The combination of IDH1 mutations and MGMT methylation status predicts survival in glioblastoma better than either IDH1 or MGMT alone.

Title pages

Title: The combination of *IDH1* mutations and *MGMT* methylation status predicts survival in glioblastoma better than either *IDH1* or *MGMT* alone

Running title: Glioblastoma survival prediction by *IDH1* and *MGMT*

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Funding: This work was supported by grants from ABC2 (AvT), Italian Association for Cancer Research (AIRC; AB), Italian Ministry of Health, Regione Piemonte (AB), Italian Ministry of University and Research, CRT Progetto Alfieri (AB), Association for International Cancer Research (AICRUK; AB) and EU FP6 contract 037297 (AB). F.E. Bleeker was supported by a NWO travel grant and a Netherlands Genomic Initiative Fellowship. R.J. Molenaar was supported by an AMC PhD Scholarship.

Conflict of interest: Alberto Bardelli is a shareholder of Horizon Discovery Limited.

Abstract

Background: (Epi)genetic profiling of glioblastomas has provided a comprehensive list of altered cancer genes of which, thus far, only MGMT methylation is used as predictive marker in a clinical setting. We investigated the prognostic significance of (epi)genetic alterations in glioblastoma patients.

Methods: We screened 98 human glioblastoma samples for (epi)genetic alterations in ten genes and chromosomal loci by polymerase chain reaction (PCR) and multiplex ligation-dependent probe amplification (MLPA). We tested the association between these (epi)genetic alterations and glioblastoma patient survival. Subsequently, we developed a two-gene survival predictor.

Results: Multivariate analyses revealed that mutations in isocitrate dehydrogenase 1 (*IDH1*), promoter methylation of O^6-methylguanine-methyltransferase (*MGMT*), irradiation dosage and Karnofsky Performance Status were independent prognostic factors. A two-gene predictor for glioblastoma survival was generated. Based on the (epi)genetic status of *IDH1* and *MGMT*, glioblastoma patients were stratified into three clinically different genotypes: glioblastoma patients with "*IDH1*mt/*MGMT*met" had the longest survival, then patients with "*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met" and last patients with "*IDH1*wt/*MGMT*unmet". This two-gene predictor was an independent prognostic factor and significantly better for survival prediction than either *IDH1* mutations or *MGMT* methylation alone. The predictor was validated in three external datasets.

Discussion: The combination of *IDH1* mutations and *MGMT* methylation outperforms either *IDH1* mutations or *MGMT* methylation alone in glioblastoma patient survival prediction.

This information will help to increase our understanding of glioblastoma biology and it may be helpful for baseline comparisons in future clinical trials.

Words: 233.

Keywords

Glioblastoma; *IDH1; MGMT;* (Epi)genetic; Molecular; Survival prediction

Introduction

Glioblastoma is the most common malignant brain tumor and has a poor prognosis. Therapeutic advances have been made in the past decade with the addition of temozolomide chemotherapy to maximal safe tumor resection and radiotherapy. However, median survival is still limited to only 15 months.[1,2] Therefore, novel therapies are urgently needed. For rational drug design, it is essential to unravel the underlying oncogenic mechanisms of glioblastoma.

Most glioblastoma are primary, meaning that they manifest rapidly *de novo*, without recognizable precursor lesions. Approximately 5% of glioblastomas are diagnosed in patients with a preceding low-grade glioma, which in years has progressed to secondary glioblastoma.[3] Both genotypes are considered to be histopathologically indistinguishable, but differences in molecular alterations are apparent. Different genes have been found to be involved in glioblastoma, by changes in gene expression, methylation, copy number alterations and/or mutations (reviewed in [4]). The understanding of molecular alterations in signaling pathways and their consequences for the pathology of glioblastoma has greatly increased in the last years.

For a long time, the most studied genetic hallmarks of glioblastoma have been *EGFRvIII*, a truncated constitutively activated form of *EGFR*, mutations in *TP53* and deletions in *PTEN*.[4] More recently, methylation of the O6-methylguanine-methyltransferase (MGMT) gene promoter appeared to be a predictive factor in glioblastoma patients for the response to temozolomide and radiotherapy and hence survival.[1,5,6] Conflicting results have been reported on the methylation status of *MGMT* as a positive prognostic marker independently of therapy.[7-9] Most recently, genome-wide sequencing of glioblastoma revealed that the *IDH1* and *IDH2* genes, encoding isocitrate dehydrogenases 1 and 2, are

mutated in a subset of glioblastoma.[10,11] Interestingly, *IDH1/2* mutations have been demonstrated predominantly in younger patients and secondary glioblastomas.[11-15] Mutations in *IDH1*, but not *IDH2*, were shown to be an independent positive prognostic marker for glioblastoma patient survival.[15-17] Gene expression analysis studies[18-20] have been able to stratify patients in Classical, Mesenchymal, Proneural and Neural subtypes that are characterized by aberrations in and gene expression of *EGFR*, *NF1*, *IDH1* and *PDGFRA* and predict prognosis.[20]

We investigated the association between (epi)genetic alterations in *IDH1/2*, *MGMT* and other genes and chromosomal loci, and survival of glioblastoma patients. These results led us to propose a novel two-gene predictor for glioblastoma survival prediction based on the combination of the *IDH1* mutational status and *MGMT* methylation status.

Materials and methods

Patients, tumor samples and DNA extraction

Glioblastoma samples were obtained from 98 patients with known follow-up. The samples were retrieved from the tumor bank maintained by the Departments of Neurosurgery and Neuropathology at the Academic Medical Center (AMC, Amsterdam, The Netherlands). Oral consent for removal of the tissue and its storage in the tumor bank for research purposes was obtained and documented in the patient's medical chart. Consent for this project was approved by the local ethics committee. The research was performed on 'waste' material, stored in a coded fashion. Tumor samples were included only if at least 80% of the sample consisted of cancer cells, as verified by H&E staining. Genomic DNA was isolated as previously described.[13] Matches between germline and tumor DNA were verified for all samples by direct sequencing of 26 single nucleotide polymorphisms (SNPs) at 24 loci (data not shown).

