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

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Background. Genetic and epigenetic profiling of glioblastomas has provided a comprehensive list of altered cancer genes of which only O^6-methylguanine-methyltransferase (*MGMT*) methylation is used thus far as a predictive marker in a clinical setting. We investigated the prognostic significance of genetic and epigenetic alterations in glioblastoma patients.

Methods. We screened 98 human glioblastoma samples for genetic and epigenetic alterations in 10 genes and chromosomal loci by PCR and multiplex ligation-dependent probe amplification (MLPA). We tested the association between these genetic and epigenetic alterations and glioblastoma patient survival. Subsequently, we developed a 2-gene survival predictor.

Results. Multivariate analyses revealed that mutations in isocitrate dehydrogenase 1 (*IDH1*), promoter methylation of *MGMT*, irradiation dosage, and Karnofsky Performance Status (KFS) were independent prognostic factors. A 2-gene predictor for glioblastoma survival was generated. Based on the genetic and epigenetic status of *IDH1* and *MGMT*, glioblastoma patients were stratified into 3 clinically different genotypes: glioblastoma patients with *IDH1*mt/*MGMT*met had the longest survival, followed by patients with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met, and patients with *IDH1*wt/*MGMT*unmet had the shortest survival. This 2-gene predictor was an independent prognostic factor and performed significantly better in predicting survival than either *IDH1* mutations or *MGMT* methylation alone. The predictor was validated in 3 external datasets.

Discussion. The combination of *IDH1* mutations and *MGMT* methylation outperforms either *IDH1* mutations or *MGMT* methylation alone in predicting survival of glioblastoma patients. This information will help to increase our understanding of glioblastoma biology, and it may be helpful for baseline comparisons in future clinical trials.

Keywords: genetic and epigenetic, glioblastoma, *IDH1*, *MGMT*, survival prediction.

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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 optimal drug development, it is essential to unravel the underlying oncogenic mechanisms of glioblastoma.

Most glioblastomas 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 that has progressed to secondary glioblastoma over a period of years.[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.[4] In the last several years, there has been a great increase in understanding molecular alterations and their consequences for the pathology of glioblastoma.

For a long time, the most studied genetic hallmarks of glioblastoma were *EGFRvIII*, a truncated constitutively activated form of *EGFR*, mutations in *TP53*, and deletions in *PTEN*.[4] More recently, methylation of the MGMT gene promoter appeared to be a predictive factor for the response of glioblastoma patients to temozolomide and radiotherapy, and hence their survival.[1,5,6] Conflicting results have been reported on the methylation status of *MGMT* as a positive prognostic marker independent of therapy.[7–9] Recently, genome-wide sequencing of glioblastoma has 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 allowed stratification of glioblastoma patients into the classical, mesenchymal, proneural, and neural subtypes, which are characterized by aberrations in and gene expression of *EGFR*, *NF1*, *IDH1*, and *PDGFRA* and predict prognosis.[20]

We investigated the association between genetic and epigenetic alterations in *IDH1/2, MGMT* and other genes, chromosomal loci, and survival of glioblastoma patients. These results led us to propose a novel 2-gene predictor for glioblastoma survival 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) in Amsterdam. Oral consent for removal of the tissue and its storage in the tumor bank for research purposes was obtained and documented in the patients' medical charts. Consent for this project was approved by the local ethics committee. Research was performed on "waste" material and stored in a coded fashion. Tumor samples were included only if at least 80% of the sample consisted of cancer cells, as verified by hematoxylin and eosin 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) cases were included, but recurrent glioblastoma cases were not included. These patients had undergone 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) were 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 reirradiation (leading to a total radiation dosage up to 78 Gy), chemoradiation (radiotherapy with concomitant and adjuvant temozolomide therapy), brachytherapy, carmustine wafers (gliadel), procarbazine/lomustine/vincristine (PCV), temozolomide, methotrexate, and nicotinamine (as an enhancer during irradiation). The preoperatively determined KFS score was used to match other studies.[2]

