Correlation between *IDH1* Gene Mutation Status and Survival of Patients Treated for Recurrent Glioma

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Abstract. *Somatic mutations in the isocitrate dehydrogenase 1 (IDH1) gene have been frequently found in low-grade glioma and secondary glioblastoma and are associated with a significantly younger age at diagnosis and a superior overall survival. We investigated the IDH1 gene mutation status by nested PCR and denaturing gradient gel electrophoresis (DGGE) on DNA extracted from archival tumor blocks of 63 glioma patients who were treated following recurrence with the epidermal growth factor receptor (EGFR)-targeted blocking monoclonal antibody cetuximab, or the vascular endothelial growth factor (receptor) (VEGF(R))-targeted agents sunitinib malate and bevacizumab. In our study population, IDH1 mutation was significantly correlated with a longer overall survival (OS) from the time of initial diagnosis. Patients with IDH1 mutation also had a superior OS from the time of recurrence when treated with sunitinib or bevacizumab but a worse OS when treated with cetuximab. Our observations support the hypothesis that IDH1 mutation may correlate with the benefit from VEGF(R)- versus EGFR-targeted therapy at the time of recurrence in glioma patients.*

Glioma patients who experience progression of their disease following treatment with radiation therapy and alkylating chemotherapy have a poor prognosis (1). No treatment has yet been demonstrated to improve the survival of patients with recurrent glioma in a randomized controlled clinical trial. Bevacizumab, a vascular endothelial growth factor (VEGF)-targeted monoclonal antibody has demonstrated activity against recurrent glioma and has been registered for this indication by the Food and Drug Administration (FDA) based on evidence from an uncontrolled phase II clinical trial (2).

Insight has been obtained in the molecular-genetic features that determine the natural prognosis of glioma. These features include the mutation status of the isocitrate dehydrogenase *(IDH)-1* and -2 genes (3-5) which were recently identified as target genes for somatic mutations in glioma through a genome-wide mutational analysis (6). IDH1, a member of the isocitrate dehydrogenase enzyme family, is located in the cytoplasm and functions in the catalytic oxidative decarboxylation of isocitrate. Somatic mutations of *IDH1* are found in up to 70% of grade II and III gliomas, and secondary glioblastomas (these are WHO grade 4 glioma by transformation of a lower-grade glioma), but are rarely detected in *de novo* glioblastomas (<10%) (5, 7-8). *IDH1* mutation is associated with a younger age at diagnosis, and a better prognosis following treatment in patients with newly diagnosed glioma (5, 9-11). However the prognostic or predictive role of *IDH1* mutation from the time of recurrence has not been established.

PCR followed by direct sequencing (5, 12), single-strand conformation polymorphism (SSCP) (8), or restriction endonuclease-based analysis (13), have been used most often to detect *IDH1* mutations. In an attempt to obtain a higher sensitivity, we utilized a hemi-nested PCR technique followed by denaturing gradient gel electrophoresis (DGGE) (14) to analyze the archival formalin-fixed paraffin embedded (FFPE) glioma samples in this study. We

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investigated the correlation of *IDH1* mutation status with the overall survival (OS) of the patients treated in three study cohorts investigating cetuximab (an epidermal growth factor receptor (EGFR)-targeted monoclonal antibody), sunitinib malate (a VEGFR-targeted small molecule tyrosine kinase inhibitor), or bevacizumab for the treatment of recurrent, alkylator- refractory, gliomas.

Patients and Methods

Study design, patients, and tumor material. The primary objective of this study was to investigate the correlation between the *IDH1* gene mutation status and the survival following treatment with cetuximab, sunitinib, or bevacizumab in patients with glioma experiencing recurrence following prior therapy with surgery, radiation , and alkylating chemotherapy. Gliomas were classified on a histopathological basis according to the WHO 2007 criteria during central review by a neuropathologist (A.M.) (15). Formalin-fixed paraffin-embedded tissue blocks were sectioned at a thickness of 10 μm (3 sections for DNA isolation) for the *IDH1* mutation analysis and at a thickness of 4 μm for hematoxylin and eosin staining.