Glioblastoma patient data

A retrospective survival analysis was performed for the 98 glioblastoma patients. Both primary (85) and secondary glioblastoma (13), but no recurrent glioblastoma patients were included. These patients underwent brain surgery at the AMC between 1988 and 2006 and were selected when both clinical follow-up and a sufficient amount of tissue for these and other analyses (previously published [16,21] and unpublished results) was available. Patient characteristics are displayed in Table 1. Overall survival was calculated as time from surgery to death. Event times were censored if the patient was alive at the time of last follow-up. Follow-up for included patients ranged from 15 days to 7.5 years (mean 384 days). Patients were treated with different regimens either in trials or with standard protocols. Patients were treated in the era before chemoradiation was standard protocol;[2] treatment consisted of

maximum safe tumor resection and radiotherapy. At relapse, patients were treated with different regimens either in trials or on the basis of local protocols including re-irradiation (leading to a total radiation dosage up to 78 Gy), chemoradiation (radiotherapy with concomitant and adjuvant temodal therapy), brachytherapy, gliadel, PCV, temodal, MTX and nicotinamine (as enhancer during irradiation).

Mutation analysis, polymerase chain reaction and sequencing details

We investigated *EGFR*, *IDH1*, *IDH2*, *PIK3CA*, *PTEN* and *TP53* for somatic mutations, genes known to be (relatively) frequently mutated in glioblastoma.[22] Sequencing results of *IDH1* and *IDH2* have been published previously.[16] Polymerase chain reaction (PCR) and sequencing primers were designed using Primer 3 and synthesized by Invitrogen (Life Technologies, Paisley, UK). PCR primers were designed to amplify the selected 50 exons and the flanking intron sequences, including splicing donor and acceptor regions of the genes (Supplementary Table 1). PCR products were approximately 400 bp in length with multiple overlapping amplimers for larger exons. On each sample, 198 PCRs were performed in 384- and 96-well formats in 5 or 10 µl reaction volumes, respectively. PCR conditions have been published previously.[13] Over 5,000 nucleotide changes were identified during this initial screening. Changes previously described as SNPs were excluded from further analyses. To ensure that the observed mutations were not PCR or sequencing artifacts, amplicons including non-silent mutations were independently re-amplified and re-sequenced in the corresponding tumors. All verified changes were re-sequenced in parallel with the matched normal DNA to distinguish between somatic mutations and SNPs not previously described.

Multiplex ligation-dependent probe amplification experiments

Multiplex ligation-dependent probe amplification (MLPA) analysis was used to detect copy number changes of multiple loci simultaneously.[23] All assays used were prepared by

MRC-Holland (Amsterdam, the Netherlands). MLPA assay P088 (lot no. 0804, 0305, 0706 or 0608) was used to detect complete or partial losses involving chromosome 1p (15–16 probes depending on lot number) and 19q (8 probes), whereas MLPA assay P105 (lot nr 0306, 0407 or 1008) was used to detect copy number changes in the genes *CDKN2A* (5 probes), *PTEN* (10–11 probes) and *EGFR* (11 probes), and identification of EGFR rearrangements (EGFRΔ), for example, *EGFRvIII*. As described previously, the sensitivity and specificity of these MLPA assays was fully validated.[24,25] As MLPA provides (semi)quantitative information on copy number, we were able to distinguish between low-level copy number gains, amplifications and high-copy number amplifications as well as hemizygous and homozygous deletions.

MLPA was performed as described by the manufacturer with minor modifications and data analysis was performed in Excel (Microsoft, Redwood, WA, USA), as described previously.[25] MLPA copy number detection thresholds were set at 1.2 and 0.8 for the detection of low-level gains and hemizygous losses, respectively. Furthermore, ratios < 0.4 were considered homozygous losses, ratios > 2.0 as amplifications and those > 10 as high copy number amplifications. As described previously,[24] *EGFRvIII* was identified by assessing the average ratio for exon 2–7 probes and comparison of ratio with the average ratio of probes for exons 1, 8, 13, 17 and 22 (*EGFRvIII* ratio). *EGFRvIII* ratios < 0.8 were considered to harbor the *EGFRvIII* deletion variant.[24] Additionally, the individual probe ratios were inspected in order to confirm the presence of *EGFRvIII* and/or to identify other *EGFRΔ* as indicated by a significant increase or decrease of the ratios identified by repeated experiments and confirmed MLPA assay P315 evaluating all *EGFR* exons. For chromosome 1p and 19q losses, a distinction was made between complete and partial losses; the latter were defined as a ratio < 0.8 for at least 3 adjacent probes but not of all probes for these chromosome arms.

MGMT promoter methylation was assessed with MS-MLPA as described previously.[26] The promoter of the *MGMT* gene was considered to be methylated when the MS-MLPA ratio was > 0.5.[27]

Statistical analysis

Statistical processing of data was performed using Excel 2002 (Microsoft) and SPSS 19 for Windows (IBM, Armonk, NY, USA). Figures were constructed in SPSS and Prism 5 (Graphpad Software, La Jolla, CA, USA). Associations between the different alterations were assessed by the Fisher's exact test. Differences in age and survival were tested by the Student's T-test and log-rank test, respectively. Associations between mutations and patient survival were tested with Cox regression analyses. Parameters with $P < 0.05$ in the univariate analyses were incorporated in the multivariate analysis, using a Wald backward selection procedure (stepwise elimination of parameters until all remaining parameters had $P < 0.05$). As many parameters were included in the multivariate model relative to the sample size, the reliability of the model was assessed in a more conservative multivariate Cox regression analysis incorporating only parameters that had $P < 0.01$ in the univariate analyses. Log minus log plots were used to evaluate the adequacy of the proportional hazards assumption.