Mutation Analysis, Polymerase Chain Reaction, and Sequencing Details

We investigated *EGFR, IDH1*, *IDH2*, *PIK3CA*, *PTEN*, and *TP53* for somatic mutations, genes known to be mutated in glioblastoma in at least 10% of cases.[22] Sequencing results of *IDH1* and *IDH2* have been published previously.[16] PCR and sequencing primers were designed using Primer 3 and synthesized by Invitrogen. PCR primers were designed to amplify the selected 47 exons and the flanking intron sequences, including splicing donor and acceptor regions of the genes (Supplementary Table S1). PCR products were ~400 base pairs in length with multiple overlapping amplimers for larger exons. *IDH2* was sequenced using an M13 sequencing primer. Fifty PCRs were performed on each sample in 384- and 96-well formats in 5 or 10 μL reaction volumes, respectively. PCR conditions have been published previously.[13] Changes previously described as SNPs were excluded from further analyses. To ensure that the observed mutations were not PCR or sequencing artifacts, amplicons (including nonsilent mutations) were independently reamplified and resequenced in the corresponding tumors. All verified changes were resequenced in parallel with the matched normal DNA to distinguish between somatic mutations and SNPs not previously described.

Table 1. Baseline characteristics of 98 glioblastoma patients

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*
KFS	Mean (range), in points	76 (50–90)	75 (50–90)	76 (50–90)	.813*
Sex	Male	53 (54%)	44	9	.796†
	Female	45 (46%)	36	9	
Surgical procedure	Gross total removal	57 (58%)	46	11	.999†
	Biopsy or partial 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	11 (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%)			

Prevalence of genetic and epigenetic alterations and cross tabulation of *IDH1* mutation status versus clinical characteristics and genetic and epigenetic 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; KFS, Karnofsky Performance Status.

Multiplex Ligation-dependent Probe Amplification Experiments

Multiple 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. MLPA assay P088 (lot number 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). MLPA assay P105 (lot number 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 to identify EGFR rearrangements (EGFRΔ) such as *EGFRvIII*. As described previously, the sensitivity and specificity of these MLPA assays were fully validated.[24,25] As MLPA provides semiquantitative 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), 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 <4:10 were considered to represent homozygous losses, ratios >2:1 to represent amplifications, and ratios >10:1 to represent high copy-number amplifications. As described previously,[24] *EGFRvIII* was identified by assessing the average ratio for exon 2–7 probes and comparing the ratio with the average ratio of probes for exons 1, 8, 13, 17, and 22 (*EGFRvIII* ratio). *EGFRvIII* ratios <8:10 were considered to harbor the *EGFRvIII* deletion variant.[24]

Additionally, the individual probe ratios were inspected 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 <8:10 for at least 3 adjacent probes but not for all probes of 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 >5:10.[27]

Statistical Analysis

Statistical processing of data was performed using Excel 2002 (Microsoft) and SPSS 19 for Windows (IBM). Figures were constructed in SPSS 19 and Prism 5 (Graphpad Software). Associations between the different alterations were assessed by the Fisher's exact test. Differences in age and survival were tested by the Student' *t* test and log-rank test, respectively. Associations between mutations and patient survival were tested with Cox regression analyses. Parameters with $P < .05$ in the univariate analyses were incorporated into the multivariate analysis using a Wald backward selection procedure (stepwise elimination of parameters until all remaining parameters had $P < .05$). Because 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 by incorporating only parameters with $P < .01$ in the univariate analyses. Log-minus-log plots were used to evaluate the adequacy of the proportional hazards assumption.

A 2-gene predictor for survival in glioblastoma was designed, incorporating both *IDH1* mutational status and *MGMT* promoter methylation status. We classified tumors in 3 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 2-gene predictor was validated internally, and externally in 3 additional glioblastoma datasets using receiver-operating characteristic (ROC) curves, multivariate Cox regression analyses, and -2 log-likelihood tests. The external datasets were obtained from Mulholland et al (2012),[28] The Cancer Genome Atlas Network (TCGA; accessed November 27, 2012),[22] and Boots-Sprenger et al (2013)[27] and contained 182, 104, and 105 glioblastoma samples, respectively. In a merged dataset containing all 4 datasets ($n = 489$ glioblastoma cases), we further evaluated the performance of the 2-gene predictor in different populations by testing for interaction between datasets and the 2-gene predictor in a multivariate Cox regression analysis.

