5,7,8} a consequence of an unbalanced translocation t(1;19)(q10;p10)^{18,19} seen in the majority of oligodendroglial tumors but only very occasionally in astrocytic tumors.^{20,21} The total 1p/19q loss has been shown to predict longer survival in patients with anaplastic oligodendroglial tumors^{22,23} as well as astrocytic tumors.²¹

DNA hypermethylation of the CpG island (CGI) of the *MGMT* gene is an important therapeutic indicator for response to alkylating agents in glioblastomas.² *MGMT* (O⁶-methylguanine-DNA methyltransferase) is a DNA repair protein that removes O⁶-methylguanine from DNA, the main lesion produced by alkylating chemotherapeutic agents such as temozolomide (TMZ).²⁴ Transcriptional silencing of *MGMT* by DNA methylation is therefore expected to increase the chemosensitivity of tumors, although recently two large clinical studies showed that patients with anaplastic gliomas and *MGMT* methylation responded to radiotherapy alone compared with those without methylation.^{25,26} Abnormal hypermethylation of *MGMT* is a common finding in various subtypes of astrocytic and oligodendroglial tumors of almost all malignancy grades (reviewed in Ref. 27). The presence of *MGMT* methylation is significantly correlated with longer overall survival for newly diagnosed glioblastoma patients as well as other types of glioma.^{27,28}

We documented the methylation status of the *MGMT* CGI in 362 astrocytic and oligodendroglial tumors of all major types using bisulfite modification of DNA and pyrosequencing. We found that *MGMT* methylation highly correlated with the presence of *IDH1/IDH2* mutations in all types of astrocytic/oligodendroglial tumors of WHO grade II and III. Our findings suggest that *MGMT* methylation may be the earliest change in the development of subtypes of gliomas.

Material and Methods

Tumor materials and DNA extraction

A total of 362 astrocytic, oligodendroglial and mixed gliomas were included in the study as follows: 22 diffuse astrocytomas grade II [A], 61 anaplastic astrocytomas grade III [AA], 172 primary glioblastomas grade IV [pGB], 10 secondary glioblastomas grade IV [sGB], 34 oligodendrogliomas grade II [O], 20 anaplastic oligodendrogliomas grade III [AO], 20 oligoastrocytomas grade II [OA], 23 anaplastic oligoastrocytomas grade III [AOA]. Six normal brain tissue samples from cerebral subcortex were also included.²⁹ All patients were operated prior to 1998 and received various adjuvant treatments but not temozolomide. The histopathological diagnosis was made according to the WHO criteria.¹ Collection, handling and DNA/RNA extraction from tumor tissues and the patients' blood samples were as described previously.³⁰ The study was approved by the Ethical Committee of Sahlgrenska University Hospital (S339:01), the Karolinska Hospital (no.

91:16) and the Cambridge Research Ethics Committee (Cambridge, UK; NRES Cambridgeshire 2 REC reference 03/115).

Pyrosequencing

Bisulfite modification and pyrosequencing were performed as previously described.²⁹ Briefly, 500 ng of genomic DNA was bisulfite-modified using an EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's recommendations. The bisulfite-modified DNA was amplified using the forward primer PC4963 (GTTTYGGA-TATGTTGGGATAG) and the biotinylated reverse primer PC5453 (AAAACCACTCRAAACTACCAC). Single-stranded templates for pyrosequencing were prepared using a PyroMark Vacuum Prep Workstation (Qiagen, Crawley, UK) according to the manufacturer's recommendations. Two assays were designed and run on this template using two pyrosequencing primers: PC4966 (GATAGTTYGYGTTTT-TAGAA) and PC4967 (GYGATTTGGTGAGTGTGTTG). Pyrosequencing was performed using PyroGold Q96 SQA Reagents and the Pyro Q-CpG software on a PyroMark ID pyrosequencer (Qiagen, Crawley, UK) according to the manufacturer's recommendations. The full details of the pyrosequencing assay are given in Supporting Information.

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) was performed essentially according to the published protocol.³¹ The bisulfite-modified DNA was directly amplified using the published primer pairs specifically designed to target completely methylated sequences.³¹ A touchdown thermal cycling program was used for amplification. PCR products were run on an agarose gel and digitally captured for visual assessment using the Labworks software and GelDoc-It Imaging System (UVP, Cambridge, UK). The MSP assay is described in details in Supporting Information.

Real-time quantitative PCR

mRNA expression levels of *MGMT* were determined with a forward primer located in Exon 3 (CTCTTCAC-CATCCCGTTTTCCA) and a reverse primer in Exon 5 (AGAAGCCATTCCTTCACG) by real-time quantitative PCR (qPCR) using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Burgess Hill, UK) and the SYBR Green I (483–533 nm) detection format on a LightCycler 480 (Roche Diagnostics, Burgess Hill, UK) as described.^{29,32} The expression level of 18S rRNA was used as an internal reference for normalization.³² Expression was measured relative to that of normal brain RNA (Applied Biosystems/Ambion, Austin, TX). Relative quantification analyses were performed using LightCycler 480 software version 1.2 (Roche Diagnostics, Burgess Hill, UK).

Genetic analysis

The copy number status of known oncogenes (*CDK4*, *CDK6*, *MDM2*, *MDM4*, *CCND1*, *CCND2*, *CCND3*, *EGFR*, *IDH1*, *IDH2*) and 1p/19q as well as mutation status of *IDH1*, *IDH2*,

TP53, *RBI*, *PTEN* and *CDKN2A* have previously been reported.^{5,21,30,33,34}

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS, Chicago, IL). Multiple test correction was performed using the Benjamini and Hochberg false discovery rate (FDR) in the R statistical analysis environment version 2.10.1 (www.r-project.org). The log-rank test was performed for univariate analysis, and Cox regression with forward likelihood ratio for multivariate analysis of potential association between overall survival and age, diagnosis or presence of *MGMT* methylation, *IDH1/2* mutations, or total 1p/19q loss, using the SPSS version 18.0.