A two-gene predictor for survival prediction in glioblastoma was designed, incorporating both *IDH1* mutational status and *MGMT* promoter methylation status. We classified tumors in three groups: "*IDH1*wt/*MGMT*unmet" (no *IDH1* mutation, no *MGMT* methylation), "*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met" (*IDH1* mutation or *MGMT* methylation) and "*IDH1*mt/*MGMT*met" (*IDH1* mutation and *MGMT* methylation). This two-gene predictor was validated internally and externally in three additional glioblastoma data sets, using receiver-operating characteristic (ROC) curves, multivariate Cox regression analyses and -2 Log Likelihood tests. The external data sets were obtained from Mulholland *et al.* (2012),[28] The Cancer Genome Atlas Network (TCGA; accessed on 27-11-2012),[22] and

Boots-Sprenger *et al.* (2013)[27] and contained 182, 104 and 105 glioblastoma samples, respectively. In a merged dataset containing all four datasets (n = 489 glioblastoma patients) we further evaluated the performance of the two-gene predictor in different populations by testing for interaction between datasets and the two-gene predictor in a multivariate Cox regression analysis.

Results

An overview of the prevalence of (epi)genetic alterations identified in 98 glioblastoma patients with known follow-up is shown in Table 1. A more detailed description on the observed *EGFR, IDH1, PIK3CA, PTEN* and *TP53* mutations is given in Supplementary Table 2. We found *IDH1* mutations in 18 of 98 glioblastoma samples, 10 in primary glioblastoma (12%) and eight in secondary glioblastoma (62%). Of these mutations, 15 were at residue R132H, and one at R132C, R132L and R132G each.[16] We did not identify any *IDH2* mutations in our set of glioblastoma (Table 1). *IDH2* mutations occur less frequently in gliomas and are mainly found in oligodendrogliomas.[29] Glioblastoma patients with *IDH1* mutations were significantly younger than patients without *IDH1/2* mutations and *IDH1* mutations were observed more often in patients previously diagnosed with a low-grade glioma (Table 1).

MGMT methylation was found in 30% of the samples. There was no correlation between *MGMT* methylation and age. We found high prevalences of *IDH1* mutations and *TP53* mutations in glioblastoma samples with *MGMT* methylation (Supplementary Table 3). Cases showing *IDH1* mutations in combination with *TP53* mutations in our set included both non-R132H (n = 3) and R132H (n = 12) mutations, in contrast to a previous report.[30]

Co-deletion of chromosomes 1p and 19q (1p/19q co-deletion) is frequently observed in chemotherapy-sensitive oligodendrogliomas and is, in those cases, associated with prolonged survival.[31,32] Although 1p/19q co-deletion has been observed in glioblastoma,[14,33] no translocations have been identified. In our dataset, co-deletions involving chromosome arms of 1p and 19q were found in 9% of the cases, with only 2% of tumors showing complete 1p/19q co-deletion (Table 1), which might be indicative for a reported

translocation.[34-36] LOH in 19q has been reported as a marker of prolonged survival in glioblastoma patients,[34] but our study did not confirm this (Table 2).

In our set of 98 glioblastomas, alterations in *PTEN* and *CDKN2A* and copy number alterations of *EGFR* were significantly more frequent in primary glioblastomas ($P = 0.001$, $P = 0.026$ and $P = 0.008$, respectively, Fisher's exact test; Supplementary Table 3), whereas *IDH1* mutations and complete loss of chromosome 1p occurred more frequently in secondary glioblastomas, as reported previously.[3,37,38] Although most secondary glioblastomas contained a *TP53* mutation (8/13), we did not observe significant differences in mutation frequency in *TP53* or *PIK3CA* mutations, or *MGMT* methylation between primary and secondary glioblastomas.

IDH1 mutations were often accompanied by mutations in *TP53* and methylation of the *MGMT* promoter. In contrast, *IDH1* mutations were negatively associated with alterations in *CDKN2A, EGFR* and *PTEN* (Table 1). These data reflect the robust genetic differences between primary and secondary glioblastoma as has been reported before (reviewed in [4]).

Survival analysis

The median survival of the patients was 252 days (8.5 months; Figure 1A) with 35% and 16% of patients alive at one and two years, respectively. Seven patients (7%) were considered to be long-term survivors with a survival over three years. Remarkably, the two patients who were still alive when the dataset was finalized (survival > 5 and 7 years) had mutations in both *IDH1* and *PIK3CA*.

First, we assessed in univariate analyses whether any of the genetic alterations was associated with survival (Table 2). The patients' age, extent of resection, Karnofsky Performance Status (KPS), the dosage (Gy) of radiotherapy received and mutations in *IDH1*,

PIK3CA, *PTEN* and *TP53*, alterations of *EGFR* and methylation of the *MGMT* promoter were found to be significantly associated with survival.

In a multivariate Cox regression analysis incorporating parameters that had $P < 0.05$ in the univariate analyses, the prognostic significance of radiotherapy, the KPS score, *IDH1* mutations and *MGMT* methylation was confirmed after correction for age, extent of resection and the aforementioned genetic statuses of *EGFR*, *PIK3CA*, *PTEN* and *TP53* (Table 2). The adequacy of the proportional hazards assumption of the Cox regression model was evaluated in log minus log plots, which showed parallel lines (Supplementary Figure 1). This indicates that the proportional hazards assumption holds and supports the reliability of the Cox regression model. A more conservative multivariate analysis incorporating only parameters that had $P < 0.01$ in the univariate analyses revealed the same parameters as independent prognostic factors (data not shown).