Results

An overview of the prevalence of genetic and epigenetic alterations identified in 98 glioblastoma patients with known follow-up is shown in Table 1. A more detailed description of the observed *EGFR, IDH1, PIK3CA, PTEN*, and *TP53* mutations is given in Supplementary Table S2. We found *IDH1* mutations in 18 of 98 glioblastoma samples, 10 in primary glioblastomas (12%), and 8 in secondary glioblastomas (62%). Of these mutations, 15 were R132H, and one each was R132C, R132L, and R132G.[16] We did not identify any *IDH2* mutations in our set of glioblastoma samples (Table 1). 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 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 S3). 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.[29]

Codeletion of chromosomes 1p and 19q (1p/19q codeletion) is frequently observed in chemotherapy-sensitive oligodendrogliomas and is associated with prolonged survival in those cases.[30,31] Although 1p/19q codeletion has been observed in glioblastoma,[14,32] no translocations have been identified. In our dataset, codeletions involving chromosome arms of 1p and 19q were found in 9% of the cases, with only 2% of tumors showing complete 1p/19q codeletion (Table 1), which might be indicative of a reported translocation.[33–35] Loss of heterozygosity in 19q has been reported as a marker of prolonged survival in glioblastoma patients,[33] 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 ($P = .001$, $P = .026$, and $P = .008$, respectively, Fisher's exact test; Supplementary Table S3), whereas

Table 2. Prognostic univariate and multivariate Cox regression analyses in 98 glioblastoma patients using a stepwise Wald backward selection procedure

Characteristic	Univariate	Multivariate			
	P value	*P* value	HR	95% CI for HR	
				Lower	Upper
Age, per year	<.001	.056	1.019	0.999	1.040
Extent of resection	.041	.089	0.651	0.397	1.068
KFS, per 10 points	.001	<.001	0.958	0.937	0.979
Radiotherapy, per Gy	<.001	<.001	0.974	0.964	0.984
Secondary glioblastoma	.075				
CDKN2A alteration	.120				
EGFR alteration	.018	.918	0.964	0.480	1.936
IDH1 mutation	<.001	.001	0.241	0.107	0.544
MGMT methylation	.009	.001	0.396	0.227	0.689
PIK3CA mutation	.025	.767	0.871	0.350	2.167
TP53 mutation	.004	.694	0.886	0.484	1.622
PTEN mutation	.125	.983	1.007	0.536	1.892
1p19q codeletion	.687				

KFS 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 $P < .05$ and $P < .01$ in the univariate analyses.

Abbreviations: Gy, gray; KFS, Karnofsky Performance Status; HR, hazard ratio.

IDH1 mutations and complete loss of chromosome 1p occurred more frequently in secondary glioblastomas, as reported previously.[3,36,37] Although most secondary glioblastomas contained a *TP53* mutation (8 of 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 reported before (reviewed in [4,38]). A new classification, based only on genetic alterations, has recently been proposed.[38]

Survival Analysis

The median survival of the patients was 252 days (8.5 months; Fig. 1A) with 35% and 16% of patients alive at 1 and 2 years, respectively. Seven patients (7%) were considered to be long-term survivors with survival longer than 3 years. Remarkably, the 2 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 whether any of the genetic alterations was associated with survival by univariate analyses (Table 2). The patients' age, extent of resection, KFS, the dosage (Gy) of radiotherapy received, mutations in *IDH1*, *PIK3CA*, *PTEN* and *TP53*, alterations of *EGFR*, and methylation of the *MGMT* promoter were found to be significantly associated with survival.