Results

Strategy for MGMT methylation analysis

The *MGMT* CGI encompasses a 762-bp region around Exon 1 of *MGMT* and contains 98 CpGs according to the UCSC Human Genome Browser GRCh37/hg19 Assembly (<http://genome.ucsc.edu/>). In this study, the CpG sites within the *MGMT* CGI are assigned with consecutive numbers (CpG1-CpG98) from 5' to 3' on the coding strand of *MGMT*. The nucleotide coordinates in the *MGMT* CGI are presented as a distance from the transcription start site (TSS, distance = 0, chr10:131,265,401) defined by Harris *et al.*³⁵ In a previous study, we pyrosequenced the whole *MGMT* CGI (from -452 to +309 relative to TSS) in glioma cell lines and xenografts, and found that the methylation status of two separate regions defined as differentially methylated region 1 (DMR1, from -242 to -63) and 2 (DMR2, from +95 to +225) were statistically significantly correlated to *MGMT* mRNA expression.²⁹ DMR2 was always methylated when DMR1 was methylated. Moreover, a luciferase reporter assay showed that the status of some of the individual CpG sites within DMR2, *i.e.* CpG83, 86, 87 and 89, may be critical for the promoter activity.²⁹ On the basis of these findings, we chose to focus on the 16 CpGs (CpG74-89, from +106 to +213, see Supporting Information Table 1) included within DMR2 in this study. This region contains all 9 CpGs that are interrogated by the widely used MSP primers (CpG76-80 for MSP-F and CpG84-87 for MSP-R, see below).³¹

MGMT methylation profiling

The methylation levels of all 16 CpGs were determined in all 362 tumors by pyrosequencing using bisulfite-modified DNA. The levels were highly variable across the 16 sites within individual cases, the maximum difference at a single CpG being 90.4% (ranging from 0% at CpG88 to 90.4% at CpG85 in GB45, Supporting Information Table 1). The methylation profiles across the 16 sites varied greatly from tumor to tumor, even among tumors of the same type. The methylation levels for all analyzed loci in all tumors (5792 data points) showed a bimodal distribution (histogram, Fig. 1). The distribution of methylation levels in normal brain generally overlapped with the first peak. Repeated

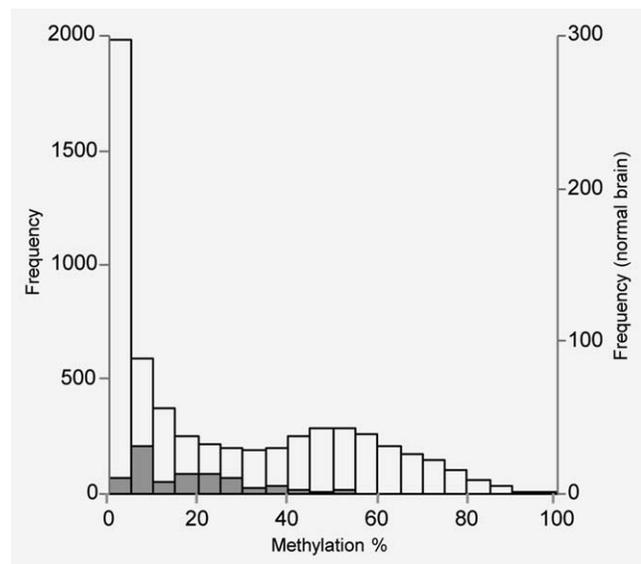


Figure 1. A histogram generated from all 5,792 data points (methylation levels) in 362 tumors (open bars, left ordinate) and 96 in 6 normal brain (grey bars, right ordinate) from each of the 16 sites in the *MGMT* CpG island.

PCR amplification and pyrosequencing of chemically unmethylated bisulfite-modified human genomic DNA (see Supporting Information) gave a mean methylation level of 4.9% (SD 2.4%, Supporting Information Table 1). We thus considered that this distribution of methylation levels represents methylation statuses which could have different functional consequences.

Given the highly heterogeneous patterns of methylation and the presence of a number of critical CpG sites as identified previously,²⁹ we considered that the methylation level at any single CpG site would not reflect the true methylation status of the region as a whole. Thus, the mean methylation level of all 16 CpG sites was used.

To further determine a level of methylation that significantly affects expression, *MGMT* expression was quantified using qPCR in 18 diffuse astrocytomas and compared with the mean methylation levels across the 16 CpGs. Two distinct groups of tumors were observed, one with high expression and low mean levels of methylation similar to normal brain and unmethylated DNA (1.6–4.9%), the other with low expression and higher methylation levels (12.1–44.4%) (Fig. 2).

Based on these findings, *MGMT* was scored as methylated when the mean methylation was 10% or higher. This cut-off value was set above the mean methylation levels + 2 × standard deviations of the four unmethylated controls (9.8%, see above). Thus the 362 tumors were scored as methylated and unmethylated (Table 1). Over 50% of all tumor types were scored as methylated.

Comparison of MSP to pyrosequencing results

To compare efficacies of methylation detection methods, a conventional direct MSP was performed on all oligodendrogliomas

and mixed gliomas (97 tumors in total). Methylation was scored as positive (3+), weakly positive (2+), very weakly positive (1+), or negative (-) by visual inspection (Fig. 3a). All cases showing a positive MSP PCR product were considered methylated. In total, 54 out of 97 tumors (56%) were recorded as methylated by MSP (3+, 30; 2+, 12; 1+, 12;

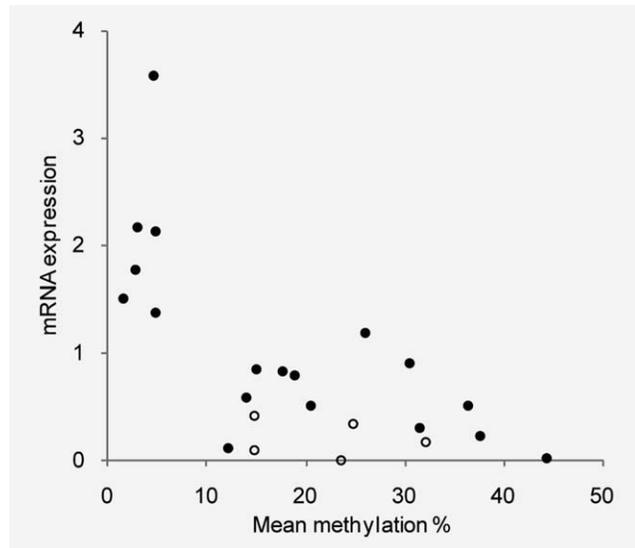


Figure 2. Correlation of *MGMT* mRNA expression to the methylation levels in 18 diffuse astrocytomas (closed circle) shows two distinct groups, with either high expression and low methylation, or vice versa. Five oligodendrogliomas grade II which are scored as methylated by pyrosequencing but not by MSP show low *MGMT* expression (open circle). The mRNA expression relative to that of normal brain is plotted against the mean methylation of the 16 CpG sites.

