Malignant astrocytomas of elderly patients lack favorable molecular markers: an analysis of the NOA-08 study collective

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Background. The number of patients age > 65 years with malignant gliomas is increasing. Prognosis of these patients is worse compared with younger patients. To determine biological differences among malignant gliomas of different age groups and help to explain the survival heterogeneity seen in the NOA-08 trial, the prevalence and impact of recently established biomarkers for outcome in younger patients were characterized in elderly patients.

Methods. Prevalences of mutations of isocitrate dehydrogenase 1 (IDH1) and histone H3.3 (H3F3A), the glioma cytosine–phosphate–guanine island methylator phenotype (G-CIMP), and methylation of alkylpurine DNA N-glycosylase (APNG) and peroxiredoxin 1 (PRDX1) promoters were determined in a representative biomarker subset (n = 126 patients with anaplastic astrocytoma or glioblastoma) from the NOA-08 trial.

Results. IDH1 mutations (R132H) were detected in only 3/126 patients, precluding determination of an association between IDH mutation and outcome. These 3 patients also displayed the G-CIMP phenotype. None of the IDH1 wild-type tumors were G-CIMP positive. Mutations in H3F3A were absent in all 103 patients sequenced for H3F3A. MassARRAY analysis of the APNG promoter revealed generally low methylation levels and failed to confirm any predictive properties.
for benefit from alkylating chemotherapy. Neither did PRDX1 promoter methylation show differential methylation or association with outcome in this cohort. In a 170-patient cohort from The Cancer Genome Atlas database matched for relevant prognostic factors, age ≥ 65 years was strongly associated with shorter survival.

Conclusions. Despite an age-independent stable frequency of O⁶-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation, tumors in this age group largely lack prognostically favorable markers established in younger glioblastoma patients, which likely contributes to the overall worse prognosis of elderly patients. However, the survival differences hint at fundamental further differences among malignant gliomas of different age groups.

Keywords: APNG methylation, G-CIMP, glioblastoma, IDH mutation, IDH1 mutation, PRDX1 methylation.

Glioblastoma (World Health Organization [WHO] grade IV) is the most common intrinsic brain tumor. The prognosis for patients suffering from this disease remains dismal. Age in particular is a strong negative predictor for survival, resulting in a population-based median survival of elderly patients (ie, >65 y) of < 6 months.¹ As glioblastoma incidence strongly increases with age, soon more than half of all glioblastoma patients will be considered elderly.² Improving the therapy of these patients is therefore one of the major challenges in neuro-oncology.³

The standard of care in elderly patients is currently ill-defined. This is in part due to the exclusion of elderly patients in many clinical trials. The trial by the European Organisation for Research and Treatment of Cancer and the National Cancer Institute of Canada, which defined concomitant and adjuvant radiochemotherapy with temozolomide (TMZ) as the standard of care, excluded patients older than 70 years.⁴ In elderly patients receiving combined radiochemotherapy, treatment-associated toxicity seems to be higher compared with younger patients.⁵ This especially holds true for radiation-related neurotoxicity, which demonstrates a clear age dependency.⁶ To date, involved-field radiation alone is recommended as the standard first-line therapy after biopsy or resection in elderly patients.⁶ However, due to the risk for radiation-induced neurotoxicity, TMZ alone has been explored as a treatment option. Several studies have reported a median overall survival (OS) comparable to radiotherapy (RT) alone with only modest toxicity of TMZ in this population.⁹ To directly compare RT alone versus TMZ alone, the German Neuro-Oncology Working Group (NOA) conducted a randomized phase III trial (NOA-08) in elderly patients. This trial demonstrated a noninferiority of TMZ (1 wk on/1 wk off regimen) to focal RT. Importantly, hypermethylation of the O⁶-methylguanine-DNA methyltransferase (MGMT) promoter was established as a very strong predictive biomarker for TMZ sensitivity in elderly patients with malignant glioma. Of note, even though median OS was short (8.6 mo in the TMZ group vs 9.6 mo in the RT group), in both treatment arms a subset of patients survived considerably longer than 2 years.¹¹

