Predictive Biomarkers and Personalized Medicine

MGMT-STP27 Methylation Status as Predictive Marker for Response to PCV in Anaplastic Oligodendrogliomas and Oligoastrocytomas. A Report from EORTC Study 26951

Martin J. van den Bent1,4, Lale Erdem-Eraslan1, Ahmed Idbaih5, Johan de Rooi2, Paul H.C. Eilers2, Wim G.M. Spliet6, Wilfred F.A. den Dunnen7, Cees Tijsen8, Pieter Wesseling9,10, Peter A.E. Sillevis Smitt1, Johan M. Kros3, Thierry Gorlia11, and Pim J. French1

Abstract

**Purpose:** The long-term follow-up results from the EORTC-26951 trial showed that the addition of procarbazine, CCNU, and vincristine (PCV) after radiotherapy increases survival in anaplastic oligodendrogliomas/oligoastrocytomas (AOD/AOA). However, some patients appeared to benefit more from PCV treatment than others.

**Experimental Design:** We conducted genome-wide methylation profiling of 115 samples included in the EORTC-26951 trial and extracted the CpG island hypermethylated phenotype (CIMP) and MGMT promoter methylation (MGMT-STP27) status.

**Results:** We first show that methylation profiling can be conducted on archival tissues with a performance that is similar to snap-frozen tissue samples. We then conducted methylation profiling on EORTC-26951 clinical trial samples. Univariate analysis indicated that CIMP+ or MGMT-STP27 methylated tumors had an improved survival compared with CIMP− and/or MGMT-STP27 unmethylated tumors [median overall survival (OS), 1.05 vs. 6.46 years and 1.06 vs. 3.8 years, both \( P < 0.0001 \) for CIMP and MGMT-STP27 status, respectively]. Multivariable analysis indicates that CIMP and MGMT-STP27 are significant prognostic factors for survival in presence of age, sex, performance score, and review diagnosis in the model. CIMP+ and MGMT-STP27 methylated tumors showed a clear benefit from adjuvant PCV chemotherapy: the median OS of CIMP+ samples in the RT and RT-PCV arms was 3.27 and 9.51 years, respectively (\( P = 0.0033 \)); for MGMT-STP27 methylated samples, it was 1.98 and 8.65 years. There was no such benefit for CIMP− or for MGMT-STP27 unmethylated tumors. MGMT-STP27 status remained significant in an interaction test (\( P = 0.003 \)). Statistical analysis of microarray (SAM) identified 259 novel CpGs associated with treatment response.

**Conclusions:** MGMT-STP27 may be used to guide treatment decisions in this tumor type. Clin Cancer Res; 19(19); 5513–22. ©2013 AACR.

Introduction

In 1995, a large European phase III clinical trial ("EORTC-26951") was initiated to examine the effects of adjuvant procarbazine, CCNU, and vincristine (PCV) chemotherapy in anaplastic oligodendrogial tumors [oligodendrogliomas (AOD) and oligoastrocytomas (OA)]; ref. 1. The long-term follow-up of this study shows that adjuvant PCV chemotherapy given after radiotherapy improves overall survival (OS) in this tumor type (2). Molecular analysis has shown that in particular patients in which the tumor has a combined deletion of the 1p and 19q chromosomal arms (1p/19q co-deleted tumors) appeared to benefit from the addition of PCV treatment. A trial with similar inclusion criteria and treatment protocol, RTOG 9402, showed comparable results: patients with oligodendroglioma who harbor tumors with a 1p/19q co-deletion showed a benefit from neoadjuvant PCV chemotherapy (3, 4). Combined, these trials have changed the standard of care for 1p/19q co-deleted tumors.

Results from both trials also indicate that a subset of patients benefit from (neo-)adjuvant PCV even though the tumor has retained 1p and/or 19q. In search for markers that identify those responsive patients, post hoc analysis on...
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Translational Relevance

In this study, we have conducted genome-wide methylation profiling on samples treated within EORTC study 26951. Although the study was set up to further explore the predictive significance of CpG island hypermethylated phenotype (CIMP) status, our results show a more predictive value of MGMT promoter methylation status as calculated from the methylation array data (MGMT-STEP27). Of note, MGMT promoter methylation status as determined using MLPA does not show such a predictive value but this technique assesses a different set of CpG sites. Our data therefore confirm that different CpG sites within the MGMT promoter have different predictive power for outcome to alkylating chemotherapy. Exploratory analysis also identified a set of 259 CpG sites that are associated with benefit from treatment. In summary, we show using clinical trial samples that methylation profiling can identify patients with anaplastic oligodendroglioma and anaplastic mixed oligoastrocytomas who benefit from adjuvant procarbazine, CCNU, and vincristine (PCV) chemotherapy.