Supporting Information Table 1). All of these 54 tumors had also been scored as methylated by pyrosequencing using the above criteria. However, 17 tumors were scored methylated only by pyrosequencing but not by MSP. No tumors were scored methylated by MSP alone. The discordant cases showed low or no methylation by pyrosequencing at several CpGs, mostly within the MSP-F region, but moderate or high methylation in other CpGs and low *MGMT* expression (Figs. 2 and 3b).

Correlation between *MGMT* methylation and genotype

Next, we investigated whether the patterns of *MGMT* methylation correlated to any of the genetic abnormalities previously identified in the same tumor series.^{5,21,30,33,36} Strikingly, we found that all but two tumors (AA15 and O41, 127/129) with *IDH1/2* mutations regardless of tumor type had *MGMT* methylation (Table 1 and Supporting Information Table 1). While mean methylation in AA15 was 8.8%, the methylation level at CpG89 was 25.0%, and O41 had mean methylation of 8.2% but showed 22.3% methylation at CpG87. In A, O, AO and AOA, all tumors with *MGMT* methylation except two (AOA4, AOA15) had *IDH1/2* mutations. Among AA, sGB and OA, the great majority of tumors with *MGMT* methylation also had *IDH1/2* mutation. In contrast, only a small fraction of pGB with *MGMT* methylation had *IDH1/2* mutations. When the levels of mean methylation were compared between tumors with or without *IDH1/2* mutations in all cases, tumors with *IDH1/2* mutations showed significantly higher levels of methylation than those without ($p < 0.001$, Mann-Whitney *U* test). Within each tumor type, there was a clear difference in the distribution of mean methylation levels

Table 1. Frequencies of *MGMT* methylation and *IDH1/2* mutations

| Tumor type | WHO grade | No. of Cases | Mean methylation (%) of all CpGs | No. of cases (incidence %) | | <i>IDH/MGMT</i> concordance | |
|--------------------------------|-----------|--------------|----------------------------------|----------------------------|------------------------|---------------------------------|---------------------------------|
| | | | | <i>MGMT</i> met | <i>IDH1/2</i> mutation | <i>MGMT</i> met/ <i>IDH</i> mut | <i>IDH</i> mut/ <i>MGMT</i> met |
| Astrocytic tumors | | | | | | | |
| PA | I | 44 | 3.2 | 1 (2.3%) | 0 (0%) | na | na |
| A | II | 22 | 18.8 | 15 (68%) | 15 (68%) | 100% | 100% |
| AA | III | 61 | 22.5 | 42 (69%) | 34 (56%) | 97% | 79% |
| pGB | IV | 172 | 23.8 | 90 (52%) | 6 (3.5%) | 100% | 6.7% |
| sGB | IV | 10 | 31.9 | 7 (70%) | 5 (50%) | 100% | 71% |
| Oligodendroglial tumors | | | | | | | |
| O | II | 34 | 30.9 | 26 (76%) | 27 (79%) | 96% | 100% |
| AO | III | 20 | 36.2 | 13 (65%) | 13 (65%) | 100% | 100% |
| Mixed gliomas | | | | | | | |
| OA | II | 20 | 16.4 | 12 (60%) | 10 (50%) | 100% | 83% |
| AOA | III | 23 | 35.2 | 20 (87%) | 19 (83%) | 100% | 95% |
| Total | | 406 | 22.8 | 226 (56%) | 129 (32%) | 98% | 52% |

MGMT met, cases with mean methylation >10% at any site. PA, Pilocytic astrocytomas. A, diffuse astrocytomas. O, oligodendrogliomas WHO grade II. OA, oligoastrocytomas WHO grade II. AA, anaplastic astrocytomas. AO, anaplastic oligodendrogliomas. AOA, anaplastic oligoastrocytomas. sGB, secondary glioblastomas. pGB, primary glioblastomas. na, not applicable.

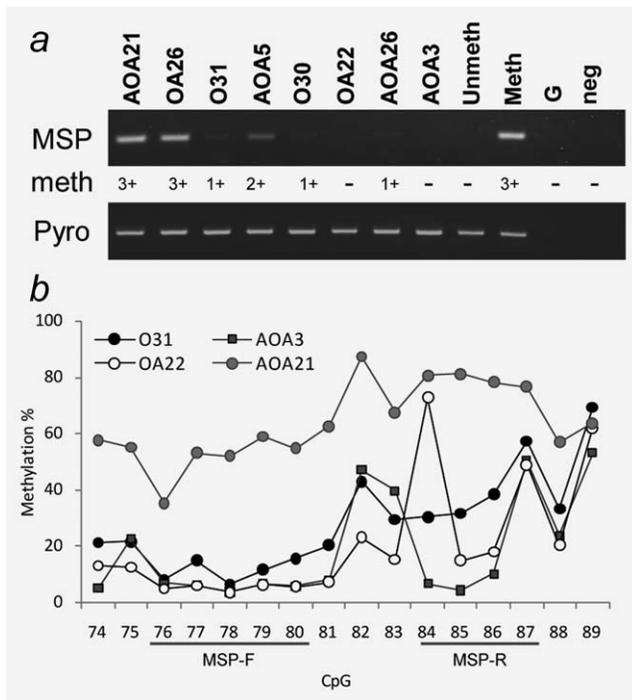


Figure 3. Comparison of MSP and pyrosequencing results. *a*, A representative result of MSP. An 81 bp PCR product of methylation-specific PCR (MSP) was amplified only from methylated DNA. meth, methylation status scored based on MSP result (3+, positive; 2+, weakly positive; 1+, very weakly positive; -, negative). A 166bp PCR product for the pyrosequencing PCR (Pyro) was amplified regardless of the methylation status and was used as a control. Unmeth, fully unmethylated control DNA. Meth, fully methylated control DNA. G, unmodified genomic DNA. neg, no template DNA. *b*, Profiles of the methylation levels by pyrosequencing in selected cases. All four cases had mean methylation above 10% by pyrosequencing and were therefore qualified as methylated. AOA21 (scored as methylated by MSP) showed more than 10% methylation at all sites, while O31 (weakly positive by MSP), OA22 and AOA3 (scored negative by MSP) had methylation levels reaching less than 10% at some or all of the CpGs in the MSP-F region.

between tumors with and without *IDH1/2* mutations in A, AO, OA ($p = 0.002$), AA and O ($p < 0.001$, Fig. 4). The difference was not significant in AOA, pGB or sGB.