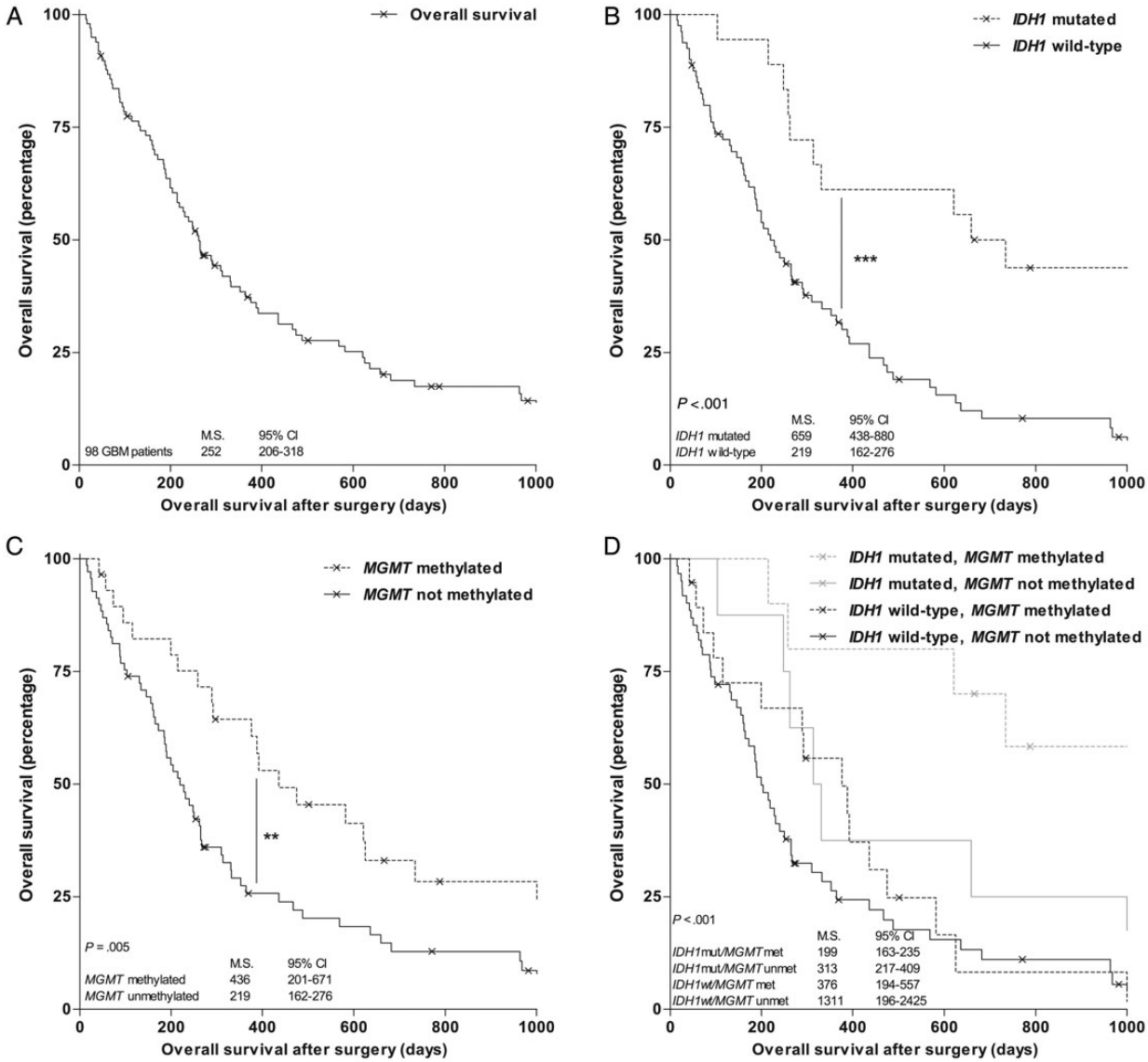

Fig. 1. Kaplan–Meier survival curves of 98 glioblastoma patients. (A) Overall survival curve. (B) Survival curves comparing *IDH1*-mutated with nonmutated glioblastoma patients. (C) Survival curves comparing *MGMT*-methylated and non-*MGMT*-methylated glioblastoma patients. (D) Survival curves of patients 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.

In a multivariate Cox regression analysis incorporating parameters with $P < .05$ in the univariate analyses, the prognostic significance of radiotherapy, the KFS 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 Fig. S1). This indicates that the proportional hazards assumption holds firm and supports the reliability of the Cox regression model. A more conservative multivariate analysis, incorporating only parameters that had $P < .01$ in the univariate analyses, revealed the same parameters to be 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 (Fig. 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 (Fig. 1C). Patients with both an *IDH1* mutation and *MGMT* methylation had the best survival, followed by patients with only an *IDH1* mutation, then by patients with only *MGMT* methylation, and last by patients without an *IDH1* mutation or *MGMT* methylation (Fig. 1D). Patients with only an *IDH1* mutation did not have 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 2-gene predictor for glioblastoma survival based on the genetic and epigenetic statuses of *IDH1* and *MGMT*. This predictor stratifies patients into 3 groups: patients with "*IDH1*wt/*MGMT*unmet" glioblastoma (61 patients), "*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met" glioblastoma (27 patients), and "*IDH1*mt/*MGMT*met" glioblastoma (10 patients). In Cox regression analyses, a significant difference was identified in overall survival between the 3 genotypes (Fig. 2A). Patients with *IDH1*mt/*MGMT*met glioblastoma had the longest survival, followed by patients with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastoma, while patients with *IDH1*wt/*MGMT*unmet glioblastoma had the shortest survival.