Recently, several other molecular markers have been reported as being either prognostic or even predictive of benefit from specific therapeutic interventions in malignant glioma patients. In 2008, Parsons et al¹² discovered mutations in the isocitrate dehydrogenase 1 gene (IDH1) in a subset of glioblastoma patients. Subsequently, mutations in either IDH1 or IDH2 have been identified in >70% of WHO grades II and III gliomas and secondary glioblastomas.¹³ In primary glioblastomas, IDH mutations are rare. Importantly, IDH mutations are associated with a significantly longer survival time compared with IDH wild-type tumors. Among grades III and IV gliomas pooled, IDH has a stronger prognostic impact than WHO grade. In the same study, the authors also found that in patients age ≥60 years with anaplastic astrocytoma and glioblastoma, IDH1 mutation was found in only 7.5% of the patients (11/146).¹⁴ Analysis of the glioblastoma epigenome revealed a distinct hypermethylator phenotype, the glioma cytosine–phosphate–guanine (CpG) island methylator phenotype (G-CIMP), which confers a good prognosis.¹⁵ Tumors positive for G-CIMP usually harbor IDH mutations, hence they are common among grades II and III gliomas and rare in primary glioblastomas. Genome- and epigenome-wide analyses of glioblastoma samples further revealed frequent mutations in the histone 3.3 gene (H3F3A) at K27 or G34.¹⁶ Just like IDH mutations, both K27M and G34R/V mutations are associated with a distinct epigenetic signature and possibly cell of origin each. In this study, tumors carrying G34 mutations show a favorable clinical course.¹⁷ Importantly, H3F3A and IDH mutations are mutually exclusive, suggesting that mutations in either of these genes represent different gliomagenic pathways. While the aforementioned alterations are prognostic, Aghiniotri et al¹⁸ recently reported on epigenetic inactivation of alkylpurine DNA N-glycosylase (APNG) as a predictive biomarker for benefit from TMZ treatment. APNG is a DNA base excision repair enzyme, which catalyzes removal of N3-methyladenine and N7-methylguanine from DNA, both of which can be caused by TMZ. APNG expression is regulated through promoter methylation, and in patients with unmethylated MGMT promoter receiving TMZ, the subset of APNG-negative tumors was reported to have a better prognosis. Peroxiredoxin 1 (PRDX1) is another interesting candidate, especially in the group of anaplastic astrocytomas. The PRDX1 promoter was found to be frequently hypermethylated in oligodendrogial tumors and secondary glioblastomas carrying a deletion of 1p/19q, leading to epigenetic downregulation of PRDX1 expression.¹⁹ In this study, silencing of PRDX1 in Hs683 glioma cells sensitized these cells both to TMZ and to RT in vitro.

The objective of our present study was to determine the prevalence and impact of recently defined biomarkers associated with survival, heretofore established in younger patients, in an elderly collective. We hypothesized that these biomarkers might help to explain the heterogeneity in survival seen in the NOA-08 population. However,
as our study revealed that they are virtually absent in elderly patients, we assume that relevant molecular differences exist among malignant gliomas of different age groups, which warrant further studies.

**Materials and Methods**

**Patients and DNA**

This study comprised 126 patients of the NOA-08 collective; 9 with anaplastic astrocytomas (7.1%) and 117 with glioblastomas (92.9%). All patients were 65 years or older.11

DNA from fresh-frozen paraffin-embedded tissue was extracted using the Invisorb Genomic DNA Kit II (Stratec Molecular). Before DNA extraction, specimens were histopathologically reviewed and tumor content > 80% was confirmed.

To complement our subset with a series of tumors from younger glioblastoma patients, we received bisulfite-converted DNA of 10 glioblastoma patients (mean age, 45 y) from the Department of Neuropathology, University of Heidelberg (W.M.).

**IDH1 and H3F3A Mutation Status**

Patients were screened for IDH1 and H3F3A mutations by direct sequencing of PCR products or immunohistochemistry. For sequencing analysis, the following primers were used: IDH1 forward: 5′-CGTCTTCA GAGAAGCCATT-3′, reverse: 5′-GCAAATCACATTA TTGCCCAAC-3′; H3F3A forward: 5′-CATGCTCCTG ACAAGACAGA-3′, reverse: 5′-CAAGAGAGACTTT GTCCGTTTTT-3′. Sequencing was performed by GATC Biotech. Immunohistochemistry detecting the IDH1R132H monoclonal antibody H09 (Dianova) on an automated immunostainer (BenchMark, Ventana Medical Systems).