Material from the EORTC-26951 trial has shown that IDH1 mutational status and MGMT promoter methylation may be correlated to increased benefit to adjuvant PCV chemotherapy (5, 6). Statistical tests for interaction remained however negative, perhaps due to the relatively small number of patients of whom material could be analyzed. Similarly, gene expression profiling showed that tumors assigned to “intrinsic glioma subtype-9” (IGS-9, containing a high percentage of tumors with 1p/19q codeletion) showed benefit from adjuvant PCV chemotherapy (7). However, tumors assigned to IGS-17 (of which the majority had retained 1p and/or 19q) also showed a trend toward benefit from adjuvant PCV. These findings suggest there may be alternative molecular factors that predict benefit from adjuvant PCV chemotherapy.

In 2011, we have reported on a study in which genome-wide methylation profiling was conducted on snap-frozen tissue samples of patients included in the EORTC-26951 trial. Similar to other studies, we found a strong correlation between a genome-wide hypermethylation phenotype (“CIMP”) and survival (8–11). However, preliminary analysis also indicated that CIMP status may be predictive for a genome-wide hypermethylation phenotype (CIMP) status, our results show a more predictive value of MGMT promoter methylation status as calculated from the methylation array data (MGMT-STEP27). Of note, MGMT promoter methylation status as determined using MLPA does not show such a predictive value but this technique assesses a different set of CpG sites. Our data therefore confirm that different CpG sites within the MGMT promoter have different predictive power for outcome to alkylating chemotherapy. Exploratory analysis also identified a set of 259 CpG sites that are associated with benefit from treatment. In summary, we show using clinical trial samples that methylation profiling can identify patients with anaplastic oligodendroglioma and anaplastic mixed oligoastrocytomas who benefit from adjuvant procarbazine, CCNU, and vincristine (PCV) chemotherapy.

Materials and Methods

Patient samples

Patients were considered eligible in the EORTC-26951, if they had been diagnosed by the local pathologist with an AOD or an AOA according to the 1993 WHO classification. Details of the eligibility criteria and the consolidated standards on reporting trials (Consort) flow diagram have been described previously (1). All samples that could be retrieved from this study were included (n = 115). A central pathology review was conducted on 345 of 368 samples, 113 of 115 for samples used in present study. The 2 cases were omitted in the multivariate analysis that included review diagnosis as factor. All analyses using histologic diagnosis made use of the review diagnosis. Patient/sample characteristics are detailed in Supplementary Table S1. Analysis of 1p/19q LOH, EGFR amplification, IDH1 mutations (as determined by direct sequencing of the c.395G mutation hotspot), and MGMT promoter methylation as assessed by MLPA on EORTC-26951 samples were described previously (1, 6, 17–19). Areas with high tumor content (>70%–80%) were highlighted by the pathologist (J.M. Kros) before conducting the IDH1 mutation analysis.

Six additional samples were collected from the Erasmus MC brain tumor tissue bank to test the performance of Illumina 450 k beadchips. One part of these tumors was fixed in formalin and embedded in paraffin (FFPE). However, recent technological advances suggest that FFPE tissues can be now used for genome-wide methylation analysis which increases the number of EORTC-26951 samples available for methylation profiling.