Another notable common genetic change that correlated to *MGMT* methylation was total 1p/19q loss. The presence of total 1p/19q loss was significantly correlated with higher mean *MGMT* methylation in O (Mann-Whitney test with FDR multiple test correction, $p < 0.001$), AO ($p = 0.002$) and AOA ($p = 0.020$). However this correlation was not observed in any other tumor types. No other genetic changes examined, including the presence of *TP53* mutation, were correlated to *MGMT* methylation after FDR multiple test correction.

The *MGMT* gene is located at 10q26, where deletions frequently occur in glioblastomas as a result of monosomy 10. We therefore compared *MGMT* methylation levels with the copy number status of 10q26 determined previously^{5,21,33} to establish whether there was a correlation between them. There

was no significant difference in the mean methylation levels between the tumors with or without 10q26 loss (all tumors $p = 0.707$, all glioblastomas $p = 0.803$, Mann-Whitney *U* test).

MGMT methylation and clinical features

There was no statistically significant difference in the incidence of *MGMT* methylation between male and female (chi-squared test or Fisher's exact test). Patients with *MGMT*-methylated tumors were significantly older than those with *MGMT*-unmethylated tumors among As ($p < 0.001$, Mann-Whitney's *U*-test, see Supporting Information Table 2 and Supporting Information Fig. 1a), OAs ($p < 0.001$), AOs ($p = 0.017$) and AOAs ($p = 0.015$). There was no statistically significant difference in age between the patients with *MGMT*-methylated tumors and unmethylated tumors in other tumor types. All patients with *MGMT*-methylated tumors were adult (18 years or older) with the exception of only two pGBs (12 and 15 years, Supporting Information Table 2) and two AAs (both 17 years).

We then investigated a correlation between *MGMT* methylation and survival. Because our previous study suggested that tumors in pediatric and adult patients of the same histological subtype may belong to biologically distinct categories,³⁷ we focused among adult patients with tumors of WHO grade II-IV (18 years or older, 334 patients) in this analysis. A and AOA were excluded from this analysis as there were not enough patients with *MGMT*-unmethylated tumors for comparison (Supporting Information Table 2). A univariate analysis using the log rank test within each subtype showed that the presence of *MGMT* methylation was associated with longer survival only among pGBs ($p = 0.014$), median survival being 301 days (95% Confidence interval (CI): 240–361 days) for methylated and 205 days (95% CI: 178–232 days) for unmethylated cases. Presence of *IDH1/2* mutation ($p = 0.003$) or total 1p/19q loss ($p = 0.028$) was also correlated with longer overall survival. A multivariate analysis using Cox regression was then performed including age and presence of either *MGMT* methylation, *IDH1/2* mutations, or total 1p/19q loss, or any combination of these genetic/epigenetic parameters within each tumor subtype. The results showed that age [$p < 0.001$, Hazard ratio (HR) 1.053, 95% CI 1.037–1.070], presence of *MGMT* methylation ($p = 0.001$, HR 1.695, 95% CI 1.236–2.323) and total 1p/19q loss ($p = 0.006$, HR 5.540, 95% CI 1.641–18.702) were independent prognostic factors among pGBs (Supporting Information Table 3). None of the genetic/epigenetic parameters appeared to be independent prognostic factors in any other tumor types.

Discussion

In this study, we measured methylation levels at 16 CpG sites which we previously determined to be strongly associated with mRNA expression of *MGMT*²⁹ in 362 astrocytic and oligodendroglial tumors of all grades using bisulfite modification and pyrosequencing. This is the most comprehensive

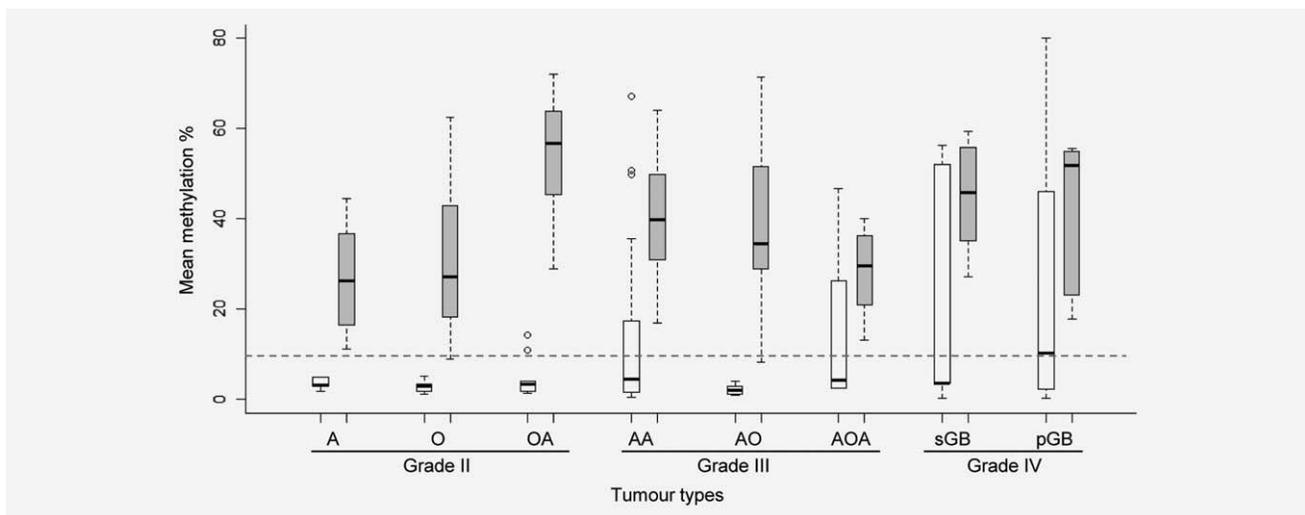


Figure 4. Box plots for the mean *MGMT* methylation levels according to the *IDH1/2* status in each tumor type. Distributions of the methylation levels are indicated by open boxes (no mutations in *IDH1/2*) or filled grey boxes (mutations in *IDH1* or *IDH2*). A, diffuse astrocytomas. O, oligodendrogliomas WHO grade II. OA, oligoastrocytomas WHO grade II. AA, anaplastic astrocytomas. AO, anaplastic oligodendrogliomas. AOA, anaplastic oligoastrocytomas. sGB, secondary glioblastomas. pGB, primary glioblastomas.