**G-CIMP Status**

Methylation-specific PCRs (MSPs) for 8 genes were performed as previously described.13 A sample was considered positive for G-CIMP when either DOCK5 was hypomethylated and 5 of the remaining 7 genes were hypermethylated or (in case of a hypermethylation of DOCK5) 6 out of the other 7 genes were hypermethylated. Supplementary Table S1 lists the primers used for MSP analysis.

**Quantitative High Resolution DNA Methylation Analysis**

APNG and PRDX1 promoter methylation was screened using the MassARRAY technique (Sequenom). This technology relies on detection of mass shifts, which are introduced through sequence changes following bisulfite treatment.19 In short, 500 ng genomic DNA was bisulfite converted using the Epitect Bisulfite Kit (Qiagen). For PCR amplification, HotStarTaq (Qiagen) and the primers listed in Supplementary Table S2 were used.

Next, DNA methylation analysis was performed on a Sequenom mass spectrometer, and the results were analyzed by Epityper software version 1.05 (Sequenom).

**Illumina 450k Methylation Array**

For genome-wide assessment of DNA methylation, we used the Human Methylation 450 Bead Chip (HM450BC; Illumina). Methylation analysis of glioblastoma samples (n = 22) was performed at the in-house Genomics and Proteomics Core Facility (German Cancer Research Center). Methylation data of additional adult glioblastoma samples (n = 74) were obtained from TCGA (http://cancergenome.nih.gov).

**TCGA Collective**

To assess the influence of age on survival in IDH wild-type patients, methylation (Illumina HM27BC, n = 294 samples; and Illumina HM450BC, n = 126 samples) and clinical data were obtained from the database of TCGA (http://cancergenome.nih.gov). Unsupervised hierarchical clustering of methylation data was performed as described previously.15,17 Briefly, probes (i) targeting the X and Y chromosomes, (ii) containing a single nucleotide polymorphism within 5 base pairs of and including the CpG site, and (iii) not mapping uniquely to the human reference genome (hg19), allowing for 1 mismatch, were removed. The 1500 (Illumina HM27BC) and 8000 (Illumina HM450BC) most variable (by SD) probes were kept, and unsupervised hierarchical clustering was performed for each platform.

A logistic regression model to estimate the probability of MGMT methylation from Illumina HMBC data was used as described by Bady et al.21 From the normalized methylated (m) and unmethylated (u) signal intensities, the normalized methylated (m) and unmethylated (u) signal intensities, the normalized methylated (m) and unmethylated (u) signal intensities, the normalized methylated (m) and unmethylated (u) signal intensities were calculated as

\[ M_{value} = \frac{M_{value}(cg12434587) + 0.9265 * M_{value}(cg12981137)}{0.5271 * M_{value}(cg12434587) + 0.358} \]

**Statistics**

MassARRAY CpG units were evaluated separately as well as averaged per amplicon. Association of quantitative MassARRAY measurements with OS and event-free survival (EFS) was assessed with univariate Cox proportional hazards regression models. Predictive factors were assessed with a Factor × Treatment interaction term. Proportional hazards assumption was tested for violation according to Grambsch and Therneau.23 Risk groups were determined based on optimal cutpoint analysis using the maximally selected log-rank statistic approach,24,25 which corrects for type I error inflation due to multiple testing. Survival of risk groups was estimated with the Kaplan–Meier method. A Wilcoxon rank-sum
test was employed to compare median Karnofsky performance scores between old and young subgroups of TCGA data; Fisher’s exact test was used to analyze the relationship between age (dichotomized as <65 y and ≥65 y) and MGMT methylation, extent of surgery, and treatment. Univariable P-values were adjusted for multiple testing using a Benjamini–Hochberg correction in order to control the false discovery rate.26 All tests were 2-sided; P < .05 was considered statistically significant. Analyses were carried out using R software version 2.14.27