Apart from the potential predictive value of CIMP status, methylation of the MGMT promoter has been established as a predictive marker for outcome to chemo-irradiation with temozolomide in gliomas (12–14). In patients treated within EORTC-26951, we were unable to establish such an association between MGMT promoter methylation and benefit from adjuvant PCV chemotherapy (6). However, the assay used for our study (MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification) interrogated different CpG sites than the assay used for the studies whereby MGMT promoter methylation status predicted benefit from chemotherapy (MS-PCR). Two regions within the MGMT promoter are correlated with mRNA expression levels, DMR1 and DMR2, of which the latter is interrogated by MS-PCR (15). Recently, a study identified 2 CpG sites that are correlated to MGMT RNA expression levels and patient survival (16). These CpGs are located on the Humanmethylshot 27 and 450 arrays. These CpG sites lie within DMR1 and DMR2 and are not assessed by the MS-MLPA assay that we used to assess MGMT promoter methylation. A prediction model, MGMT-STEP27, was generated to determine the MGMT promoter methylation status from the methylation data. We used this model as an alternative method to assess MGMT promoter methylation and compared it with our MS-MLPA findings.

Nucleic acid isolation and array hybridization

For FF tissues, gDNA was isolated from 5 to 40 cryostat sections of 40-μm thickness using the QIAamp DNA Mini...
Kit (Qiagen) according to the manufacturer’s instructions. For FFPE tissue, gDNA was isolated from 8 to 10 sections of 10-μm thickness from paraffin blocks using the QIAamp FFPE DNA Kit. Methylation profiling was conducted using 1 μg of gDNA which was subjected to bisulfite modification using the EZ DNA Methylation Kit (Zymo Research Company). Bisulfite-converted DNA was then hybridized to Illumina Infinium HumanMethylation27 arrays (Illumina) by Service XS or to Infinium HumanMethylation 450 arrays (Illumina) run by ArosAB according to standard Illumina protocols. Data from 50 Infinium HumanMethylation 27 arrays samples were previously reported (11). All array data is available via the NCBI GEO datasets, accession number GSE48462. Infinium HumanMethylation 27 arrays interrogate 27,578 CpG sites across 14,476 genes; the 450 arrays interrogate >485,000 CpG sites and contain most, but not all, of 27 k array content. The 450 k array contains 1,432 of 1,503 probesets used to determine CIMP status.

Statistical analysis

Samples were assigned to either the CIMP+ or CIMP− subtype using ClusterRepro (an R package) based on the nearest centroid as described previously (20) according to the 1,503 CpGs described by the TCGA (10). The MGMT promoter methylation status (MGMT-STM27) was extracted from 2 CpG sites on the methylation array (cg12434587 and cg12981137) as described (16). Differences between the Kaplan–Meier survival curves were calculated by the log-rank (Mantel–Cox) test using GraphPad Prism version 5.00 for Windows, GraphPad Software. Comparisons between frequencies were calculated by the Fisher exact test in which the P < 0.05 was considered to indicate significant differences. Progression-free survival (PFS) and overall survival (OS) were computed from randomization to date of event (progression and/or death) or censored at the date of last visit. To assess interfactor relationship, we used the well-established Spearman correlation coefficient (SCC) which provided for binary and ordinal factors the same measures of association as the phi coefficient. With the available sample size, clinically relevant coefficient (SCC ≥ 0.4) all had a significance lower than a conservative threshold of 1% (P < 0.01). The log-rank test for interaction was used to compare treatment effect in different molecular subsets. Significance analysis of microarrays (SAM) was conducted using SAMR, an R package, making use of censored survival outcome (21, 22). M-values of methylation were used for SAM analysis (23); CpGs located on the X or Y chromosomes were removed from all analysis. The variance inflation factor (VIF) was assessed to determine the severity of multicolinearity between factors. For a factor, $VIF = 4 (\sqrt{4} = 2)$ means that the SE of the coefficient of that factor is 2 times as large as it would be if it was uncorrelated to other factors. A VIF bigger than 2 is considered as large. Pathway analysis was conducted using IPA (Ingenuity Systems).

Results

Evaluation of the suitability of FFPE material for genome-wide methylation profiling

Most clinical trial samples are fixed in formalin and embedded in paraffin. Our first experiments therefore evaluated the suitability of FFPE material for genome-wide methylation profiling. For this, we generated methylation profiles of 6 glioma samples, using 3 to 5 replicates per sample. Replicates included (i) fresh-frozen (FF) samples (n = 4), (ii) FFPE of sections cut from a paraffin block (n = 6), (iii) FFPE of sections that were scraped from microscope slides to allow selecting for the area with highest tumor density (n = 6), (iv) a technical replicate of ii (n = 3), and (v) technical replicates of iii (n = 4). The time in paraffin of these samples was between 15 and 17 years.