DNA extraction, hemi-nested PCR amplification, DGGE and sequencing. DNA was isolated from tumor sections using a QIAamp DNA FFPF Tissue kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions and finally resuspended in 60μl of the elution buffer. A PCR-based test was designed to identify mutations between nucleotide c.330A and c.414+10G. Two pairs of primers were designed in Primer3 system (http://frodo.wi.mit.edu/primer3/) based on the gene sequence from Ensembl ENSG00000138413 (hPFS://www.ensembl.rog). For the first step PCR, a 164 bp long fragment spanning the catalytic domain of *IDH1* including codon 132 was amplified with the forward primer ACCAAATGGCACCAT ACGAA and the reverse primer GCAAAATCACATTATTGCCAAC. A standard PCR was performed in a total volume of 25 μl and comprising DNA (1 μl), 1× PCR buffer, 1 μg/μl bovine serum albumin (BSA), 0.8 mM dNTPs, 0.025 unit/μl Taq DNA polymerase (Qiagen; 5 units/μl) and 2 ng/μl of each primer. The PCR consisted of 35 cycles with denaturing at 94˚C for 1 min, annealing at 60˚C for 1 min, extension at 72˚C for 1 min. Another fragment of 135 bp length with 40 bp GC clamp which also spans codon 132 was amplified under the same standard conditions but with 6 ng/μl of the forward primer GTGGCACGGTCTTCAGAGA fused to a GC clamp (40 bp) and 2 ng/μl of the reverse primer GCAAAATCACATTATTGCCAAC in a second step PCR of 25 cycles using 1 μl of the first PCR product as template DNA. DGGE screening for mutations was performed with the INGENT PhorU electrophoresis system (INGENY Company, Goes, Netherlands) based on a published method (16) with a 35%-55% gradient gel. After running, the gel was stained using ethidium bromide. Comparing with the control, the variant homoduplex or heteroduplex bands were cut and another 25 cycles PCR and DGGE analysis were applied to enrich for the mutant variant until ready for sequence analysis. Analysis of every tumor sample was performed in triplicate. For the purpose of DNA sequencing, the PCR product of each sample with mutation was purified with the High Pure PCR Product Purification kit (Roche, Penzberg, Germany) and subjected to sequencing using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA).

Clinical data and statistical analysis. Clinical data were retrieved from the case report form (CRF) of the patients, included in three clinical studies, respectively two interventional clinical trials with cetuximab, and sunitinib (17, 18), and an observational study with bevacizumab (M. Huylebrouck, not yet published). Clinical data included in this study were the baseline demographics, glioma type (histopathology and WHO grading), *EGFR* amplification status [limited to the cetuximab trial patient cohort, detected by fluorescence *in situ* hybridization (FISH) (17)], the platelet-derived growth factor receptor (*PDGFRA*), stem cell growth factor receptor (*KIT*) and *VEGFR2* gene amplification status [limited to the sunitinib patient cohort, detected by chromogenic *in situ* hybridization (CISH) (18)], date of birth, date of first diagnosis, date of cetuximab/sunitinib/bevacizumab treatment initiation, date of progression and date of death or latest follow-up. The progression-free survival (PFS) during treatment for recurrence was calculated from the date of recruitment to the date of progression or death. The OS was calculated from the date of first diagnosis and from the date of recruitment for treatment with cetuximab/sunitinib/bevacizumab until the date of death or date of latest follow-up.

The Kaplan-Meier methodology was used to estimate the survival probability and to investigate the correlations of baseline factors with survival outcome in a univariate analysis (using the log-rank test). Cox's proportional hazard models were used to analyze the correlation between *IDH1* mutation and the patients' and tumor baseline characteristics. The relationship between *IDH1* mutations and patient age was analyzed by the independent sample *t*-test. The association between the glioma WHO grade and *IDH1* mutation status was examined by crosstabs statistics. All reported *p*-values are two-sided, and values of less than 0.05 were considered to indicate statistical significance.