Results

Study Population

In total, 126 patients of the NOA-08 trial (the NOA-08 biomarker cohort) were analyzed. Table 1 lists the patient characteristics of this study collective and the NOA-08 collective. Patients with a resection rather than a biopsy were overrepresented in this cohort due to the requirement of a sufficient amount of tissue. MGMT promoter methylation status was similar between both groups. Median EFS times were comparable between our study population (4.4 mo; 95% confidence interval [CI], 3.7–5.4) and the entire NOA-08 collective (4.1 mo; 95% CI, 3.7–4.5), while median OS was higher (11.2 mo; 95% CI, 9.5–13.6 vs 8.9 mo; 95% CI, 8.0–9.9). Long-term OS data show a group of patients with a considerably longer than average survival mainly in the TMZ arm (Fig. 1A). As expected from the increased median OS, these patients were overrepresented in our cohort due to enrichment of patients having undergone resection instead of biopsy. Supplementary Table S3 lists the EFS and OS data for all 126 patients included in this study.

Table 1. Comparison of patient characteristics of our study population (n = 126) and the NOA-08 collective (n = 373)

<table>
<thead>
<tr>
<th></th>
<th>Our Study Population, n (%)</th>
<th>NOA-08, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>9 (7)</td>
<td>40 (11)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>117 (93)</td>
<td>331 (89)</td>
</tr>
<tr>
<td>Not confirmed</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMZ</td>
<td>62 (49)</td>
<td>195 (52)</td>
</tr>
<tr>
<td>RT</td>
<td>64 (51)</td>
<td>178 (48)</td>
</tr>
<tr>
<td><strong>Resection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>44 (35)</td>
<td>104 (28)</td>
</tr>
<tr>
<td>Partial</td>
<td>52 (41)</td>
<td>123 (33)</td>
</tr>
<tr>
<td>Biopsy</td>
<td>30 (24)</td>
<td>145 (39)</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>MGMT promoter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>42 (35)</td>
<td>73 (35)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>77 (65)</td>
<td>136 (65)</td>
</tr>
<tr>
<td>Missing/inconclusive</td>
<td>7</td>
<td>164</td>
</tr>
</tbody>
</table>

In the NOA-08 trial, histology (anaplastic astrocytoma vs glioblastoma) did not significantly influence EFS or OS.11 This was recapitated in the present biomarker cohort, where histology had no influence on EFS or OS. Kaplan–Meier plots are depicted in Supplementary Fig. S1. Importantly, the predictive value of MGMT promoter methylation for response to TMZ was recapitated in this NOA-08 biomarker cohort (interaction P = .03)
[OS] and \( P < .001 \) [EFS]). In a Cox regression model, MGMT promoter hypermethylation significantly prolonged EFS and OS in patients treated with TMZ (\( P < .0001 \) and \( P = .0014 \), respectively), while in the RT group, the influence of MGMT promoter methylation on survival was not significant (\( P = .34 \) for EFS and \( P = .14 \) for OS) (Fig. 1B and C).

**IDH1 Mutation, G-CIMP Status, and H3F3A Mutations**

In this study cohort, 3 patients carried an IDH1 mutation in their tumor tissue as determined by sequencing. Of these, 2 patients had glioblastomas (out of 117 glioblastoma patients) and 1 had an anaplastic astrocytoma (out of 9 patients). The 2 glioblastoma patients had OS of 582 and 924 days, respectively. This is above the median OS (272 d; range, 231–315) in the NOA-08 group, whereas the patient with an anaplastic astrocytoma had shorter than median survival (patient #37, OS 196 d). Notably, MGMT promoter analysis revealed hypermethylation in all 3 patients. On the other hand, only patient #110 received TMZ as first-line treatment (Table 2).

To expand the sample size, we performed IDH1 immunohistochemistry on another 50 patients from the NOA-08 trial, for whom only unstained paraffin-embedded slides, but no tumor DNA for further analysis were available. In this group, no IDH1(R132H) mutant tumor was detected. In summary, the low frequency of IDH1 mutations precludes a predictive role of this marker in elderly patients with glioblastoma, although individual patients may have a better course than the average.