A first evaluation showed that the signal intensities of the methylated and unmethylated probes of the array were higher in the FF samples than in the FFPE samples (6.882 ± 931 vs. 3.946 ± 1,436, P = 0.002; Supplementary Fig. S1). Within the FFPE samples, scraped sections showed a significantly lower intensity than the whole FFPE sections (2.797 ± 962 vs. 5.096 ± 672, P < 0.001). Despite of these differences in signal intensities, there was no obvious difference between the different tissue sources in the beta values [methylated/(methylated + unmethylated), Supplementary Fig. S1]. Indeed, reproducibility between the beta values of technical replicates was high: $R^2 = 0.987 ± 0.009$ (range, 0.960–0.994). Reproducibility between the beta values of whole tissue sections and scraped microscope slides was also high: $R^2 = 0.976 ± 0.012$ (range, 0.960–0.985), which indicates that the enrichment for areas with high tumor content only marginally influences results in these samples.

When comparing the beta values of the FF samples with those of the FFPE samples, we also observed a high correlation between the replicates $R^2 = 0.961 ± 0.023$ (range, 0.919–0.987) when using all the 480 k CpG sites. This is remarkably high, especially when considering the time samples were stored in paraffin (15–17 years). This long storage time therefore does not significantly compromise the performance of the platform. The high correlation between all replicates is also shown in an unsupervised clustering analysis conducted on the 2,000 most variable CpG sites in which samples from the same replicate clustered closely together (Fig. 1). These data show that the Illumina humanmethylaton450 platform can be used to study CpG methylation on both FF and FFPE samples.

Previous methylation data on EORTC-26951 samples were generated using the HumanMethylation27 beadchip. To determine whether the methylation array used for FFPE profiling (HumanMethylation450) is comparable to the HumanMethylation27 beadchip, we analyzed 48 FFPE samples on both platforms. A high sample correlation in all samples across the 2 array types was observed when using the beta values of probesets that are overlapping on both platforms (n = 25,978), $R^2 = 0.960 ± 0.020$ (range, 0.854–0.982). These data show that the performance of the
HumanMethylation450 beadchip is similar to the HumanMethylation27 beadchip.

We then conducted methylation profiling on 66 samples of the EORTC-26951 trial. Of these, 8 were FF and run on HumanMethylation27 beadchips, 59 were FFPE and run on HumanMethylation450 beadchips. Data were combined with 50 samples that were analyzed previously using FF samples on HumanMethylation27 beadchips (11). Data are available via NCBI GEO datasets, GSE48462. Samples 21 and 224 were run on both platforms, data from the HumanMethylation450 beadchips for these two samples were used in the analysis. This sample cohort did not differ from the entire EORTC patient cohort with respect to age, sex, performance status, diagnosis, tumor location, entire EORTC patient cohort with respect to age, sex, performance status, diagnosis, tumor location, performance status, diagnosis, tumor location, performance status, diagnosis, tumor location.

Univariate analysis indicates that patients with CIMP+ tumors have a more favorable prognosis than CIMP− tumors (6.65 vs. 1.05 years for OS and 3.69 vs. 0.50 years for PFS; Table 1 and Fig. 2). Similarly, MGMT−STP27 methylated samples have a more favorable prognosis than MGMT−STP27 unmethylated samples (3.6 vs. 1.06 years for OS and 3.69 vs. 0.50 years for PFS; Table 1 and Fig. 3). Multivariate analysis indicates that CIMP and MGMT−STP27 status are prognostic factors for survival in the presence of clinical and pathological parameters (age,

CIMP and MGMT methylation status are predictive for benefit from PCV chemotherapy

Of the 115 samples that were profiled, 66 were CIMP+ and 49 CIMP−. The MGMT promoter methylation status (MGMT−STP27) extracted from the genome-wide methylation data identified 88 of 115 samples with a methylated MGMT promoter and 27 of 115 with an unmethylated promoter. CIMP and MGMT−STP27 status were correlated: of the 66 CIMP+ samples, 60 had a methylated MGMT promoter, 6 were unmethylated. Of the 49 CIMP- samples, 28 had a methylated MGMT promoter, 21 were unmethylated. 1p/19q codeletion was identified in 29 of 111 samples. All of the 48 CIMP− had retained 1p/19q as did 34 of 63 CIMP+ samples. Twenty-six of 27 MGMT−STP27 unmethylated samples had retained 1p/19q as did 56 of 84 MGMT−STP27 methylated samples. Correlation of CIMP and MGMT−STP27 status with other molecular markers and associated survival data are shown in Supplementary Table S3.