Glioblastoma patients with *IDH1* mutations had a median overall survival of 659 days versus 219 days for patients without an *IDH1* mutation (Figure 1B), as we described previously.[16] Methylation of the *MGMT* promoter was associated with a median overall survival of 436 days versus 219 days in patients without a methylated *MGMT* promoter (Figure 1C). Patients with both an *IDH1* mutation and *MGMT* methylation had the best survival, followed by patients with only an *IDH1* mutation, then patients with only *MGMT* methylation, and then patients without an *IDH1* mutation or *MGMT* methylation (Figure 1D). Patients with only an *IDH1* mutation had not a significantly different survival from patients with only *MGMT* methylation.

Two-gene predictor

Based on recent reports[1,39,40] and our results confirming the independent prognostic importance of mutations in *IDH1 (IDH1*mt vs. *IDH1*wt*)* and methylation of *MGMT*

*(MGMT*met vs. *MGMT*unmet*)*, we generated a two-gene predictor for glioblastoma survival prediction, based on the (epi)genetic statuses of *IDH1* and *MGMT*. This predictor stratifies glioblastoma patients in three groups: patients with "*IDH1*wt/*MGMT*unmet" glioblastoma (61 patients), "*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met" glioblastoma (27 patients) or "*IDH1*mt/*MGMT*met" glioblastoma (10 patients). In Cox regression analyses a significant difference in overall survival between the three genotypes was identified (Figure 2A). Patients with *IDH1*mt/*MGMT*met glioblastoma had the longest survival, then patients with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastoma whereas patients with *IDH1*wt/*MGMT*unmet glioblastoma had the shortest survival.

To investigate whether this two-gene predictor was able to predict glioblastoma patient survival better than the individual (epi)genetic statuses of *IDH1* and *MGMT* alone, ROC curves were generated for one and two-year survival. Higher area under the curve (AUC) values were retrieved for the two-gene predictor compared to both *IDH1* mutation and *MGMT* methylation (Supplementary Table 4) alone, indicating a better association for the two-gene predictor with glioblastoma patient survival. To further investigate the performance of the two-gene predictor for glioblastoma patient survival prediction, -2 Log Likelihood tests were conducted comparing the two-gene predictor with the (epi)genetic statuses of *IDH1* and *MGMT* alone. These tests indicated that the combined consideration of both *IDH1* mutations and *MGMT* methylation provides better survival predictions than *IDH1* mutations ($P < 0.0001$) or *MGMT* methylation ($P = 0.008$) alone.

Next, this two-gene predictor was validated in three external datasets containing different prevalences of *IDH1* mutations and *MGMT* methylation (Supplementary Table 5). Also in these datasets, the patients with *IDH1*wt/*MGMT*unmet, *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met and *IDH1*mt/*MGMT*met glioblastoma had the shortest, intermediate and longest survival, respectively (Figure 2B-D).[22,27,28] A multivariate Cox regression analysis

confirmed the independent prognostic significance of the two-gene predictor in two of the three external datasets after correction for radiotherapy and the patients' age (Table 3). Other possible confounding factors were not or only limitedly available and could not be corrected for. Log minus log plots were in agreement with the proportional hazards assumption of the Cox models (Supplementary Figure 2). The two-gene predictor outperformed the individual statuses of *IDH1* and *MGMT* in survival prediction in these three external datasets as well ($P = 0.001$ versus *IDH1* mutations and $P = 0.004$ versus *MGMT* methylation; -2 Log Likelihood test and using AUC values (Supplementary Table 4)).

In addition, a multivariate Cox regression analysis on the combined three external datasets was conducted. No interaction between the datasets and the predictor was identified ($P = .403$; Supplementary Table 6). This suggests that the performance of the two-gene predictor is independent of the population and strengthens its significance. Additionally, the predictive value of the two-gene predictor was compared with *IDH1* mutations and *MGMT* methylation alone. In two multivariate Cox regression analyses controlling for either *IDH1* mutation or *MGMT* methylation, the two-gene predictor was an independent prognostic factor (Supplementary Table 7). This indicates that the two-gene predictor harbors significant additional prognostic information to the individual (epi)genetic statuses of *IDH1* and *MGMT*.

Discussion

Here, we present a novel two-gene predictor for glioblastoma survival based on mutations in *IDH1* and methylation of *MGMT*, which is a better predictor of survival than either *IDH1* mutations or *MGMT* methylation alone.

We initially screened 98 human glioblastoma samples for (epi)genetic alterations in ten genes and chromosomal loci. The survival outcome of our patient cohort reflects the dismal prognosis of glioblastoma patients as described in the literature. Because chemoradiation was not standard treatment at the time patients were included, most patients in our cohort did not receive chemoradiation according to the Stupp protocol.[2] Thus, our cohort does not reflect the predictive virtue of *MGMT* methylation to treatment with temozolomide.[5] The median survival in our study (8.5 months) is shorter than reported in other studies (9-12 months) in which patients did not have temozolomide as standard treatment yet.[1,2] However, seven patients (7%) were considered to be long-term survivors with a survival over three years, which is slightly higher than reported previously (2-5%).[32,34] Performance status and age have similar distributions in other studies[2,32] and prevalence of identified genetic alterations were in concordance with previous reports,[4] except for *EGFR*. In *EGFR*, the mutation frequency is lower than reported previously, due to the fact that we sequenced only exons belonging to the kinase domain, whereas Lee *et al.* found mutations predominantly in the extracellular domain.[41] In multivariate analyses, we found the mutational status of *IDH1*, the methylation status of *MGMT* promoter, the KPS score and dosage of irradiation to be independent prognostic factors (Table 2), which is in concordance with previous studies.[6,42-48]

In contrast to other studies,[49-52] the extent of tumor resection was not an independent prognostic factor in our population. This may be the result of differential methods in

determining the extent of the resection. In our study, this information was derived from the surgeon's post-operative report. In other studies,[49-52] the extent of resection is defined on the basis of a post-operative MRI, which is a more objective method.