In line with earlier reports demonstrating a strong correlation between IDH1 mutations and G-CIMP, the 8-gene MSP panel revealed that these 3 IDH1 mutant patients were also G-CIMP positive. In the remaining IDH1 wild-type group of 123 samples, 4 samples could not be analyzed due to insufficient DNA left for bisulfite conversion, and the other 119 were G-CIMP negative.

To screen our biomarker cohort for the recently described H3F3A K27M and G34R/V mutations, we amplified and sequenced a 170-bp fragment spanning the 2 mutation sites of the H3F3A gene in all 103 patients for which enough suitable DNA was left. We detected neither K27M nor G34R/V mutations in our cohort.

**APNG and PRDX1 Methylation**

CpG methylation was assessed by MassARRAY technology. In total, 99 (APNG) and 73 (PRDX1) tumors of the 126-sample cohort were analyzed. Methylation levels across the 20 CpGs that were examined (measured as 13 distinct CpG units) in the promoter/intron 1 of the APNG locus were low, apart from a few exceptions (median 6%, interquartile range 5%–9%). The heatmap of APNG methylation is depicted in Fig. 2A. Mean quantitative CpG methylation levels of APNG were not associated with EFS (\( P = .54 \)) or OS (\( P = .61 \)). Similarly, discrimination between APNG methylated and APNG unmethylated patients at cutoff values of 10% (EFS, \( P = .71 \)) and 3% (OS, \( P = .42 \)), respectively, which optimally separated the curves, did not yield a significant risk stratification independent of treatment. When analyzing the predictive effect of APNG methylation in patients with an unmethylated MGMT promoter who were treated with TMZ, the low methylation levels precluded identification of a biologically meaningful cutoff (7.2% meth of APNG for OS and 8.5% meth of APNG as cutoff for EFS, respectively).

Since APNG has been proposed as a biomarker predictive of benefit from TMZ, with average methylation levels varying between 30% and 40% (APNG expressers) and 70% and 80% (APNG non-expressers), we next aimed at investigating reasons for that discrepancy to our findings. To exclude APNG as a biomarker relevant only for younger patients, we also did MassARRAY analyses on 10 patients with an average age of 45 years. This analysis yielded the same homogeneously low methylation level as in the NOA-08 biomarker cohort (data not shown). In addition, Illumina 450k methylation arrays were performed for 22 patients from the NOA-08 biomarker collective. Further, samples were included from the project by TCGA, which were analyzed on the same Illumina 450k platform \((n = 74; \text{mean age}, 61 \text{y} \text{[range, 23–85]})\). Two probes (cg05397937 and cg15768556) surveyed a total of 3 CpGs, which were also included in our MassARRAY amplicon and bisulfite sequencing performed by Agnihotri and colleagues (see Fig. 2C). Consistent with the MassARRAY findings, homogeneously low levels of methylation were demonstrated across all 96 samples (<0.2, see Fig. 2D and E). Even though 5 TCGA samples clustered into the G-CIMP group, they did not show increased levels of APNG methylation compared with non–G-CIMP samples. Of note, comparing MassARRAY data and 450k data for the NOA-08 patients of whom both data sets were available showed good agreement between the methods.

Promoter methylation analysis of PRDX1 revealed moderately low methylation, with most samples exhibiting a mean methylation between 10% and 20% across the 6 CpGs examined (measured as 5 distinct CpG units; Fig. 2B). Only 8 out of 73 samples had a mean methylation >30%. PRDX1 methylation did not show an association with EFS or OS, either in the whole study population or by treatment.