To investigate whether this 2-gene predictor could predict glioblastoma patient survival better than the individual genetic and epigenetic statuses of *IDH1* and *MGMT* alone, ROC curves were generated for 1- and 2-year survival. Higher area under the curve (AUC) values were retrieved for the 2-gene predictor compared with both *IDH1* mutation and *MGMT* methylation alone (Supplementary Table S4), which indicated stronger association for the 2-gene predictor with glioblastoma patient survival. To further investigate performance of the 2-gene predictor for glioblastoma patient survival prediction, -2 log-likelihood tests were conducted to compare the 2-gene predictor with the genetic and epigenetic 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 ($P < .0001$) or *MGMT* methylation ($P = .008$) alone.

Next, this 2-gene predictor was validated in 3 external datasets containing different prevalences of *IDH1* mutations and *MGMT* methylation (Supplementary Table S5). 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 survivals, respectively (Fig. 2B–D).[22,27,28] A multivariate Cox regression analysis confirmed the independent prognostic significance of the 2-gene predictor in 2 of the 3 external datasets after correction for radiotherapy and patient's age (Table 3). Other possible confounding factors were not or only limitedly available and could not be corrected for. Log-minus-log plots confirmed the proportional hazards assumption of the Cox models (Supplementary Fig. S2). The 2-gene predictor outperformed the individual statuses of *IDH1* and *MGMT* in survival prediction in these 3 external datasets as well ($P = .001$ vs *IDH1* mutations and $P = .004$ vs *MGMT* methylation; -2 log-likelihood test using AUC values (Supplementary Table S4)).

In addition, multivariate Cox regression analysis was conducted on the combined 3 external datasets. No interaction between the datasets and the predictor was identified ($P = .403$; Supplementary Table S6). This suggests that the performance of the 2-gene predictor is independent of the population and increases its significance. Additionally, the predictive value of the 2-gene predictor was compared with *IDH1* mutations and *MGMT* methylation alone. In 2 multivariate Cox regression analyses controlling for either *IDH1* mutation or *MGMT* methylation, the 2-gene predictor was an independent prognostic factor (Supplementary Table S7). This indicates that the 2-gene predictor harbors significant additional prognostic information to the prognostic information of the genetic and epigenetic statuses of *IDH1* and *MGMT*.

Discussion

Here, we present a novel 2-gene predictor for glioblastoma survival based on mutations in *IDH1* and methylation of *MGMT*, which is more predictive of survival than either *IDH1* mutations or *MGMT* methylation alone.

We initially screened 98 human glioblastoma samples for genetic and epigenetic alterations in 10 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 these patients were included, most patients in our cohort did not receive chemoradiation according to the Stupp protocol.[2] Thus, our cohort may not completely reflect the predictive virtue of *MGMT* methylation to treatment with temozolomide.[5] However, a methylated *MGMT* promoter was an independent prognostic factor in our study (Table 2), as was shown previously in some other studies.[7–9] There is still controversy about the limit of the predictive and prognostic value of *MGMT*, and further research is warranted.[41] The median survival in our study (8.5 months) is shorter than that reported in other studies (9–12 months) in which patients did not yet have temozolomide as the standard treatment.[1,2] However, 7 patients (7%) were considered to be long-term survivors with survival over 3 years, which is slightly higher than reported previously (2%–5%).[31,33] KFS and age have similar distributions in other studies,[2,31] and the prevalence of identified genetic alterations was in concordance with previous reports,[4] except for *EGFR*. In *EGFR*, the mutation frequency is