study of *MGMT* methylation analysis using pyrosequencing performed on diverse types of gliomas published to date. Pyrosequencing provides quantitative and highly reproducible levels of methylation at each CpG over multiple sites in a time- and labor-efficient way.³⁸ It has advantages over other methods such as MSP, where heterogeneous methylation patterns may result in erroneous results. Our study showed that 24% (17/71) of tumors with significant methylation and scored as positive by pyrosequencing were not detected by MSP. The methylation levels measured by pyrosequencing in these tumors were very low at some of the CpGs within the regions where MSP primers bind, in particular the forward primer (Fig. 3 and Supporting Information Table 1). Thus, pyrosequencing can precisely profile heterogeneous methylation which may not be detected by MSP. To properly assess the efficacy of *MGMT* methylation as a molecular marker to predict clinical outcome, we believe it is crucial to utilize an accurate assay for clinical testing. On the other hand, the quantitative output at individual CpGs with pyrosequencing sets several challenges in determining a significant overall methylation status, an issue especially relevant in the context of clinical testing where a binary classification, *i.e.*, methylated or unmethylated, is required.²⁷ They are as follows.

First, a biologically relevant and clinically practicable (*i.e.*, reasonably small) region within the CGI needs to be defined for the methylation analysis. We have previously studied the whole *MGMT* CGI using a combination of pyrosequencing, real-time quantitative PCR and luciferase reporter assay, and identified a region where the methylation status was most strongly correlated with mRNA expression.²⁹ The pyrosequencing analysis in the present study examines the 16 CpG sites in this region.

Second, the methylation data from multiple sites need to be compiled into a single measure. We found that the patterns of methylation among the 16 sites were very heterogeneous. We have also reported that several individual CpGs, including CpG83, 86, 87 and 89, may be critical for the transcriptional regulation of *MGMT*.²⁹ Thus, focusing on single CpGs carries an inherent risk of missing methylation at other sites. We therefore used the mean methylation level across the 16 sites to represent the methylation status of a case. The mean of the methylation levels across multiple CpG sites measured by pyrosequencing is commonly used to interpret pyrosequencing data.^{39,40}

Finally, a biologically and clinically relevant cut-off value to score methylation needs to be determined. The methylation levels for all data points showed a bimodal distribution (histogram, Fig. 1). Thus, it appears that there are two distinct statuses of methylation in this particular region of the *MGMT* CGI, which supports the idea of converting variable methylation levels into a binary classification, *i.e.*, methylated or unmethylated. We have used 10% as cut-off value (see Results). Among 18 diffuse astrocytomas, this cut-off value clearly separates tumors with low and high expression (Fig. 2).

Ultimately, the optimal criteria and cut-off value have to be those which best predict clinical outcome. In our tumor series, we found age, *MGMT* methylation and total 1p/19q loss as independent prognostic markers among pGBs. One limitation in our investigation is the retrospective nature of the study with the uncontrolled patient cohort treated with various adjuvant regimens before TMZ was introduced in routine clinical practice. We have shown that pyrosequencing can more accurately determine *MGMT* methylation than

MSP, which is currently the most widely used method for clinical testing. However, the efficacy and criteria of MGMT methylation assessment by pyrosequencing as a clinically relevant method to predict prognosis and treatment response for glioma patients needs to be evaluated in a prospectively treated large patient cohort with a sufficient number of tumors representing each subtype in comparison to MSP. Other confounding factors such as the amount of nonneoplastic cells in the samples, which could influence the observed methylation levels, should also be considered when determining criteria in a clinical setting. The pyrosequencing protocol used in this study is currently being assessed in several independent clinical studies including the translation part of the BR12 Clinical Trial (Ref. 41 and Collins *et al.*, in preparation) and the French Multi-centre Study (Quillien *et al.*, in preparation).

When MGMT methylation was scored using the above criteria, the most striking finding in our tumor series was the strong correlation with the presence of IDH1/2 mutations in all types of adult astrocytic and oligodendroglial tumors. All tumors with either IDH1 or IDH2 mutations except two had been independently scored as having significant methylation (mean methylation of 16 CpG sites >10%) of the MGMT gene. Thus, almost all 129 tumors with IDH1/2 mutations also showed increased methylation at one or more CpG sites of MGMT. Moreover, almost all tumors with MGMT methylation had IDH1/2 mutations among A, O, AO and AOA, meaning IDH1/2 mutations and MGMT methylation are nearly 100% concordant in these tumors (Table 1). In other types (AA, OA and sGB), several tumors had MGMT methylation without IDH1/2 mutations, while only a small minority of pGB with MGMT methylation had IDH1/2 mutations. The association between IDH1/2 mutations and MGMT methylation has also been observed by others.^{10,42} However, no data for individual cases were presented in these studies and the virtually invariable presence of MGMT methylation in IDH1/2-mutated tumors as established in our study has not been previously documented. Our observation may also be attributable to the fact that pyrosequencing was more sensitive than MSP to detect methylation.