Results

Patient baseline characteristics. Archival tumor material obtained from a total of 63 patients was used for *IDH1* mutation analysis. The baseline demographic characteristics of this patient population are shown in Table I. Thirty-six patients were treated with cetuximab (in this trial patients were stratified at baseline according to the EGFR gene amplification status) (17), 16 patients with sunitinib (18), and 11 patients with bevacizumab. At the time of treatment for recurrence, the most recent histopathological diagnosis was *de novo* glioblastoma in 45 patients, grade II or grade III glioma in 9 patients, and secondary glioblastoma in an additional 9 patients.

IDH1 mutation detection in tumor samples. *IDH1* point mutations were detected in 17 out of 63 (26.9%) patients (Table I). All mutations were located at amino acid residue 132, and most (16/17) were c.395G>A, p.Arg 132His. Only one glioma carried a c.394C>T, p. Arg 132Cys mutation (Figure 1). Seven patients had two metachronous tumor samples available for *IDH1* mutation analysis. Intra-patient mutation analysis was consistent for these seven patients. Mutation analysis was successful in more than 95% of the tumor samples, including some biopsies smaller than 1 mm^2.

Table I. *Patient characteristics*.

Characteristic	N=63	*IDH1*	
		Mut. N=17 (26.9%)	Wt. N=46 (73.1%)
Gender (M/F)	38/25	11/6	27/19
Age, years; median (range)	48.7 (30-73)	39.4 (30-50)	53.1 (34-73)
Histology at first diagnosis			
WHO grade II			
Astrocytoma	3	2 (66.6%)	1 (33.3%)
Oligoastrocytoma	2	1 (50.0%)	1 (50.0%)
WHO grade III			
Anaplastic astrocytoma	8	3 (37.5%)	5 (62.5%)
Anaplastic oligoastrocytoma	5	4 (80.0%)	1 (20.0%)
WHO grade IV (*de novo* glioblastoma)	45	7 (15.6%)	38 (84.4%)
Histology at the time of treatment for recurrence			
WHO grade II & III	9	5 (55.5%)	4 (44.5%)
Secondary glioblastoma	9	5 (55.5%)	4 (44.5%)
De novo glioblastoma	45	7 (15.6%)	38 (84.4%)
Treatment at recurrence			
Cetuximab	36	6 (16.6%)	30 (83.3%)
Sunitinib	16	6 (37.5%)	10 (62.5%)
Bevacizumab	11	5 (45.5%)	6 (54.5%)
KPS at study recruitment			
100	3	0	3 (100%)
90-80	23	7 (30.4%)	16 (69.6%)
70-60	37	10 (27.0%)	27 (73.0%)

N, number of patients; Mut., mutant; Wt., wild type; KPS, Karnofsky performance status.

In order to complement our mutation detection method, we also performed PCR- and restriction endonuclease-based mutation detection (13) (results not shown). The six tumor samples which were found to carry an *IDH1* mutation with the PCR- and restriction endonuclease-based technique were also positive with our DGGE-based method; however, three samples which were found negative after restriction endonuclease-based mutation detection were tested positive as in our DGGE methodology, providing evidence that the hemi-nested PCR-DGGE analysis is more sensitive, especially for samples with a low tumor cell percentage.

Correlation between IDH1 mutations and tumor characteristics. *IDH1* mutations were identified in 15.6% (7/45) of *de novo* glioblastoma, 55.5% (5/9) of secondary glioblastoma, and 55.5% (5/9) of WHO grade II and III glioma (Table I), The age at diagnosis of patients identified with an *IDH1*-mutated glioma was significantly younger as compared to those without *IDH1* mutations (median age was 39.4 and 53.1 years respectively; $p<0.001$) (Table I).

IDH1 mutation was rarely detected in patients with *EGFR* amplification (10%, 2/20 patients with *EGFR* amplification were *IDH1* mutant). The two patients with a *PDGFRA, KIT* and *VEGFR2* gene amplification both had an *IDH1* mutation; an additional two out of three patients which had an increased *PDGFRA, KIT* and *VEGFR2* gene copy number, without true amplification, also carried an *IDH1* mutation (Table II).

Table II. *IDH1 mutation status, EGFR, PDGFRA, VEGFR2 and KIT amplification status*.