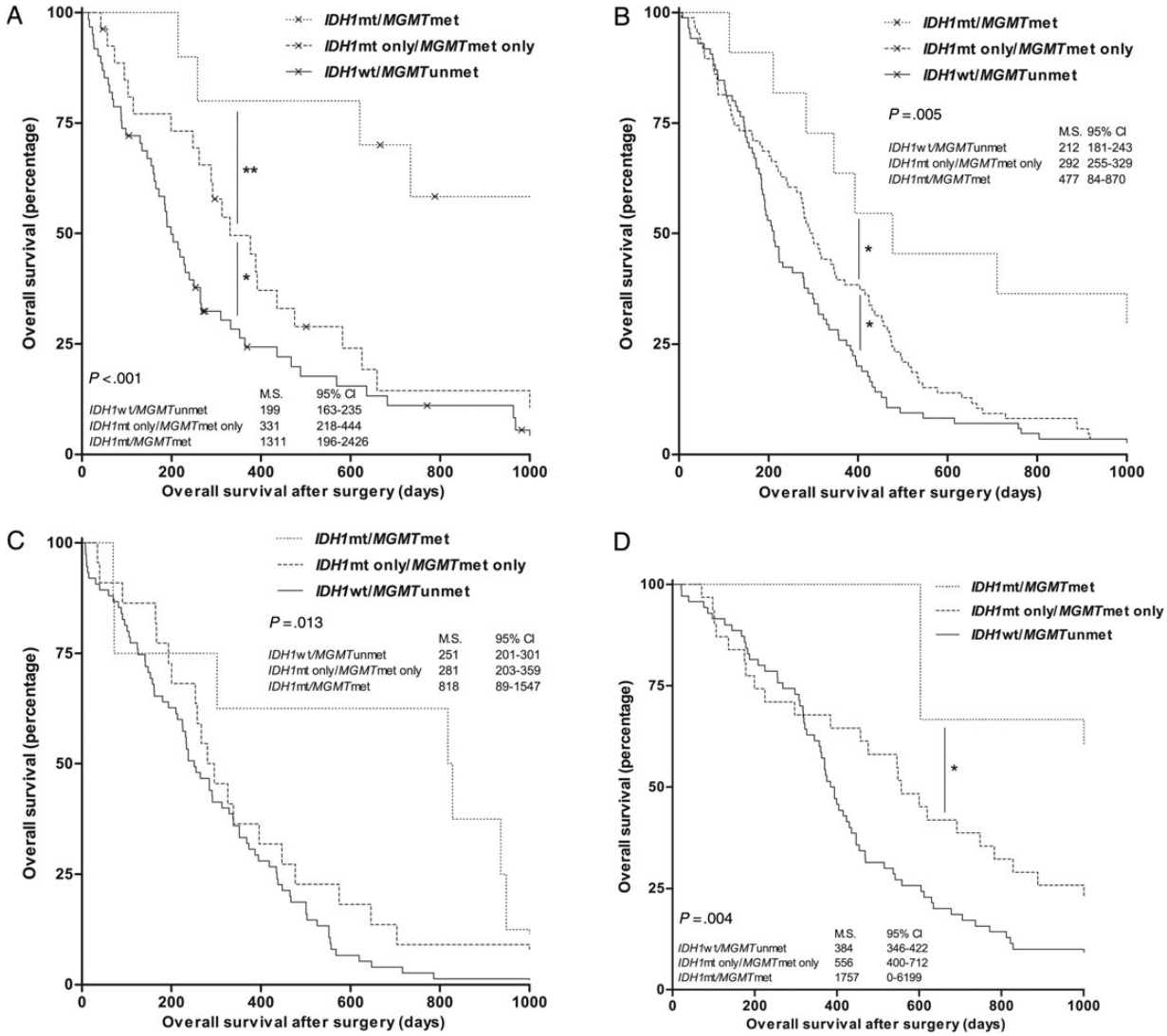

Fig. 2. Kaplan–Meier survival curves of a 2-gene prognostic model in which glioblastoma patients are stratified into 3 groups (*IDH1*wt/*MGMT*unmet, *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met, and *IDH1*mt/*MGMT*met). (A) Survival curves comparing 98 glioblastoma patients who were stratified using the 2-gene predictor. (B–D) Survival curves comparing the 3 groups of the 2-gene predictor in 3 external datasets: (B) Mulholland et al, 2012,[28] (C) Boots-Sprenger et al, 2013,[27] and (D) TCGA, 2012.[22] *P* values were calculated by Cox proportional hazard models. Abbreviations: M.S., median survival.