Univariate analysis indicates that patients with CIMP+ tumors have a more favorable prognosis than CIMP− tumors (6.65 vs. 1.05 years for OS and 3.69 vs. 0.50 years for PFS; Table 1 and Fig. 2). Similarly, MGMT−STP27 methylated samples have a more favorable prognosis than MGMT−STP27 unmethylated samples (3.6 vs. 1.06 years for OS and 3.69 vs. 0.50 years for PFS; Table 1 and Fig. 3). Multivariate analysis indicates that CIMP and MGMT−STP27 status are prognostic factors for survival in the presence of clinical and pathological parameters (age,
sex, performance score, type of surgery, and review diagnosis. Table 1). They are no longer independent prognostic variables when all clinical and molecular parameters and histology (Clinical) or including all molecular and clinical parameters and review diagnosis (All). Calculations are based on 107, 112, and 104 observations, respectively (between brackets: number of events). Depicted are the results for OS (top) and PFS (bottom). Type of surgery categories included: biopsy, partial resection, and total resection. Performance status is based on WHO-ECOG scoring (0, 1, or 2); all categories were used as variables in the analysis. Age was used as continuous variable. Values in bold are significant if \( P < 0.05 \).
When stratified for treatment, patients with CIMP+ tumors showed a clear benefit from adjuvant PCV chemotherapy, both for OS and PFS (Table 2, Fig. 2). For example, the median OS of CIMP+ samples in the RT and RT-PCV arms was 3.47 and 9.51 years, respectively. For CIMP-/C0 tumors, there was no such benefit for OS, although there was a slight increase in PFS 0.68 vs. 0.47 years. These data show that CIMP-positive status is predictive, at least for OS, for benefit from adjuvant PCV in patients treated within EORTC-26951. The interaction test, however, remained above the threshold of significance (P = 0.07, Fig. 4).

A more pronounced benefit from treatment was observed in MGMT-STP27 methylated samples (Fig. 3): Median OS of MGMT-STP27 methylated samples in the RT and RT-PCV arms was 1.98 and 6.85 years, respectively. For PFS, it was 0.73 and 5.36 years. For MGMT-STP27 unmethylated, there was no such benefit, neither for OS nor for PFS. MGMT-STP27 status was highly significant in the interaction test (P = 0.003, Fig. 4).

Identification of CpG sites associated with survival and response to treatment

SAM analysis identified a total of 13,852 CpG sites that are associated with survival when using the entire dataset. This large number of CpGs (approximately half of all probesets on the array) is related to the tumors’ CIMP status and is illustrative for the large differences between these 2 subtypes. SAM analysis also identified 13 probesets and 3,921 probesets associated with survival in the CIMP-/C0 and in the CIMP+ subtype, respectively. Hierarchical clustering based on these CpGs identified 2 distinct subtypes that indeed correlated with survival in our dataset (Supplementary Fig. S3).

We next aimed to identify CpG sites associated with benefit from treatment. For this, we first conducted SAM analysis to identify CpG sites associated with survival in the RT-PCV arm and then removed all CpGs that were associated with survival in the RT-only arm. To increase stringency, SAM analysis on the RT-only arm was conducted used...
relaxed criteria (false discovery rate (fdr) cutoff of 0.05), whereas stringent criteria were used to identify CpGs in the RT-PCV arm (fdr cutoff of 0.001). Our analysis identified 13,912 and 3,308 CpGs associated with survival in the RT and RT-PCV arms, respectively. Most of the 3,308 CpGs identified by analysis of the RT-PCV arm were also present in the 13,912 CpGs identified by analysis of the RT arm. However, 259 CpGs were uniquely associated with survival in the RT-PCV arm (Supplementary Table S4). The strongest differentially methylated CpG site identified this way was MGMT- STP27 methylated.