Recently, several reports have suggested an association between the presence of IDH1/2 mutation and a CpG island methylator phenotype (CIMP).^{14–17} Noushmehr *et al.* profiled DNA methylation genome-wide in 272 GBs in the context of The Cancer Genome Atlas and observed hypermethylation in a distinct set of genes in a subset of tumors, to which the term glioma-CpG island methylator phenotype (G-CIMP) was applied.¹⁶ They further studied methylation of eight selected genes using Methylight, seven of which were found to be methylated in the G-CIMP phenotype, in an independent set of gliomas of various subtypes and malignancy grades, and found that the G-CIMP phenotype was strongly associated with the presence of IDH1 mutations. They suggested that the transcriptional silencing of as yet unknown G-CIMP targets may provide an advantageous environment for the ac-

quisition of IDH1 mutation.¹⁶ Figueroa *et al.* studied for methylation status genome-wide in 385 AMLs, the other human malignancy that frequently has IDH1/2 mutations and found that IDH1/2-mutant AML display global DNA hypermethylation and a specific hypermethylation signature.¹⁴ They suggested that mutation of IDH1/2 may lead to increased methylation through impairment of TET2, which converts 5-methylcytosine to 5-hydroxymethylcytosine in an α -ketoglutarate dependent manner. It has been suggested that 5-hydroxymethylcytosine may act as an intermediate in the pathway that actively demethylates 5-methylcytosine.⁴³ Mutations of IDH1/2 may suppress TET2 through depleting or antagonizing α -ketoglutarate with D-2-hydroxyglutarate, which is a metabolite specific to mutated IDHs.^{12,13,44}

In the light of these reports, it is possible that MGMT methylation may occur as part of the G-CIMP phenotype and thus associated with the presence of IDH1/2 mutations. It has to be pointed out, however, that the causal relationship between IDH1/2 mutations and the methylator phenotype has yet to be experimentally demonstrated. The reduction of α -ketoglutarate in IDH1/2-mutated gliomas has not been reproduced.¹² It is noteworthy to recall that α -ketoglutarate can also be converted from glutamate, which exists in abundance in the brain. No TET2 mutations have so far been reported in gliomas.⁶ Until the direct role of IDH1/2 mutations in increased methylation is established, the chronological order of these two events remains an open question.

In our study, a number of tumors had MGMT methylation but no IDH1/2 mutations, indicating that MGMT methylation may occur independently of IDH status. Based on these observations, an alternative hypothesis would be that MGMT methylation may precede IDH1/2 mutations in gliomas, at least under some circumstances. A biological basis for this may be explained as follows.

The main function of MGMT is to repair O⁶-methylguanine, which is one of the most toxic, mutagenic and carcinogenic DNA adducts.⁴⁵ Although O⁶-methylguanine is best known to be formed by various carcinogens and alkylating agents, it is also generated by endogenous sources such as S-adenosylmethionine (SAM), a reactive methyl group donor physiologically present in living cells.⁴⁵ Persistence of unrepaired O⁶-methylguanine due to MGMT methylation may cause misreading by DNA polymerase and mispairing with thymine instead of cytosine (reviewed in Ref. 46). It has been shown that silencing of MGMT induced by DNA methylation may lead to an increased incidence of G:C to A:T transition mutations.⁴⁶ The vast majority of IDH1/2 mutations in our tumor series are G:C>A:T transitions (119/129, 92.2%, Supporting Information Table 1). The high incidence of such transition mutations in IDH1/2 has also been seen in other large scale studies.^{7,47} It is thus possible that early MGMT methylation may have allowed transition mutations in IDH1/2 to persist. In addition, it has been reported that the incidence of G:C>A:T transition mutations in TP53 is significantly higher in tumors with MGMT methylation as opposed

to those without.⁴⁸ In this study, among all *TP53* point mutations, G:C>A:T transition mutations was 24.2% in *MGMT* methylation cases compared with 13.7% in cases lacking methylation ($p = 0.017$, chi-squared test, see also Supporting Information Table 1). This suggests that *MGMT* methylation may play a role in the early development of glioma through acquisition of a mutator phenotype, thus conferring a selective advantage to tumor cells. However, the number of grade II or III gliomas with *MGMT* methylation and without *IDH* mutation which supports this hypothesis is limited, and our findings need to be confirmed in a larger series of samples. The hypothesis also requires experimental testing.

We have previously proposed a model of glioma development in which *IDH1/2* mutations precede total 1p/19q loss or *TP53* mutations.⁵ This was based on the observation that *IDH1/2* mutations are strongly associated with the presence of either total 1p/19q loss or *TP53* mutations in these tumors, as has also been reported by others.^{5,7,8,11} It now appears that a multistep acquisition of *MGMT* and *IDH1/2* mutations, whichever comes first, followed by either total 1p/19q loss or *TP53* mutations may play a critical role in the early stage of gliomagenesis for astrocytic and oligodendroglial tumors. The previously reported correlations between *MGMT* methylation and either total 1p/19q loss or *TP53* mutations may in fact merely reflect their association with *IDH1/2* mutations.^{48,49} A number of studies have shown that the presence of *IDH1/2* mutations, *MGMT* methylation or total 1p/19q loss predicts therapy response and/or longer survival of the patients.^{2,10,22,23,26,42} Our results, showing that these three abnormalities are strongly associated, raise the question as to which event is a biologically independent predictive/prognostic marker. Wick *et al.* reported in their study of 274 anaplastic gliomas of three different subtypes that *IDH1/2* mutation and *MGMT* methylation were independent prognostic markers, *IDH1/2* mutation providing the best prognostic model, but not total 1p/19q loss in a multivariate analysis.²⁶ On the other hand, van den Bent *et al.* showed in their study of 154 gliomas of mixed grades and subtypes that *IDH1/2* mutation and total 1p/19q loss are independent prognostic factors but not *MGMT* methylation.¹⁰ Sanson *et al.* investigating almost the same number and spectrum of gliomas as the present study showed that *IDH1* mutation was an independent prognostic marker.⁴² *MGMT* methylation data were however not available for all cases. Variations in the methodologies used to detect the genetic/epigenetic abnormalities may partially explain these divergent findings.

References

- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. WHO classification of tumours of the central nervous system, 4th ed. Lyon, France: International Agency for Research on Cancer, 2007.
- Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 2009;10:459–66.
- Riemenschneider MJ, Jeuken JW, Wesseling P, Reifenberger G. Molecular diagnostics of gliomas: state of the art. *Acta Neuropathol* 2010;120:567–84.
- Tabatabai G, Stupp R, van den Bent MJ, Hegi ME, Tonn JC, Wick W, Weller M. Molecular diagnostics of gliomas: the clinical perspective. *Acta Neuropathol* 2010; 120:585–92.