lower than reported previously because we sequenced only exons belonging to the kinase domain, whereas Lee et al found mutations predominantly in the extracellular domain.[42] In multivariate analyses, we found the mutational status of *IDH1*, the methylation status of the *MGMT* promoter, the KFS score, and the dosage of irradiation to be independent prognostic factors (Table 2), which concurs with previous studies.[6,43–49] We used the preoperatively determined KFS score in this analysis. This could have led to selection bias because patients with higher pretreatment KFS scores may receive more aggressive treatment. As there was no significant difference between the preoperative and 2–3 week postoperative KFS scores in our study (data not shown), this suggests that the independent prognostic status of the KFS score was not the result of selection bias.

In contrast to other studies,[50–53] the extent of tumor resection was not an independent prognostic factor in our population. This may be the result of different methods for determining the extent of resection. In our study, this information was derived from the surgeon's postoperative report. In other studies,[50–53] the extent of resection is defined on the basis of a postoperative 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 S3).[36,54] This can be due to the small number of secondary glioblastomas in our set or the assumption that some of our "primary" glioblastomas were actually secondary glioblastomas, for which no clinical, radiological, or histological evidence of evolution from a low-grade glioma was found.[38]

Table 3. Prognostic multivariate Cox regression analyses in the datasets of Mulholland et al (A), The Cancer Genome Atlas (B), and Boots-Sprenger et al (C)

Characteristic	Multivariate analysis			
	P value	HR	95% CI for HR	
			Lower	Upper
A				
Age, per year	<.001	1.041	1.026	1.055
Radiotherapy	<.001	0.518	0.370	0.727
2-gene prognostic model:	.001	Reference		
*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met	.003	0.626	0.459	0.854
*IDH1*mt/*MGMT*met	.006	0.384	0.194	0.763
B				
Age, per year	.016	1.019	1.003	1.035
Radiotherapy	.000	0.129	0.054	0.309
2-gene prognostic model:	.002	Reference		
*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met	.002	0.470	0.291	0.759
*IDH1*mt/*MGMT*met	.029	0.242	0.068	0.862
C				
Age, per year	.023	1.023	1.003	1.042
Radiotherapy	.249	0.789	0.527	1.181
2-gene prognostic model:	.081	Reference		
*IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met	.061	0.612	0.366	1.023
*IDH1*mt/*MGMT*met	.162	0.543	0.231	1.277

P values are calculated by Cox proportional hazard models.
Abbreviations: Gy, gray; HR, hazard ratio.

Two-gene Predictor

We developed a novel 2-gene predictor that comprises both genetics and epigenetics and outperforms either mutations in *IDH1* or methylation of the *MGMT* promoter alone for prediction of glioblastoma survival. This predictor stratifies glioblastoma patients into 3 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 glioblastomas had the shortest survival. Patients with *IDH1*mt/*MGMT*unmet or *IDH1*wt/*MGMT*met glioblastomas had a longer survival than the *IDH1*wt/*MGMT*unmet genotype but shorter than the *IDH1*mt/*MGMT*met genotype. Between the patient groups with *IDH1*mt/*MGMT*unmet and *IDH1*wt/*MGMT*met glioblastomas, there was no significant difference in survival. The 2-gene predictor performs well in different populations with various prevalences of alterations in *IDH1* and *MGMT* and different median ages and overall survival. The 2-gene predictor was an independent prognostic factor in 2 of 3 external datasets. In multivariate analysis, the 2-gene predictor was not significant 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 was available ($n = 68$).

Biological Significance of the 2-gene Predictor

In contrast to our genetic and epigenetic alterations study, distinct molecular prognostic subclasses (proneural, neural, classical, and mesenchymal) in glioblastoma have previously been identified by expression-profiling studies.[19,20,55] Proneural glioblastomas resemble secondary glioblastomas in that they are characterized by *IDH1* mutations and *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 as being tightly associated with *IDH1* mutations.[56] 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.[57] This suggests that *IDH1* mutations may directly or indirectly promote *MGMT* methylation. A substantial number of patients with *IDH1*-mutated glioblastomas in our study (Table 1) and those of others[22,27,43,58] did not have *MGMT* methylation. Notably, *IDH1* mutations are very early events in the development of glioma,[59] and both the *IDH1* mutational[60,61] 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.[62] This robustness of *MGMT* methylation status may suggest that there are biological differences between glioblastomas in which an *IDH1* mutation has directly or indirectly promoted *MGMT* methylation compared with glioblastomas in which an *IDH1* mutation has not established *MGMT* methylation.