Other molecular status	N	*IDH1* status	
		Wt. (%)	Mut. (%)
EGFR			
Amplification	20	18 (90%)	2 (10%)
Wild type	16	12 (75%)	4 (25%)
PDGFRA gene copy			
Increase	4	1 (25%)	3 (75%)
Wild type	86 (75%)	2 (25%)	
VEGFR2 gene copy			
Increase	4	1 (25%)	3 (75%)
Wild type	10	7 (70%)	3 (30%)
KIT gene copy			
Increase	5	1 (20%)	4 (80%)
Wild type	9	7 (78%)	2 (22%)

EGFR, Epidermal growth factor receptor; *PDGFRA*, platelet-derived growth factor receptor alpha; *VEGFR2*, vascular endothelial cell growth factor receptor 2; *KIT*, stem cell growth factor receptor; Mut., mutant; Wt., wild-type.

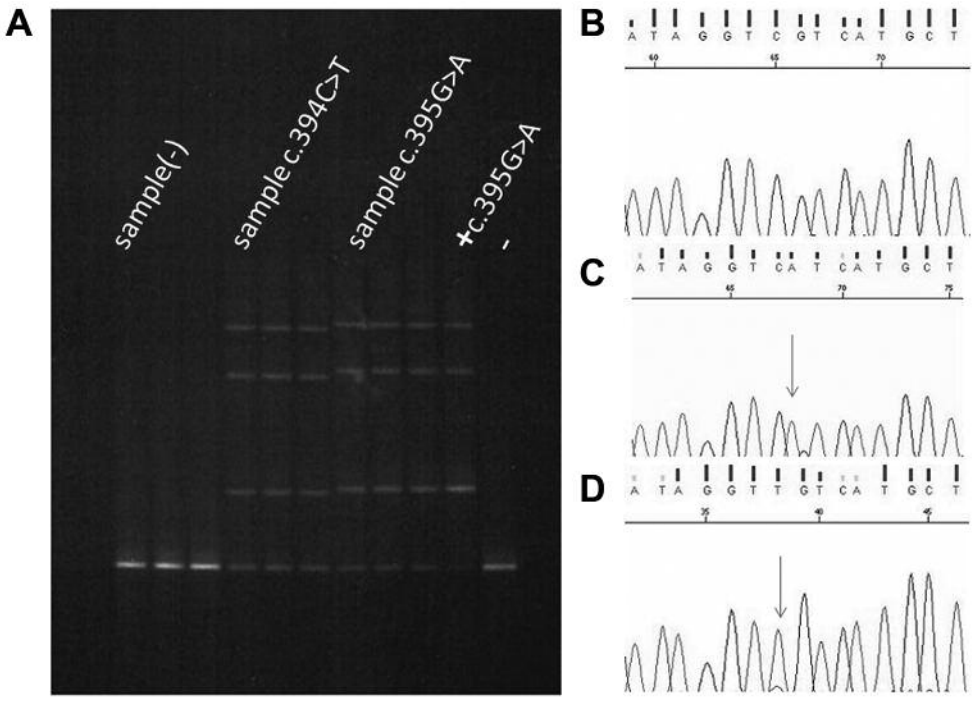

Figure 1. *Denaturing gradient gel electrophoresis (DGGE) and sequencing images. A: DGGE migration pattern corresponding to the two kinds of IDH1 point mutations found in the current study. The PCR/DGGE was performed triplicate for each sample. +c, Positive control, –, wild-type IDH1; B: wild-type IDH1 on sequencing; C: c.395G>A, p. R132H mutation on sequencing; D: c.394C>T, p. R132C mutation on sequencing.*

Table III. *Kaplan-Meier survival estimates for progression free survival (PFS) and overall survival (OS) of the patient study population according to treatment for recurrence and IDH1 mutation status.*