We found that patients with *MGMT*-methylated tumors were significantly younger than those with *MGMT*-unmethylated tumors in As, OAs, AOs and AOAs (Supporting Information Table 2 and Supporting Information Fig. 1a). As expected from the very close association between *MGMT* methylation and *IDH1/2* mutations, a similar finding was observed regarding the *IDH* status (Supporting Information Fig. 1b). Moreover, almost all tumors with *MGMT* methylation except four (222/226, 98.2%) were from patients aged 18 or older (Supporting Information Table 2). The youngest patients with *IDH1/2* mutations among all tumors were 17 years (2 AAs, Supporting Information Table 1). These findings suggest that pediatric astrocytic/oligodendroglial tumors of grade II–III may belong to biologically different entities as compared to their adult counterparts, further corroborating the observations of our previous reports.³⁷

In pGB, the frequency of *MGMT* methylation is comparable to other astrocytic tumors. However, *IDH1/2* mutations are very rare among pGBs, and the great majority of pGBs with *MGMT* methylation do not have *IDH1/2* mutations. We did not find a significant correlation between *MGMT* methylation and any other known genetic abnormalities in pGBs. This lack of correlation makes the timing of *MGMT* methylation in pGB development difficult to establish. It thus appears that if *MGMT* methylation contributes to the development of pGB, it does so with a timing and mechanism different from the other glioma subtypes. These findings further depict pGB as a distinct entity of glioma separate from other astrocytic tumors.

In summary, we quantitatively assessed *MGMT* methylation using bisulfite modification of DNA followed by pyrosequencing at the 16 CpG sites in 362 gliomas of various subtypes and WHO grades. We showed that pyrosequencing is more sensitive than conventional MSP in detecting heterogeneous methylation which predicts decreased mRNA expression. A comprehensive *MGMT* methylation analysis using pyrosequencing identified that almost all tumors with *IDH1/2* mutations had *MGMT* methylation. Although the chronological order of these two events needs to be experimentally established, it would seem probable that *MGMT* methylation may be one of the earliest changes in the development of astrocytic and oligodendroglial tumors of WHO grade II and III. Prospective trials will help validate a clinically practicable pyrosequencing protocol for *MGMT* methylation testing.

Acknowledgements

S.M. is a recipient of the grant from the Molecular Pathology Training Programme sponsored by Cancer Research UK.