**Age-related Survival Differences**

Given the paucity of positive prognostic factors in older glioblastoma patients, we sought to determine whether...
Fig. 2. Analysis of APNG and PRDX1 methylation. (A and B) Heatmaps of APNG (A) and PRDX1 (B) promoter methylation. Each column represents a sample, each row a CpG unit. Methylation values range from 0 (totally unmethylated) to 1 (fully methylated) and are color coded; the legend is shown left of the heatmaps. (C) Representation of the spatial relationship between the assessed CpGs of APNG. (D and E) Display of average methylation levels (beta values) for samples from (D) TCGA and (E) NOA-08.
the relative absence of these known factors alone might explain the survival differences seen among different age groups using the data set from TCGA. We determined G-CIMP (as a surrogate marker for IDH mutation) and MGMT promoter methylation status from Illumina HMBC data as previously described\(^{15,17,21}\) and complemented these molecular data with clinical information from the TCGA database. In total, 170 patients had a complete clinical and molecular data set. In this cohort, we detected 18 patients with a hypermethylator phenotype (10.5%), of which only 2 were older than 65 years. G-CIMP status was associated with significantly prolonged survival (median OS, 22.7 mo [95% CI, 8.4–not reached] vs 15.9 mo [95% CI, 14.1–17.6], log-rank \(P = .0085\); Supplementary Fig. S2a). MGMT promoter hypermethylation as predicted through a logistic regression model occurred in 70 cases (41%) and was associated with improved median OS in patients who initially received combined RT and chemotherapy (19.7 mo [95% CI, 15.7–23.9] vs 14.5 mo [95% CI, 12.2–16.6], log-rank \(P = .0288\), Supplementary Fig. S2b). For further analysis, we excluded patients positive for G-CIMP. Table 3 summarizes the baseline characteristics of both groups (G-CIMP–negative patients age <65 and \(\geq 65\) y). Notably, the 2 groups showed no significant differences with regard to relevant prognostic or predictive parameters, albeit there was a trend toward a higher KPS in the younger cohort. Despite the balance between the groups, older patients had a significantly shorter OS (12.5 mo [95% CI, 7.6–14.4] vs 17.6 mo [95% CI, 15.7–20.3], log-rank \(P = .0007\); Fig. 3A). To account for a potential confounding effect of the KPS, we performed a multivariate Cox regression analysis including age (as a dichotomous variable, <65 y vs \(\geq 65\) y) and KPS (as a categorical covariate). Conforming the above result, this analysis yielded a hazard ratio of 2.25 (95% CI, 1.46–3.47, \(P = .0085\)) for patients age \(\geq 65\) years adjusted for KPS.

**Table 3.** Baseline characteristics of TCGA collective

<table>
<thead>
<tr>
<th>Patients Age</th>
<th>Patients Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>Mean age, y</td>
</tr>
<tr>
<td>&lt;65 y, n = 110</td>
<td>(\geq 65) y, n = 42</td>
</tr>
<tr>
<td>51.6 (23–64)</td>
<td>71.9 (65–83)</td>
</tr>
<tr>
<td>Median KPS</td>
<td>Median KPS</td>
</tr>
<tr>
<td>(range)</td>
<td>(range)</td>
</tr>
<tr>
<td>80 (40–100)</td>
<td>80 (40–100)</td>
</tr>
<tr>
<td>Extent of</td>
<td>Extent of</td>
</tr>
<tr>
<td>operation, n</td>
<td>operation, n</td>
</tr>
<tr>
<td>Resection</td>
<td>Resection</td>
</tr>
<tr>
<td>97 (88%)</td>
<td>37 (88%)</td>
</tr>
<tr>
<td>Biopsy</td>
<td>Biopsy</td>
</tr>
<tr>
<td>13 (12%)</td>
<td>5 (12%)</td>
</tr>
<tr>
<td>Initial treatment, n</td>
<td>Initial treatment, n</td>
</tr>
<tr>
<td>RT + TMZ</td>
<td>RT + TMZ</td>
</tr>
<tr>
<td>91 (83%)</td>
<td>32 (65%)</td>
</tr>
<tr>
<td>RT alone</td>
<td>RT alone</td>
</tr>
<tr>
<td>19 (17%)</td>
<td>10 (24%)</td>
</tr>
<tr>
<td>MGMT</td>
<td>MGMT</td>
</tr>
<tr>
<td>promoter, n</td>
<td>promoter, n</td>
</tr>
<tr>
<td>Methylated</td>
<td>Methylated</td>
</tr>
<tr>
<td>39 (35%)</td>
<td>16 (38%)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>71 (65%)</td>
<td>26 (62%)</td>
</tr>
</tbody>
</table>