Cohort	N	PFS (months)				OS from treatment (months)			
		Median (95% CI)	*IDH1*			Median (95% CI)	*IDH1*		
			Mut.	Wt.	*P*-value (log-rank)		Mut.	Wt.	*P*-value (log-rank)
Cetuximab	36	1.80 (1.55-2.05)	1.17	1.83	0.18	4.73 (3.95-5.51)	3.07	4.73	0.07
Sunitinib	16	1.03 (0.93-1.13)	1.03	1.03	0.72	4.10 (1.94-6.26)	6.40	3.87	0.30
Bevacizumab	11	2.30 (0.00-5.38)	3.23	1.37	0.05	7.53 (3.37-11.68)	10.16	4.90	0.09
Bev.and Sun. combined	27	1.67 (0.91-2.43)	2.07	1.10	0.06	5.23 (4.43-6.03)	7.53	4.83	0.04
De novo GB in cetuximab trial	27	1.80 (1.46-2.14)	0.67	1.80	0.39	5.13 (2.87-7.39)	0.90	5.13	0.035
Total population	63	1.80 (1.56-2.03)	1.80	1.80	0.32	4.93 (4.37-5.48)	5.57	4.83	0.45

N, Number of patients; Mut., mutant; Wt., wild type; Bev., bevacizumab; Sun., sunitinib; GB, glioblastoma.

Correlation between IDH1 mutation status and survival. Fifty-nine patients had died at the time of this analysis, all due to progression of their glioma; four patients were alive and were censored at the date of their last follow-up (11/05/2011). The median OS from first diagnosis was 25.73 months (95% Confidence interval (CI)=20.49-30.96 months), and 4.93 months (95% CI=4.37-5.48 months) from the time of initiation of a treatment for recurrence. Patients with an *IDH1* mutation had a significantly longer OS from first diagnosis (56.50 *vs.* 21.30 months; p=0.001) (Figure 2A), but not from the time of treatment for recurrence (5.57 *vs.* 4.83 months; p=0.45). In the total study population, the median PFS from the time of treatment for recurrence was 1.8 months (95% CI= 1.56-2.03 months), and there was no significant