In our study, the occurrence of a secondary glioblastoma was a significant prognostic factor for progression-free survival but not for overall survival, as described in other studies (Supplementary Table 3).[37,53] This can be due to the small number of secondary glioblastomas in our set, or the assumption that some of our 'primary' glioblastomas are actually secondary ones, for which no clinical, radiological or histological evidence of evolution from a low-grade glioma was found.

Two-gene predictor

We developed a novel two-gene predictor that comprises both genetics and epigenetics and outperforms either mutations in *IDH1* or methylation of the *MGMT* promoter alone for glioblastoma survival prediction. This predictor stratifies glioblastoma patients into three groups and is an independent prognostic factor for overall survival in our dataset and external datasets. *IDH1*mt/*MGMT*met glioblastoma patients had the longest survival, whereas patients with *IDH1*wt/*MGMT*unmet glioblastoma had the shortest survival. Patients with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastoma had a longer survival than the *IDH1*wt/*MGMT*unmet genotype, but shorter than the *IDH1*mt/*MGMT*met genotype. Within the patient group with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastoma, there was no significant survival difference between *IDH1*mt/*MGMT*unmet and *IDH1*wt/*MGMT*met glioblastoma. The two-gene predictor performs well in different populations with various prevalences of alterations in *IDH1* and *MGMT* and different median age and overall survival. The two-gene predictor was an independent prognostic factor in two of three external datasets. The two-gene predictor was insignificant in the Boots-Sprenger dataset (P = .081). This may be due to the fact that this dataset has a relatively small number

of patients for which complete follow-up information on the dosage of radiotherapy was available (n = 68).

Biological significance of the two-gene predictor

In contrast to our (epi)genetic alterations study, distinct molecular prognostic subclasses (proneural, neural, classical and mesenchymal) in glioblastoma have previously been identified by expression profiling studies.[19,20,54] Proneural glioblastomas resemble secondary glioblastoma as they are characterized by *IDH1* mutations, *TP53* and *PDGFRA* alterations and correlate with a better prognosis and younger age.[20] The favorable prognosis of this proneural subtype is restricted to tumors that have the glioma CpG island methylator phenotype (G-CIMP), which has been described to be tightly associated with *IDH1* mutations.[55] Indeed, *IDH1* mutations have been shown to be 'sufficient to establish the glioma hypermethylator phenotype'.[40] In addition, the G-CIMP status correlates well with *MGMT* promoter methylation in low-grade glioma,[40] and glioblastoma.[56] This suggests that *IDH1* mutations may (in)directly promote *MGMT* methylation. A substantial number of patients with *IDH1*-mutated glioblastomas in our study (Table 1) and that of others[22,27,42,57] did not have *MGMT* methylation. Notably, *IDH1* mutations are very early events in the development of glioma[58] and both the *IDH1* mutational[59,60] and *MGMT* methylation status generally do not change during treatment. In 89% of glioblastoma patients the methylation status of *MGMT* in the primary tumor was retained at recurrence.[61] This robustness of the *MGMT* methylation status may suggest that there are biological differences between glioblastoma in which an *IDH1* mutation has (in)directly promoted *MGMT* methylation compared with glioblastoma in which an *IDH1* mutation has not established *MGMT* methylation.

In addition, our results suggest that there are also differences between *IDH1*-mutated glioblastoma with and without *MGMT* methylation in terms of prognosis: The survival time of glioblastoma patients with only *IDH1* mutations is shorter than that of patients with both *IDH1* mutations and *MGMT* methylation (Figure 1D), as previously reported.[33,42,43] These results suggest that the group of *IDH1*-mutated patients is not homogenous and that the prognosis is not only dependent on the *IDH1* mutation status, but also on *MGMT* methylation or potentially G-CIMP status. As the survival advantage in our study is quite large (median 326 days (95% CI 264-388 days) versus 818 days (95% CI 532-1104 days in 489 glioblastoma patients), there may be a synergistic effect between *IDH1* and *MGMT*, which needs to be further (mechanistically) explored in other studies.

Comparison with other prognostic predictors

Other prognostic predictors based on both genetic and epigenetic alterations have been described recently. Models combining 1p/19q co-deletion and either *IDH1* mutational status[62] or G-CIMP[63] were described in (anaplastic) oligodendroglioma. In support of our two-gene predictor, other studies have described a correlation with (progression-free) survival based on the (epi)genetic statuses of *IDH1* and *MGMT* in both low-grade and high-grade glioma and (secondary) glioblastoma.[33,42,43,64] According to the data presented here, the two-gene predictor is valid for both primary and secondary glioblastoma patients. Our dataset and the three external datasets all contain both *de novo* and secondary glioblastoma.

Possible improvements for the two-gene predictor

Recently, a combined analysis of MGMT protein expression and *MGMT* promoter methylation was described, which optimizes prognostic predictions for glioblastoma patient survival.[65] It is possible that a predictor with more prognostic power is conceived by combining the mutational status of *IDH1* with this combined *MGMT* analysis.

Only a few cases of *IDH2* mutations have been described in glioblastoma thus far.[27,33] In WHO grade III glioma, *IDH2* is considered to have the same prognostic effect as *IDH1*.[11] Therefore, *IDH1* and *IDH2* mutations are handled as one in most of the glioma literature. However, definitive confirmation on the prognostic status of *IDH2* in glioblastoma is not available. A single *IDH2* mutation was found in the Boots-Sprenger *et al.* dataset,[27] but none was found in our dataset and the other external validation datasets. We did not include the *IDH2* mutation from the Boots-Sprenger *et al.* dataset in the results described here.[27] Whether we chose to combine both *IDH1* and *IDH2* mutations or to discard the *IDH2* mutation, the validation results of the two-gene predictor did not change. Tests in datasets with more patients with *IDH2* mutations are needed to confirm whether this two-gene predictor should be extended with *IDH2* mutations to a three-gene predictor.