Figure 3. MGMT- STP27 status is prognostic for survival in samples of the EORTC-26951 clinical trial. Kaplan–Meier survival curves show that patients harboring MGMT- STP27 methylated tumors have a better prognosis than patients that with MGMT- STP27 unmethylated tumors. A, PFS, B, OS. C–F, MGMT- STP27 status is predictive for benefit to adjuvant PCV chemotherapy. Kaplan–Meier survival curves showing that patients harboring MGMT- STP27 methylated tumors benefit both in PFS (C) and in OS (D) following adjuvant PCV chemotherapy. Patients harboring MGMT- STP27 unmethylated tumors do not show benefit from adjuvant PCV chemotherapy both in PFS (E) and in OS (F). For all graphs, black lines indicate the MGMT- STP27 methylated samples, gray lines indicate the MGMT- STP27 unmethylated samples. Dashed lines are RT only, uninterrupted lines RT-PCV.

Table 2. CIMP and MGMT- STP27 status predict benefit from adjuvant PCV treatment

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<td>0.68</td>
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<td>0.95 (0.44–2.10)</td>
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cg12981137, a probeset that is 1 of the 2 CpGs used to determine the tumors’ MGMT-STOP27 status. The top networks associated with the 259 CpGs associated with treatment response involve ‘lipid metabolism,’ ‘cancer,’ ‘cellular development,’ and ‘cellular movement’ as determined by Ingenuity Pathway Analysis.

Discussion
In this study, we have conducted genome-wide methylation profiling on samples treated within EORTC study 26951 on adjuvant PCV chemotherapy in anaplastic oligodendroglial tumors. Although the study was set up to further explore the predictive significance of CIMP status, the results show a more predictive value of MGMT promoter methylation status as calculated from the methylation array data. Our data expand on the predictive power of MGMT promoter methylation status by showing it not only predicts benefit from temozolomide chemotherapy in patients with glioblastoma (12–14) but also predicts benefit from PCV chemotherapy in patients with AOA and AOD. Of note, the MGMT-STOP27 has thus far only been tested on snap-frozen tissue samples (16).

Interestingly, previous experiments on EORTC-26951 clinical trial material failed to identify a predictive effect of MGMT promoter methylation status. In the previous study, however, MGMT promoter methylation status was determined using MLPA which interrogates a different set of CpG sites than MGMT-STOP27 (16, 24). Indeed, the Spearman correlation between MS-MLPA and MGMT-STOP27 was only 0.56. This confirms that different CpG sites within the MGMT promoter have different predictive power for outcome to alkylating chemotherapy. Other studies that confirmed the predictive power of MGMT promoter methylation also examined CpG sites that are different from those examined by the MLPA assay (12–14, 25). For example, the NOA-08 study has validated the role of MGMT determination in newly diagnosed glioblastoma (14). In addition to examining different CpG sites, the previous OS analysis of the MS-MLPA on MGMT promoter methylation was based on the outcome data of the 2006 report on EORTC-26951. With longer follow-up, a long-term benefit of adjuvant PCV chemotherapy has been established, in particular of the 1p1/9q co-deleted tumors.

Bady and colleagues have conducted an analysis of the best CpG sites to predict MGMT promoter methylation status on the Illumina Beadchip27 and 450 platforms (16). However, it is possible that other CpGs that lie within the MGMT promoter provide an even greater predictive power than those of MGMT-STOP27. To determine this, a further detailed analysis of the MGMT promoter region needs to be conducted in which methylation on all CpGs, but especially those in DMR1 and DMR2, are correlated to patient outcome. Data should then be validated on material derived from an independent trial.

Our observation that CIMP+ tumors benefit from adjuvant PCV chemotherapy is in line with other data obtained in anaplastic oligodendrogliomas: 1p/19q codeleted tumors are virtually all CIMP+, and we and others have reported that anaplastic oligodendrogliomas with 1p/19q codeletion benefit from (neo) adjuvant PCV (4, 19, 26; see also ref. 27). Finally, the gene expression subtypes in the EORTC-26951 trial that benefit from adjuvant PCV (IGS-9 and IGS-17) are also CIMP+ (7).