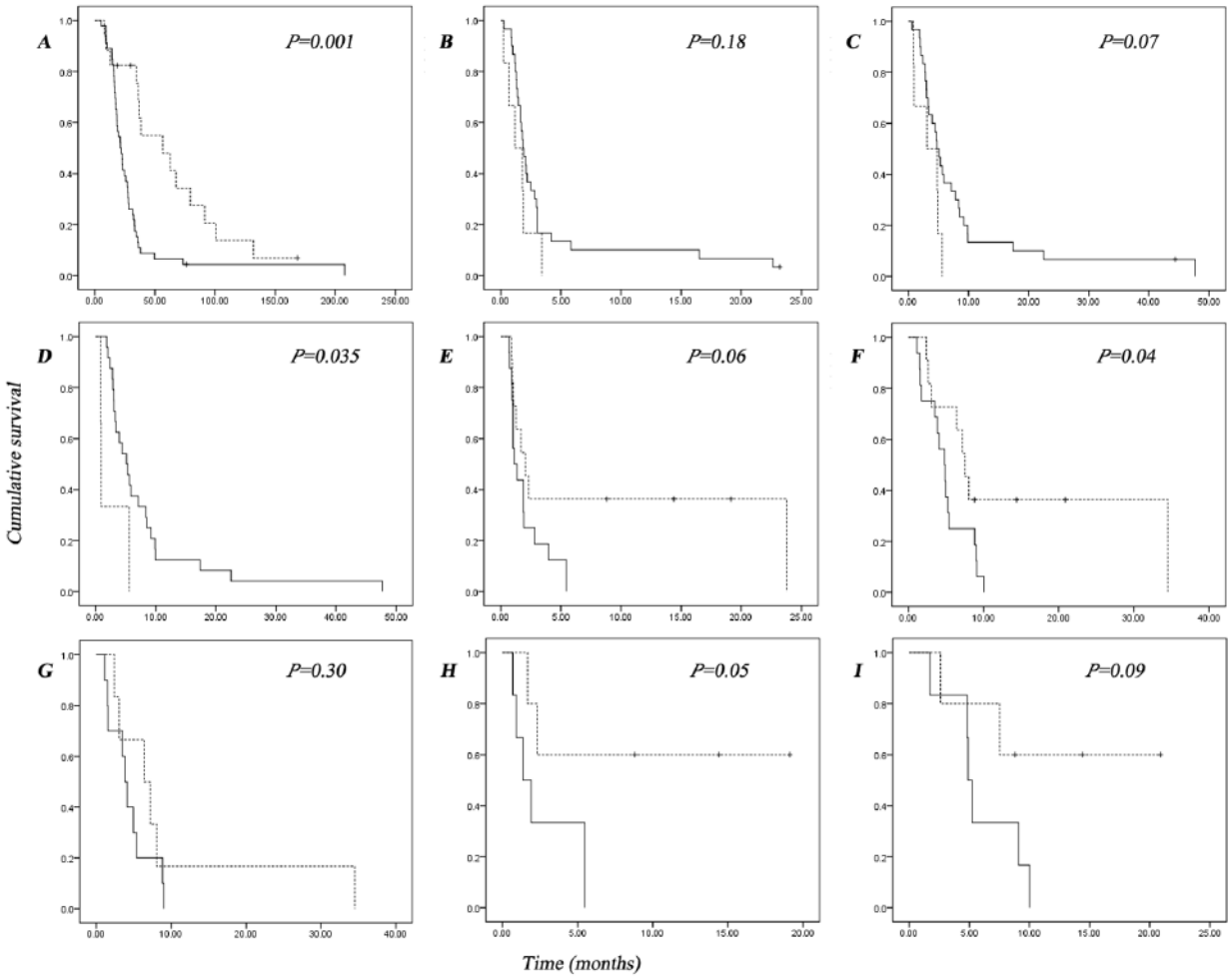

Figure 2. *Kaplan-Meier survival estimates according to IDH1 mutation status (p-value according to the log-rank test). Dashed line, IDH1 mutation; solid line, wild-type IDH1; +, censored. A: Overall survival (OS) from initial diagnosis based on IDH1 mutation status in the global study population (p=0.001); B and C: Progression free survival (PFS) and OS from the time of treatment with cetuximab for recurrence based on IDH1 mutation status (p=0.18 and 0.07); D: OS from the time of treatment for recurrence based on IDH1 mutation status in patients with de novo glioblastoma treated with cetuximab (p=0.035); E and F: PFS and OS from the time of treatment for recurrence based on IDH1 mutation status in combined cohorts of sunitinib and bevacizumab (p=0.06 and 0.04); G: OS from the time of treatment for recurrence based on IDH1 mutation status in the sunitinib cohort (p=0.3); H and I: PFS and OS from the time of treatment for recurrence based on IDH1 mutation status in the bevacizumab cohort (p=0.05 and 0.09).*

difference between *IDH1* mutant and wild-type patients (1.80 *vs*. 1.80 months; p=0.32). By multivariate Cox regression analysis (both forward and backward), only WHO tumor grade was significantly associated with OS from diagnosis (p<0.001). WHO grade however was strongly correlated with *IDH1* mutation.

In the cetuximab-treated cohort, a trend was observed in favor of a superior PFS (1.83 *vs*. 1.17 months, p=0.18) and OS (4.73 *vs*. 3.07 months, p=0.07) for patients with wild-type *IDH1* (Figure 2B and 2C). This correlation was even stronger in the subgroup of *de novo* glioblastoma patients with a significant difference in OS between patients with wild-type IDH1 and those with mutation (5.13 *vs*. 0.90 month, p=0.035) (Figure 2D). An opposite trend was found favoring OS of patients with IDH1 mutation in the survival analysis of the combined cohorts of patients treated with the VEGF(R) inhibitors sunitinib and bevacizumab (PFS 2.07 *vs*. 1.10 months, p=0.06; and OS 7.53 *vs*. 4.83 months, p=0.04) (Figure 2E and 2F). Within the sunitinib-treated cohort, a numerically superior OS was found for patients with an *IDH1* mutation (6.40 *vs*. 3.87 months, p=0.30) (Figure 2G) but not in PFS (1.03 *vs*. 1.03 months, p=0.72). In the bevacizumab-treated cohort, a superior PFS and OS were observed for patients with IDH1 mutation (PFS 3.23 *vs*. 1.37 months, p=0.05; and OS 10.16 *vs*. 4.90 months, p=0.09) (Figure 2H and 2I) (Table III).