**Discussion**

Elderly persons will soon account for more than half of all glioblastoma patients in the Western countries.\(^2\) Despite this development, elderly patients are still underrepresented in clinical trials, leaving the standard of care for this population currently ill-defined.\(^3\) There is increasing evidence to suggest fundamental molecular differences among malignant gliomas of different age groups. In 2004, Batchelor et al.\(^{28}\) demonstrated age-dependent effects of the prognostic impact of key genomic alterations (\(TP53\) mutation, \(CDK2NA/p16\) deletion, and loss of chromosome 1p) in glioblastoma. Recently, analysis of common genomic aberrations in glioblastoma (\(TP53\) mutation, epidermal growth factor receptor amplification and variant III mutation, deletion of phosphatase and tensin homolog and mutation of \(IDH\)) has revealed distinctive differences in the distribution of these aberrations in young adults (age 19–40 y) and older adults (age >40 y).\(^{29}\) In the pediatric population, somatic mutations in the H3.3-ATRX-DAXX chromatin remodeling pathway have been discovered in 44% of glioblastomas.\(^{15}\) Tumors carrying a mutation in this pathway were associated with a distinct gene expression profile. It has recently been shown that tumors with \(H3F3A\) mutations indeed have a distinct methylation, gene expression, mutation, and copy number variation profile and possibly cell of origin.\(^{17}\) Molecular analysis of glioblastomas across the age continuum showed that \(H3F3A\) mutations (K27M and G34R/V) occurred predominantly in children and young adults and that \(IDH\) mutations occurred primarily in young adults, while older patients were classified mostly into the remaining 3 subtypes (mesenchymal subtype, receptor tyrosine kinase [RTK] 1 “platelet-derived growth factor receptor A,” and RTK 2 “classic”). In agreement with this, \(IDH\) mutations are very rare in patients with malignant gliomas above the age of 60.\(^{14}\) The lack of \(IDH\) mutations in these tumors might partially contribute to the worse prognosis of elderly patients with malignant gliomas, even though the analysis of the collective from TCGA suggests that
other factors also play a role in these age-related survival differences. Along this line, we found only 3 IDH1-mutated patients in our cohort of 126 patients, and no H3F3A mutations. In agreement with the recently discovered causative role of IDH mutations in epigenetic remodeling resulting in a G-CIMP phenotype, our 3 IDH1 mutated samples proved to be positive for G-CIMP as well.10 With IDH1 mutations accounting (i) for more than 90% of IDH mutations in glioma,13 (ii) for the causality between IDH mutation and the G-CIMP phenotype, and (iii) for the lack of a G-CIMP–positive tumor in the remaining 119 IDH1 wild-type samples, we decided against testing for IDH2 mutations. Importantly, while the 2 glioblastoma patients carrying an IDH1 mutation and G-CIMP had an OS above average (see Table 2), 15 IDH wild-type/G-CIMP–negative patients in this study population had a comparable or even longer OS than patient #110, who was positive for IDH mutation/G-CIMP. Of these 15 patients, 1 displayed a longer OS than the IDH mutation/G-CIMP–positive patient #125 (see Supplementary Table S3).

The rationale for the further selection of biomarkers investigated in this study was to explain the survival heterogeneity seen in the NOA-08 trial population, where a group of patients had a considerably longer survival than average.11 MGMT promoter methylation alone is not sufficient to account for this. In glioblastoma, key chromosomal, genetic, and epigenetic aberrations have been defined, including EGFR amplification, TP53 mutation, CDK4 amplification, CDKN2A homozygous deletion, and IDH mutations.12,13 However, with the notable exception of IDH mutations,13 these molecular aberrations have no significant effect on survival. With regard to the aim of our study, we therefore limited the selection of biomarkers to prognostically relevant markers.