CIMP status is also highly correlated with IDH1 mutation status (Spearman correlation, ρ = 0.77), although 14/60 CIMP+ tumors are IDH1 wild-type. Recent evidence indicates that the hypermethylated phenotype is caused by metabolic changes due to mutations in the IDH1 gene (28–30). Previous analyses on EORTC-26951 trial material also showed an association between IDH1 mutations and treatment response. However, impact on outcome to PCV from both IDH1 mutation status and CIMP status remained nonsignificant in tests for interaction (5, 11). Apart from the present observation that
CIMP status is predictive for benefit from adjuvant PCV chemotherapy, we also show that CIMP status is prognostic for survival. These data have been corroborated in other studies (8, 10, 11).

The 14 samples in our study that were IDH1 wt but CIMP+ may be the result of assay sensitivity (either in the determination of IDH1 or CIMP status), presence of other mutations producing R-2-hydroxyluretate [either rare mutations in IDH1 (ref. 31) or mutations in IDH2] or a true positive finding that there are indeed CIMP+ tumors that are IDH1 wt. It should be noted that other groups have also identified CIMP+ tumors that are IDH1 wt. For example, in their original manuscript, the TCGA has identified 2 of 12 CIMP+ tumors that are IDH1 wt (10). This ratio of CIMP+ but IDH1 wt tumors is highly similar to that found in present study (14 of 63 or 22%).

Exploratory analysis of our dataset identified a set of CpGs that are associated with benefit from adjuvant PCV chemotherapy. One of the CpG sites identified by our analysis was cg12981137 which was the strongest candidate indentified in this analysis. This probset is located within the MGMT promoter region and is a part of the previously defined MGMT-STP27 2 CpG site predictor profile of response to chemotherapy in glioblastoma. The second CpG of the MGMT-STP27 predictor was associated with survival in both treatment arms and therefore was not specific for OS benefit after RT-PCV. For all of the identified 259 CpGs associated with treatment response, it was always the methylated form that was associated with benefit from treatment.

One limitation of our study is that we were only able to analyze a subset of patients treated within EORTC-26951, although most characteristics of samples included versus those not included are similar. However, treatment effect of patients included is larger than patients not included (Supplementary Fig. S2). Also, our sample cohort is relatively modest in size (115) and our analysis is post hoc (retrospective testing). Our results therefore require validation in an additional independent cohort to firmly establish the predictive effect of CIMP status, MGMT-STP27 status, and the CpGs associated with treatment response.

The practical consequence of our study is that the MGMT promoter methylation status needs to be determined in AOD. At present, there are different techniques available the promoter methylation status needs to be determined in AOD. Although CIMP status is correlated to benefit to PCV chemotherapy, our data show a significant predictive effect of the MGMT-STP27 status, although validation on an independent data-set is warranted. As only the MGMT-STP27 methylated samples show benefit from PCV, MGMT-STP27 could be used to guide treatment decisions in this tumor type.

Disclosure of Potential Conflicts of Interest
M.J. van den Bent has received Commercial Research Grant from Roche, Honorarium from Speakers Bureau of MSD, and is a Consultant/Advisory Board member of 283 and Abbott. No potential conflicts of interest were disclosed. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

Authors’ Contributions
Conception and design: M.J. van den Bent, J.J. de Rooi, C. Tijssen, J.M. Kros, P.J. French
Development of methodology: M.J. van den Bent, J.J. de Rooi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. van den Bent, W. Spliet, W.F. den Dunnen, C. Tijssen, P. Wesseling, J.M. Kros, P.J. French
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J. van den Bent, A. Idbaih, J.J. de Rooi, P.H. Eilers, C. Tijssen, P. Wesseling, T. Gorlia, P.J. French
Writing, review, and/or revision of the manuscript: M.J. van den Bent, L.E. Erazlan, A. Idbaih, P.H. Eilers, W. Spliet, C. Tijssen, P. Wesseling, P.A.S. Smitt, J.M. Kros, T. Gorlia, P.J. French
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. van den Bent, W.F. den Dunnen, J.M. Kros
Study supervision: M.J. van den Bent, P.J. French

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Martin J. van den Bent, Lale Erdem-Eraslan, Ahmed Idbaih, et al.


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