5. Ichimura K, Pearson DM, Kocalkowski S, Backlund LM, Chan R, Jones DT, Collins VP. IDH1 mutations are present in the majority of common adult gliomas but are rare in primary glioblastomas. *Neurol Oncol* 2009;11:341–7.
6. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* 2008;321:1807–12.
7. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 2009;360:765–73.
8. Balsl J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathol* 2008;116:597–602.
9. Dubbink HJ, Taal W, van Marion R, Kros JM, van Heuvel I, Bromberg JE, Zonnenberg BA, Zonnenberg CB, Postma TJ, Gijtenbeek JM, Boogerd W, Groenendijk FH, et al. IDH1 mutations in low-grade astrocytomas predict survival but not response to temozolomide. *Neurology* 2009;73:1792–5.
10. van den Bent MJ, Dubbink HJ, Marie Y, Brandes AA, Taphoorn MJ, Wesseling P, Frenay M, Tijssen CC, Lacombe D, Idhah A, van Marion R, Kros JM, et al. IDH1 and IDH2 mutations are prognostic but not predictive for outcome in anaplastic oligodendroglial tumors: a report of the European Organization for Research and Treatment of Cancer Brain Tumor Group. *Clin Cancer Res* 2010;16:1597–604.
11. Watanabe T, Nobusawa S, Kleihues P, Ohgaki H. IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *Am J Pathol* 2009;174:1149–53.
12. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 2009;462:739–44.
13. Zhao S, Lin Y, Xu W, Jiang W, Zha Z, Wang P, Yu W, Li Z, Gong L, Peng Y, Ding J, Lei Q, et al. Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1 α . *Science* 2009;324:261–5.
14. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 2010;18:553–67.
15. Laffaire J, Everhard S, Idhah A, Criniere E, Marie Y, de Reynies A, Schiappa R, Mokhtari K, Hoang-Xuan K, Sanson M, Delattre JY, Thillet J, et al. Methylation profiling identifies 2 groups of gliomas according to their tumorigenesis. *Neurol Oncol* 2010;13:84–98.
16. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, Pan F, Pelloski CE, Sulman EP, Bhat KP, Verhaak RG, Hoadley KA, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 2010;17:510–22.
17. Christensen BC, Smith AA, Zheng S, Koestler DC, Houseman EA, Marsit CJ, Wiemels JL, Nelson HH, Karagas MR, Wrensch MR, Kelsey KT, Wiencke JK. DNA methylation, isocitrate dehydrogenase mutation, and survival in glioma. *J Natl Cancer Inst* 2010;103:143–53.
18. Griffin CA, Burger P, Morsberger L, Yonescu R, Swierczynski S, Weingart JD, Murphy KM. Identification of der(1;19)(q10;p10) in five oligodendrogliomas suggests mechanism of concurrent 1p and 19q loss. *J Neuropathol Exp Neurol* 2006;65:988–94.
19. Jenkins RB, Blair H, Ballman KV, Giannini C, Arusell RM, Law M, Flynn H, Passe S, Felten S, Brown PD, Shaw EG, Buckner JC. A t(1;19)(q10;p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. *Cancer Res* 2006;66:9852–61.
20. Idhah A, Marie Y, Lucchesi C, Pierron G, Manie E, Raynal V, Mosseri V, Hoang-Xuan K, Kujas M, Brito I, Mokhtari K, Sanson M, et al. BAC array CGH distinguishes mutually exclusive alterations that define clinicogenetic subtypes of gliomas. *Int J Cancer* 2008;122:1778–86.
21. Vogazianou AP, Chan R, Backlund LM, Pearson DM, Liu L, Langford CF, Gregory SG, Collins VP, Ichimura K. Distinct patterns of 1p and 19q alterations identify subtypes of human gliomas that have different prognoses. *Neurol Oncol* 2010;12:664–78.
22. Cairncross G, Berkey B, Shaw E, Jenkins R, Scheithauer B, Brachman D, Buckner J, Fink K, Souhami L, Laperriere N, Mehta M, Curran W. Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: intergroup radiation therapy oncology group trial 9402. *J Clin Oncol* 2006;24:2707–14.
23. van den Bent MJ, Carpentier AF, Brandes AA, Sanson M, Taphoorn MJ, Bensen HJ, Frenay M, Tijssen CC, Grisold W, Sipos L, Haaxma-Reiche H, Kros JM, et al. Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European organisation for research and treatment of cancer phase III trial. *J Clin Oncol* 2006;24:2715–22.
24. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer* 2004;4:296–307.
25. van den Bent MJ, Dubbink HJ, Sanson M, van der Lee-Haarloo CR, Hegi M, Jeuken JW, Idhah A, Brandes AA, Taphoorn MJ, Frenay M, Lacombe D, Gorlia T, et al. MGMT promoter methylation is prognostic but not predictive for outcome to adjuvant PCV chemotherapy in anaplastic oligodendroglial tumors: a report from EORTC brain tumor group study 26951. *J Clin Oncol* 2009;27:5881–6.
26. Wick W, Hartmann C, Engel C, Stoffels M, Felsberg J, Stockhammer F, Sabel MC, Koepfen S, Ketter R, Meyermann R, Rapp M, Meisner C, et al. NOA-04 randomized phase III trial of sequential radiochemotherapy of anaplastic glioma with procarbazine, lomustine, and vincristine or temozolomide. *J Clin Oncol* 2009;27:5874–80.
27. Weller M, Stupp R, Reifenberger G, Brandes AA, van den Bent MJ, Wick W, Hegi ME. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nat Rev Neurol* 2010;6:39–51.
28. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352:997–1003.
29. Malley DS, Hamoudi RA, Kocalkowski S, Pearson DM, Collins VP, Ichimura K. A distinct region of the MGMT CpG island critical for transcriptional regulation is preferentially methylated in glioblastoma cells and xenografts. *Acta Neuropathol* 2011;121:651–61.
30. Ichimura K, Bolin MB, Goike HM, Schmidt EE, Moshref A, Collins VP. Deregulation of the p14ARF/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G1-S transition control gene abnormalities. *Cancer Res* 2000;60:417–24.
31. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999;59:793–7.
32. Zeng N, Liu L, McCabe MG, Jones DT, Ichimura K, Collins VP. Real-time quantitative polymerase chain reaction (qPCR) analysis with fluorescence resonance energy transfer (FRET) probes reveals differential expression of the four ERBB4 juxtamembrane region variants between medulloblastoma and pilocytic astrocytoma. *Neuropathol Appl Neurobiol* 2009;35:353–66.
33. Jones DT, Ichimura K, Liu L, Pearson DM, Plant K, Collins VP. Genomic analysis of pilocytic astrocytomas at 0.97 Mb resolution shows an increasing tendency toward chromosomal copy number change with age. *J Neuropathol Exp Neurol* 2006;65:1049–58.
34. Schmidt EE, Ichimura K, Goike HM, Moshref A, Liu L, Collins VP. Mutational profile of the PTEN gene in primary human astrocytic tumors and cultivated xenografts. *J Neuropathol Exp Neurol* 1999;58:1170–83.
35. Harris LC, Potter PM, Tano K, Shiota S, Mitra S, Brent TP. Characterization of the promoter region of the human O6-methylguanine-DNA methyltransferase gene. *Nucleic Acids Res* 1991;19:6163–7.
36. Jones DT, Kocalkowski S, Liu L, Pearson DM, Backlund LM, Ichimura K, Collins VP. Tandem duplication producing a novel oncogenic BRAF fusion gene defines the majority of pilocytic astrocytomas. *Cancer Res* 2008;68:8673–7.
37. Jones DT, Mulholland SA, Pearson DM, Malley DS, Openshaw SW, Lambert SR, Liu L, Backlund LM, Ichimura K, Collins VP. Adult grade II diffuse astrocytomas are genetically distinct from and more aggressive than their paediatric counterparts. *Acta Neuropathol* 2011;121:753–61.
38. Tost J, El Abdalaoui H, Gut IG. Serial pyrosequencing for quantitative DNA methylation analysis. *Biotechniques* 2006;40:721–2, 4, 6.
39. Dunn J, Baborie A, Alam F, Joyce K, Moxham M, Sibson R, Crooks D, Husband D, Shenoy A, Brodbelt A, Wong H, Liloglou T, et al. Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. *Br J Cancer* 2009;101:124–31.
40. Karayan-Tapon L, Quillien V, Guilhot J, Wager M, Fromont G, Saikali S, Etcheverry A, Hamlat A, Loussouarn D, Campion L, Campone M, Vallette FM, et al. Prognostic value of O6-methylguanine-DNA methyltransferase status in glioblastoma patients, assessed by five different methods. *J Neurooncol* 2010;97:311–22.

41. Brada M, Stenning S, Gabe R, Thompson LC, Levy D, Rampling R, Erridge S, Saran F, Gattamaneni R, Hopkins K, Beall S, Collins VP, et al. Temozolomide versus procarbazine, lomustine, and vincristine in recurrent high-grade glioma. *J Clin Oncol* 2010; 28:4601–8.
42. Sanson M, Marie Y, Paris S, Idbaih A, Laffaire J, Ducray F, El Hallani S, Boisselier B, Mokhtari K, Hoang-Xuan K, Delattre JY. Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J Clin Oncol* 2009;27:4150–4.
43. Wu SC, Zhang Y. Active DNA demethylation: many roads lead to Rome. *Nat Rev Mol Cell Biol* 2010;11:607–20.
44. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* 2011;19:17–30.
45. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis* 2004;19:169–85.
46. Esteller M, Herman JG. Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. *Oncogene* 2004;23:1–8.
47. Hartmann C, Meyer J, Bals J, Capper D, Mueller W, Christians A, Felsberg J, Wolter M, Mawrin C, Wick W, Weller M, Herold-Mende C, et al. Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta Neuropathol* 2009; 118:469–74.
48. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H. Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C → A:T mutations of the TP53 tumor suppressor gene. *Carcinogenesis* 2001;22:1715–9.
49. Mollema M, Wolter M, Felsberg J, Collins VP, Reifenberger G. Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. *Int J Cancer* 2005;113:379–85.