A recent report demonstrated a role for the DNA repair enzyme APNG in conferring resistance to TMZ in glioblastoma.14 Patients without hypermethylation of the MGMT promoter were subgrouped into APNG expressers and non-expressers (assessed by immunohistochemistry), where tumors that expressed APNG had a worse prognosis when treated with a TMZ-containing regimen. To investigate the role of promoter methylation in regulation of APNG expression, the authors18 performed bisulfite sequencing of an approximately 200-bp fragment located in the promoter/intron 1 region of APNG. Comparing APNG expressers and non-expressers, differences were found in mean methylation levels across the investigated fragment (37% ± 5% for expressers and 77% ± 6% for non-expressers). In vitro treatment of human glioblastoma cell lines with the demethylating agent 5-azacytidine resulted in upregulation of APNG mRNA levels. This led the authors to propose that APNG expression is regulated through promoter hypermethylation. As the NOA-08 study and the present biomarker cohort clearly demonstrate the predictive effect of MGMT promoter hypermethylation described earlier,14,15 we sought to determine whether APNG methylation also has the predictive value as proposed before. In contrast to Agnihotri and colleagues,18 we performed MassARRAY analysis of the same fragment, a well-established technique for quantitative assessment of methylation.20 Surprisingly, we detected only <20% levels of APNG promoter methylation across all samples and CpG units (Fig. 2) and did not observe the separation of patients into 2 groups. Since methylation is known to be at least partially age dependent,35 we expanded our MassARRAY analysis to include 10 younger glioblastoma patients (mean age, 45 y) and obtained similar results. Our findings were confirmed through a technically and (partly) biologically independent analysis of Illumina 450k methylation arrays of 22 NOA-08 samples and 74 TCGA samples. The low methylation of APNG is not an age-specific effect, as evidenced by TCGA samples, which included patients age 23–85 years (mean age, 61) and showed homogeneously low methylation levels across all samples. Furthermore, the methylation-specific predictive effect of MGMT promoter methylation is highly significant in both our subset and the whole NOA-08 cohort. A possible explanation for the conflicting results regarding APNG methylation may lie in the use of nonquantitative bisulfite sequencing, which requires clonal amplification prior to sequencing, thus introducing an additional step for potential bias. Our APNG amplicon was marginally larger than that examined by Agnihotri et al18 (233 bp vs 193 bp), yet addressed exactly the same CpGs (Fig. 2C). We carefully reinspected our amplicon sequence with respect to possible single nucleotide polymorphisms (SNPs) in primer sequences and guanine–cytosine content, being 41% after bisulfite treatment in case all CpGs would be methylated, and found no obvious hint for any PCR bias in our setting. Further studies will be required to assess the role of epigenetic regulation in APNG expression.

Analysis of PRDX1 promoter methylation, a novel marker for sensitivity toward TMZ or RT, yielded no significant effect on survival. This is in line with the original report on PRDX1 methylation, which suggested that hypermethylation occurs mostly in oligodendroglial tumors and secondary glioblastomas.31,32

Although the biomarker cohort analyzed in the present study was representative for the NOA-08 study population (Table 1), subgroup analyses have well-known limitations. Further, some DNA samples extracted from fresh-frozen paraffin-embedded tissue proved to be too fragmented to allow for PCR amplification of the desired MassARRAY amplicons (each >200 bp), explaining the discrepancy in analyzed samples between G-CIMP MSP (n = 126, with average amplicon size of 100 bp) and MassARRAY (n = 99 and n = 73 samples for APNG and PRDX1, respectively). To assess the influence of age on survival, we analyzed survival in a large cohort from TCGA, which was well matched for all relevant prognostic factors. However, the median survival times of the collective from TCGA are subject to a selection bias due to the tissue requirements for molecular analysis (exemplified by a 90% resection rate [Table 3] vs 60% in NOA-0811) and thus cannot be externally compared to, for example, the NOA-08 survival times. Nonetheless, in the intragroup comparison, patients age ≥65 years still had significantly shorter OS than their younger counterparts.
In summary, favorable prognostic biomarkers such as IDH or H3F3A mutation, G-CIMP, or PRDX1 methylation are virtually absent in malignant astrocytic tumors of the elderly, which may partially account for the worsened prognosis of these patients. However, even in tumors negative for G-CIMP (and hence IDH wild type), which are matched for known prognostic factors, older patients have a significantly shorter OS. On the other hand, several long-term surviving patients in our cohort lack the aforementioned molecular markers and sometimes even MGMT promoter hypermethylation. These 2 observations strongly hint at the existence of thus far unknown prognostic factors in these patients. Further studies are necessary to broaden our insight into the molecular aberrations in elderly patients in order to stepwise replace chronological age as the most important negative prognostic marker and potentially therapy-decisive variable in malignant astrocytomas with defined and hopefully actionable molecular mechanisms.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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