Future implications

The two-gene predictor uses two well-established (epi)genetic alterations that are most likely playing a role in future therapeutic options for glioblastoma patients. Recently, two independent clinical trials in elderly patients with *MGMT* methylated WHO grade III astrocytoma[66] or glioblastoma[66,67] reported that temozolomide treatment alone resulted in a better outcome than only radiotherapy. In contrast, in patients with unmethylated *MGMT* radiotherapy alone was better than temozolomide alone. These results suggest that the epigenetic status of *MGMT* allows individualized therapy in elderly patients who are not treated with a combination of temozolomide and radiotherapy. In addition, second-line treated glioblastoma patients are being stratified in different arms on the basis of *MGMT* methylation status in clinical trials with new agents.[68,69] Very recently, an *IDH1* mutant-specific inhibitor was described,[70] which is expected to become available for clinical trials in the near future.[71] This context underlines the clinical significance of both *IDH1* mutations and *MGMT* methylation. It may become reality soon that molecular changes provide a

rationale for newly diagnosed glioblastoma treatment. The two-gene predictor illustrates that the group of glioblastoma patients with *IDH1* mutations or *MGMT* methylation is not homogenous in terms of prognosis: it matters whether there is co-occurrence of the other alteration. Further research on the underlying mechanism may increase our biological understanding of glioblastoma. In addition, we propose that clinical trials that include glioblastoma patients with *IDH1* mutations or *MGMT* methylation report the frequencies of *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastoma and *IDH1*mt/*MGMT*met glioblastoma in each arm. We believe this will be a helpful addition to baseline comparisons in future clinical trials.

Acknowledgements

The authors thank Drs. W. Tigchelaar for sample handling.

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Figure legends

Fig. 1. Kaplan-Meier survival curves of 98 glioblastoma patients.

A: Overall survival curve. **B**: Survival curves comparing *IDH1* mutated with non-mutated glioblastoma patients. **C**: Survival curves comparing *MGMT*-methylated and non *MGMT*-methylated glioblastoma patients. **D**: Survival curves of patients with *IDH1* wild-type and unmethylated *MGMT* promoter; *IDH1* wild-type and methylated *MGMT* promoter; *IDH1* mutation and unmethylated *MGMT* promoter; *IDH1* mutation and methylated *MGMT* promoter. *P* values were calculated by the log-rank test. Abbreviations: M.S., median survival.

Fig. 2. Kaplan-Meier survival curves of a two-gene prognostic model in which glioblastoma patients are stratified in three groups (*IDH1*wt/*MGMT*unmet, *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met and *IDH1*mt/*MGMT*met).

A: Survival curves comparing 98 glioblastoma patients that were stratified using the two-gene predictor. **B-D**: Survival curves comparing the three groups of the two-gene predictor in three external datasets: Mulholland *et al.*, 2012 (**B**),[28] Boots-Sprenger *et al.*, 2013 (**C**),[27] and TCGA, 2012 (**D**).[22] *P* values were calculated by Cox proportional hazard models. Abbreviations: M.S., median survival.

Table 1

Characteristic	**Specification**	**Outcome**	**Wild type *IDH1/2*** **(n=80)**	**Mutated *IDH1*** **(n=18)**	***P*** **value**
Age	Mean (range), in years	55 (27-80)	58 (27-80)	41 (28-62)	<.001*
Irradiation dosage	Mean (range), Gy	41 (0-78)	39 (0-78)	48 (0-66)	.193*
KPS	Mean (range), in points	76 (50-90)	75 (50-90)	76 (50-90)	.813*
Gender	Male	53 (54%)	44	9	.796†
	Female	45 (46%)	36	9	
Surgical procedure	Gross total removal	57 (58%)	46	11	1.000†
	Biopsy or irradical resection	41 (42%)	34	7	
Tumor occurrence	Primary glioblastoma	85 (87%)	75	10	<.001†
	Secondary glioblastoma	13 (13%)	5	8	
Overall survival	Median (95% CI), in days	252 (206-318)	204 (157-250)	659 (565-752)	<.001‡
Progression free survival	Median (95% CI), in days	131 (105-157)	115 (88-142)	258 (54-462)	.001‡

CDKN2A	alteration	total 72 (74%)	65	7	.003†
	hemizygous loss	27 (28%)			
	homozygous loss	43 (45%)	40	3	.028†
	gain	2 (2%)			
EGFR	alteration	total 70 (72%)	64	6	.001†
	gain	40 (42%)			
	amplification	4 (4%)			
	high CNA	26 (27%)	26	0	.005†
	point mutation	5 (5%)			
	EGFRvIII	5 (5%)			
	EGFR deletion other than vIII	10 (10%)			
IDH1	mutation	18 (18%)			
IDH2	mutation	0 (0%)			
MGMT	methylation	29 (30%)	19	10	.011†
PIK3CA	mutation	10 (10%)			
PTEN	alteration	total 69 (70%)	16	11	<.001†

	mutation	24 (25%)	24	0	.005†
	hemizygous loss	64 (67%)	59	5	.002†
	homozygous loss	4 (4%)			
	no CNA	28 (29%)	17	11	<.001†
TP53	mutation	38 (39%)	23	15	<.001†
1p/19q	1p and 19q loss (partial or complete)	9 (9%)	5	4	.056†
	complete 1p and 19q loss	2 (2%)			

Table 1. Baseline characteristics of 98 glioblastoma patients. Prevalence of (epi)genetic alterations and cross tabulation of *IDH1* mutation status versus clinical characteristics and (epi)genetic alterations are depicted. For genetic alterations, only significant findings are shown. Data are mean (range), number (%) or median (95% CI). *P* values were calculated by the * Student's T-test (2-sided), † Fisher's exact test (2-sided) and ‡ log-rank test. Abbreviations: CNA, copy number alteration; Gy, Gray; KPS, Karnofsky Performance Status.