In addition, our results show that there are also differences in terms of survival between *IDH1*-mutated glioblastomas with and without *MGMT* methylation. The survival time of glioblastoma patients with only an *IDH1* mutation is shorter than that for patients with both *IDH1* mutations and *MGMT* methylation (Fig. 1D), as previously reported.[32,43,44] These results suggest that the group of *IDH1*-mutated patients is not homogenous and that the prognosis is not only dependent on the *IDH1* mutational 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* that needs to be further (mechanistically) explored. It was recently suggested that there may be a mechanistic link between *IDH1* mutations and *MGMT* methylation.[63] *MGMT* methylation is predictive for the response to temozolomide in *IDH1*-mutated high-grade glioma, and is prognostic in *IDH1* wild-type WHO high-grade glioma.[63] Because the patients in our cohort and the external validation cohorts did not all receive chemoradiation according to the Stupp protocol,[2] the predictive power of the 2-gene predictor may be different in our study compared with cohorts treated according to the Stupp protocol. As *MGMT* methylation is predictive for temozolomide response,[5] this may suggest that the results we present here underestimate the predictive power of the 2-gene predictor in cohorts treated with chemoradiation. More research is needed in such cohorts to investigate this assumption.

Comparison with Other Prognostic Predictors

Other prognostic predictors based on both genetic and genetic and epigenetic alterations have been described recently.

Predictors combining 1p/19q codeletion and either *IDH1* mutational status[64] or G-CIMP[65] were described in (anaplastic) oligodendroglioma. In support of our 2-gene predictor, other studies have described a correlation with progression-free survival based on the genetic and epigenetic statuses of *IDH1* and/or *MGMT* in both low-grade and high-grade glioma and secondary glioblastoma.[32,43,44,63,66,67] According to the data presented here, the 2-gene predictor is valid for both primary and secondary glioblastoma patients. Our dataset and the 3 external datasets all contain both primary and secondary glioblastoma.

Possible Improvements of the 2-gene Predictor

Recently, a combined analysis of MGMT protein expression and *MGMT* promoter methylation was described, which optimized prognostic predictions for glioblastoma patient survival.[68] It is possible that a predictor with more prognostic power could be 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.[27,32] *IDH2* mutations occur less frequently in gliomas and are mainly found in oligodendrogliomas.[69] 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 is not available on the prognostic status of *IDH2* in glioblastoma. 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 *IDH2* mutations are included in the 2-gene predictor or not, the results of the 2-gene predictor do not change. Tests in datasets with more patients with *IDH2* mutations are needed to confirm whether this 2-gene predictor should be extended with *IDH2* mutations to a 3-gene predictor.

Future Implications

The 2-gene predictor uses 2 well-established genetic and epigenetic alterations that will most likely play a role in future therapeutic options for glioblastoma patients. Recently, 2 independent clinical trials in elderly patients with *MGMT* methylated WHO grade III astrocytoma[70] or glioblastoma[70,71] reported that temozolomide treatment alone resulted in a better outcome than radiotherapy only. In contrast, radiotherapy alone was better than temozolomide alone in patients with unmethylated *MGMT*. These results suggest that the genetic and 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 into different arms on the basis of *MGMT* methylation status in clinical trials with new agents.[72,73] Very recently, an *IDH1* mutant-specific inhibitor was described,[74] which is expected to become available for clinical trials in the near future.[75] This context underlines the clinical significance of both *IDH1* mutations and *MGMT* methylation. It may soon become reality that molecular changes provide a rationale for treatment of newly diagnosed glioblastoma patients. The 2-gene predictor demonstrates 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. Further research on the underlying mechanisms may increase our biological understanding of glioblastoma. In addition, we propose that clinical trials, which 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. This will be a helpful addition to baseline comparisons in future clinical trials.

Supplementary Material

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

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