Discussion

In this study, we investigated the correlation between *IDH1* gene mutation status and the clinical outcome of patients with alkylator-refractory glioma treated at recurrence with the EGFR monoclonal antibody cetuximab, the VEGFR-targeted tyrosine kinase inhibitor sunitinib, or the VEGF-targeted monoclonal antibody bevacizumab. Our analysis reproduces the established correlation between *IDH1* gene mutation, younger patient age at diagnosis, and more favorable OS from diagnosis (5, 19). Surprisingly, no correlation was found between *IDH1* mutation status and survival following the initiation of experimental treatment for recurrence. Moreover, within the cetuximab-treated cohort, an unexpected negative correlation was found, particularly in the subgroup of patients with *de novo* glioblastoma. This observation raises the hypothesis that cetuximab might have a beneficial effect in gliomas with genomic activation of *EGFR* (increased gene copy number) and a wild-type *IDH1*. EGFR is known to play an important role in the biology of an important proportion of *de novo* glioblastoma, where it is identified as a driver oncogene that is frequently mutated and/or amplified (20). As such, it may serve as a candidate molecular target for inhibition. An alternative hypothesis would be that cetuximab may have a deleterious impact on the outcome of patients with *IDH1*-mutant glioma treated for recurrence. The presence of an *EGFR* amplification and the presence of an IDH1 mutation are most often mutually exclusive, which is consistent with a previous report (21), EGFR is therefore not suspected to have an oncogenic driver function in *IDH1*-mutant glioma. Inhibition of the 'physiological' role of EGFR signaling in *IDH1*-mutant glioma may interrupt a cell differentiation pathway and lead to dedifferentiation and more malignant cell behavior.

We observed a correlation between *IDH1* mutation and superior survival of patients treated with VEGF(R) blocking agents such as bevacizumab and sunitinib malate. Although this observation would also be consistent with the presumed natural superior prognosis of *IDH1*-mutant glioma, it might also be possible that patients with an *IDH1*-mutant tumor may obtain a greater benefit from VEGF(R) inhibition as compared to patients with wild-type *IDH1*. This hypothesis is supported by the observation that the increase in copy number of *VEGFR2*, most often along with *PDGFRA*, and *KIT*, was found more frequently in glioma with an *IDH1* mutation (in 3/4, 3/4 and 4/5 patients with increased *PDGFRA, VEGFR2* and *KIT* gene copy number, respectively). Our observations are consistent with other reports indicating that *PDGFRA* amplification and *IDH1* mutation identify a molecularly distinct subgroup of glioma (22). As such, *IDH1* wild-type glioma may be more often dependent on the function of these three genes. We hypothesize that the genomic activation of these treatment target genes may sensitize the receptors to inhibition of the VEGFR pathway. *IDH1* mutations have been shown to alter the enzymatic activity of the protein, resulting in an increased production of alpha-ketoglutarate and up-regulation of hypoxia inducible factor-1α (*HIF-1α*) (23). HIF-1α plays an important role in the process of angiogenesis and can also support tumor cell survival and proliferation (23-24). *IDH1*-mutated glioma may therefore be more dependent on the VEGF/HIF-1 pathway as opposed to the EGFR pathway and be more sensitive to VEGF(R)-targeted agents. Alternatively, *in vitro* down-regulation of HIF-1α has been identified to predict sensitivity to EGFR inhibition by cetuximab (25). This might be a complementary mechanism underlying the resistance of *IDH1*-mutated glioma to treatment with cetuximab.

In conclusion, our analysis suggests that *IDH1* mutation status strongly correlates with the survival from diagnosis but not from the time of treatment for recurrence following prior treatment with radiation and alkylating chemotherapy. Our study supports the hypothesis that *IDH1* mutation status may serve as a predictive factor for benefit from treatment at recurrence with EGFR or VEGF(R) inhibitors.

Conflict of Interest Statement

None declared.

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