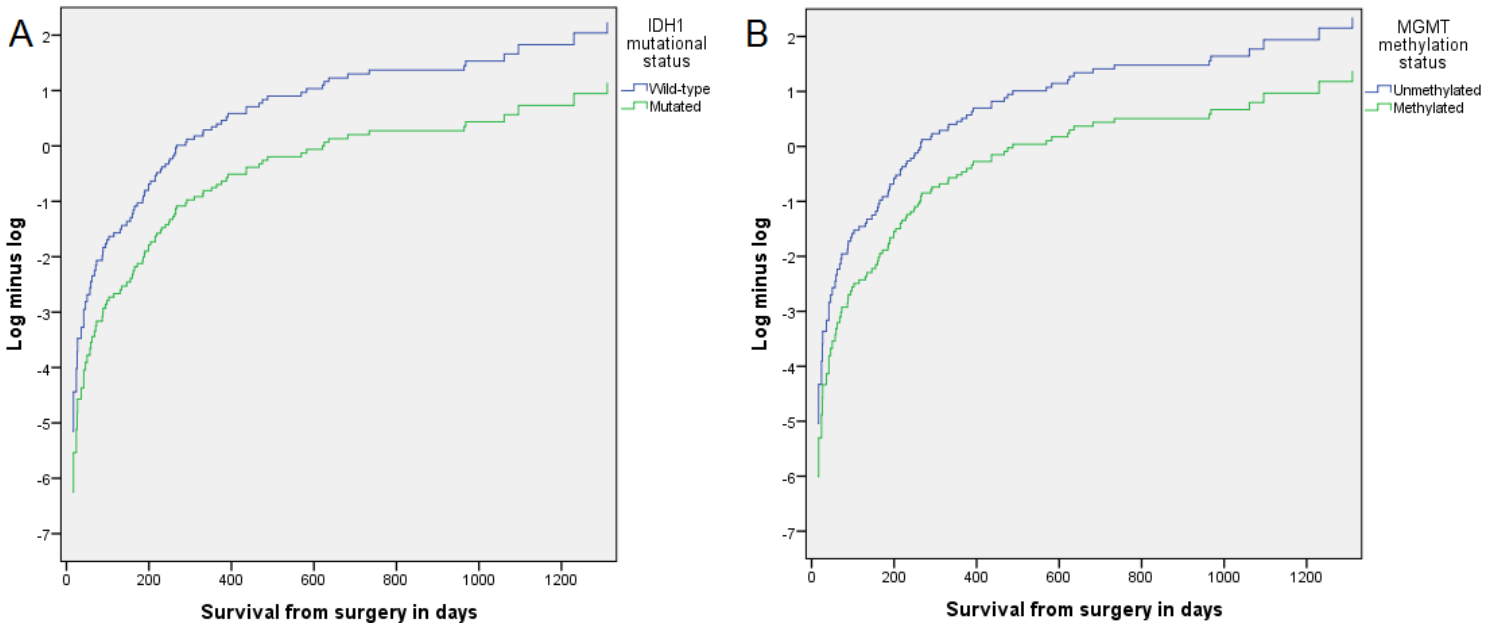
Table 2

Characteristic	**Univariate**	**Multivariate**			
	P value	*P* value	HR	95% CI for HR	
				Lower	Upper
Age, per year	<.001	.056	1.019	.999	1.040
Extent of resection	.041	.089	.651	.397	1.068
KPS, per 10 points	.001	<.001	.958	.937	.979
Radiotherapy, per Gy	<.001	<.001	.974	.964	.984
Secondary glioblastoma	.075				
CDKN2A-alteration	.120				
EGFR-alteration	.018	.918	.964	.480	1.936
IDH1-mutation	<.001	.001	.241	.107	.544
MGMT-methylation	.009	.001	.396	.227	.689
PIK3CA-mutation	.025	.767	.871	.350	2.167
TP53-mutation	.004	.694	.886	.484	1.622

PTEN-mutation	.125	.983	1.007	.536	1.892
1p19q codeletion	.687				

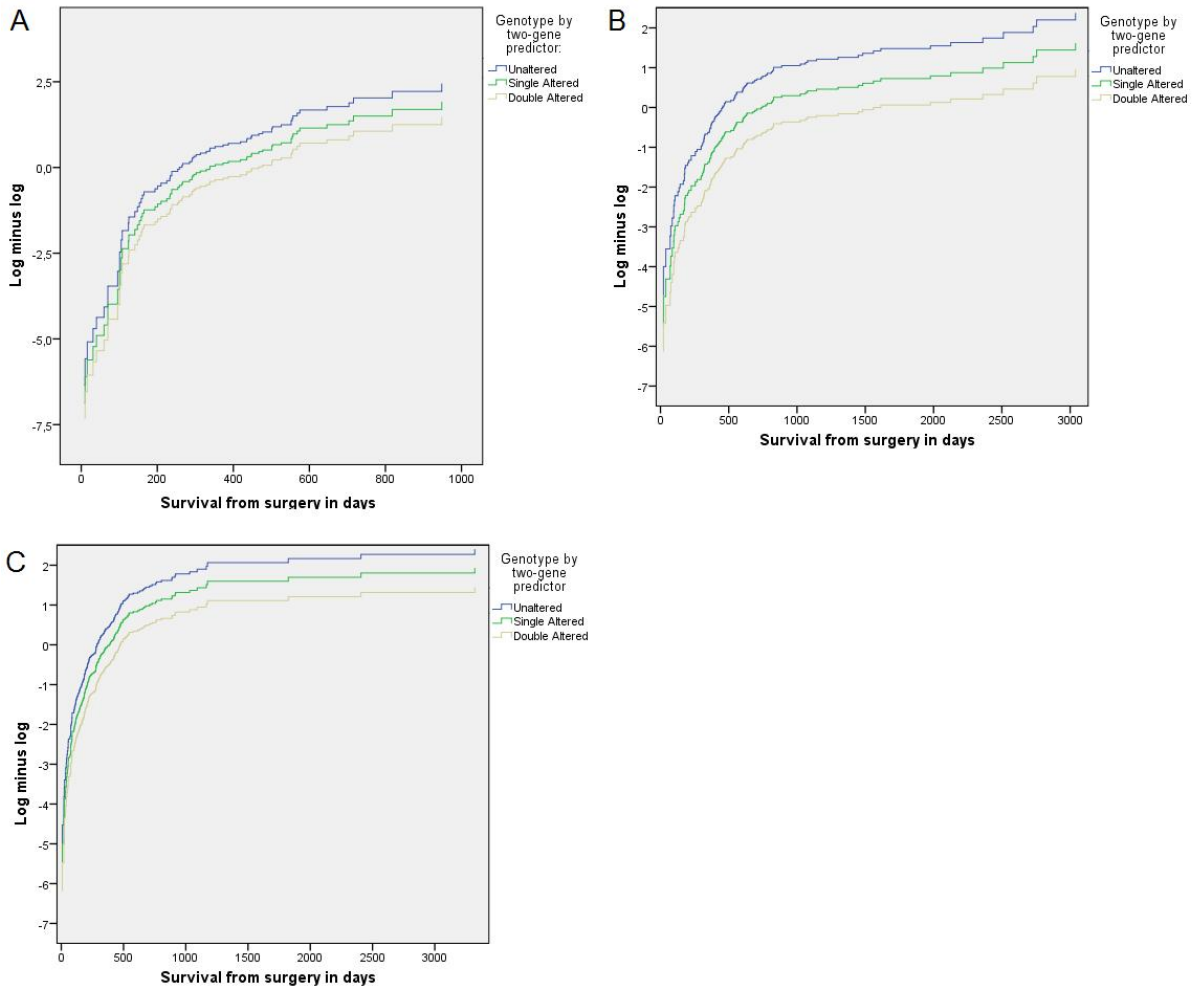
Table 2. Prognostic univariate and multivariate Cox regression analyses in 98 glioblastoma patients, using a stepwise Wald backward selection procedure. KPS score, dosage of irradiation, *IDH1*-mutation and *MGMT*-methylation were significant in the final step of the Wald procedure. For all other patient characteristics, the values depicted are calculated in the step prior to their removal. The normal and conservative multivariate analyses included parameters that had $P < 0.05$ and $P < 0.01$ in the univariate analyses. Abbreviations: Gy, Gray; KPS, Karnofsky Performance Status; HR, Hazard Ratio.

Supplemental Figure 1



Supplemental Figure 1. Log minus log function of Cox proportional hazard models of the mutational status of *IDH1* (**A**) and the methylation status of *MGMT* (**B**) in the present dataset (*n* = 98 patients). The parallel lines of the *IDH1* mutated/wild-type and *MGMT* unmethylated/methylated patient groups support the adequacy of the proportional hazard assumption.

Supplemental Figure 2



Supplemental Figure 2. Log minus log function of Cox proportional hazard models of the two-gene predictor in the three external datasets: Boots-Sprenger *et al.* (**A**), Mulholland *et al.* (**B**) and The Cancer Genome Atlas (**C**). The parallel lines of the three genotypes from the two-gene predictor support the adequacy of the proportional hazard assumption.

Supplementary table 3

Characteristic	**Specification**	***MGMT* not methylated (n=69)**	***MGMT* methylated (n=29)**	***P* value**	**Primary glioblastoma (n=85)**	**Secondary glioblastoma (n=13)**	***P* value**
Age (in years)	Mean (range)	56 (27-80)	53 (27-80)	.519 *	57 (27-80)	42 (27-80)	<.001 *
Irradiation dosage (in Gy)	Mean (range)	41 (0-78)	39 (0-66)	.654 *	43 (0-78)	27 (0-66)	.132 *
KPS (in points)	Mean (range)	76 (50-90)	74 (50-90)	.355 *	76 (50-90)	75 (50-90)	.968 *
Overall survival (in days)	Median (95% CI)	215 (166-264)	392 (287-497)	.008 †	231 (176-286)	313 (49-577)	.069 †
Progression free survival (in days)	Median (95% CI)	115 (90-140)	215 (157-237)	.010 †	126 (103-149)	258 (136-380)	.049 †
Gender	Male	41	12	.123 ‡	40 (47%)	5 (39%)	.766 ‡
	Female	28	17		45 (53%)	8 (62%)	
Tumor occurrence	Primary	62	23	.196 ‡			

	glioblastoma						
	Secondary glioblastoma	7	6				
EGFR	No alteration				19 (22%)	7 (64%)	.008 ‡
	Alteration				66 (78%)	4 (36%)	
IDH1	Wild type	61	19	.011 ‡	75 (88%)	5 (38%)	<.001 ‡
	Mutation	8	10		10 (12%)	8 (62%)	
MGMT	Not methylated				62 (73%)	7 (54%)	.196 ‡
	Methylated				23 (27%)	6 (46%)	
PTEN	No alteration				19 (22%)	8 (73%)	.001 ‡
	Alteration				66 (78%)	3 (27%)	
TP53	Wild type	54	6	<.001 ‡			
	Mutation	15	23				

Supplementary table 3. Cross tabulation of *MGMT* promoter methylation versus clinical characteristics and genetic alterations of 98 glioblastoma patients. For genetic alterations, only *MGMT* methylation, *IDH1* mutations and significant findings are shown. *P* values were

calculated by the * Student's T-test, † log-rank test and ‡ Fisher's exact test (2-sided where applicable). Abbreviations: LOH, loss of heterozygosity.