Original Article

Qualitative and Quantitative Analysis of IDH1 Mutation in Progressive Gliomas by Allele-Specific qPCR and Western Blot Analysis

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Abstract

To date, diagnosis of IDH1 mutation is based on DNA sequencing and immunohistochemistry, methods limited in terms of sensitivity and ease of use. Recently, the diagnosis of IDH1 mutation by real-time polymerase chain reaction was introduced as an alternative method. In this study, real-time polymerase chain reaction was validated as a tool for detection of IDH1 mutation, and expression levels were analyzed for correlation with course of the disease. A total of 113 tumor samples were obtained intraoperatively from 84 patients with glioma having a diagnosis of diffuse glioma (World Health Organization II), anaplastic glioma (World Health Organization III), secondary glioblastoma $\pm$ chemotherapy, primary glioblastoma $\pm$ chemotherapy (World Health Organization IV). Tumor samples were snap frozen and processed for sectioning and RNA and protein isolation. Presence of IDH1 mutation was determined by DNA sequencing. Hereafter, quantitative expression of IDH1 messenger RNA was assessed using real-time polymerase chain reaction with specific primers for IDH1 mutation and −wt; protein expression was verified by Western Blot analysis and immunohistochemistry. Additionally, 19 samples of low-grade glioma and their consecutive high-grade glioma were analyzed at different time points of the disease. IDH1 mutation was identified in 63% of samples by DNA sequencing. In correlation with the real-time polymerase chain reaction results, a cutoff value was determined. Above this threshold, sensitivity and specificity of real-time polymerase chain reaction in detecting IDH1 mutation were 98% and 94%, respectively. Quantitative analysis revealed that IDH1 mutation expression is upregulated in secondary glioblastoma (mean $\pm$ standard error of mean: 3.52 ± 0.55) compared to lower grade glioma (II $= 1.54 \pm 0.22$; III $= 1.67 \pm 0.23$). In contrast, IDH1 wt expression is upregulated in all glioma grades (concentration >0.1) compared to control brain tissue (0.007 ± 0.0016). Western Blot analysis showed a high concordance to both sequencing and real-time polymerase chain reaction results in qualitative analysis of IDH1 mutation status (specificity 100% and sensitivity 100%). Moreover, semiquantitative protein expression analysis also showed higher expression levels of mutated IDH1 in secondary glioblastoma. In our study, real-time polymerase chain reaction and Western Blot analysis were found to be highly efficient methods in detecting IDH1 mutation in glioma samples. As cost-effective and time-saving methods, real-time polymerase chain reaction and Western Blot analysis may therefore play an important role in IDH1 mutation analysis in the future. IDH1 mutation expression level was found to correlate with the course of disease to a certain extent. Yet, clinical factors as recurrent disease or prior radiochemotherapy did not alter IDH1 mutation expression level.

Keywords

chemotherapy, glioblastoma, isocitrate dehydrogenase 1, qPCR, sequencing

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Introduction

Somatic mutations of the isocitrate dehydrogenase 1 (IDH1) gene are frequent genetic alterations in grade II to III gliomas and secondary glioblastomas.[1,2] IDH1 mutations (IDH1mut) occur in more than 70% of these tumors. On the contrary, IDH1muts are rare or absent in primary glioblastomas and other nervous system tumors.[3,4]

Identification of IDH mutations help in the delineation of gliomas from other tumor types. IDH1 mutations are heterozygous point mutations leading to a substitution of arginine in codon 132. More than 90% of these IDH1mut are based on a basepair exchange of guanine to adenine (G395A) resulting in a specific amino acid replacement of arginine by histidine (R132H).[3] The gene product of IDH1 is isocitrate dehydrogenase 1, an enzyme catalyzing the cytosolic oxidative decarboxylation of isocitrate to α-ketoglutarate. IDH1 mutations not only inactivate the normal enzymatic activity of IDH1 but, additionally, also lead to a gain of function. Thus, mutated IDH1 catalyzes the reduction of α-ketoglutarate to 2-hydroxyglutarate.[5,6] On a cellular level, 2-hydroxyglutarate is believed to act as an oncometabolite leading to epigenetic aberrations that represent early events in gliomagenesis.[7,8]

Nevertheless, the precise downstream targets of 2-hydroxyglutarate, and its clinical relevance are yet to be defined. On a clinical level, the presence of IDH1mut is associated with a better prognosis in patients with glioma of all grades.[9,10] Moreover, in conjunction with O-6-Methylguanine-DNA methyltransferase (MGMT) promotor methylation status, IDH1 mut seems to be prognostic and maybe even predictive value for the efficacy of chemotherapeutic treatment.[11] Thus, clinical treatment decisions may increasingly become based on IDH1 mutational status. Therefore, determining the IDH1mut status with high precision is becoming even more important. To date, gene sequencing and immunohistochemistry (IHC) are the most commonly used techniques in analyzing IDH1mut status.[10,12,13] Hence, there is growing interest in acquiring new techniques for fast and reliable detection of IDH1mut. Over the years, several methods have been published to detect IDH1mut in gliomas, including polymerase chain reaction (PCR)-restriction fragment polymorphism, PCR-single-strand conformation polymorphism, pyrosequencing, and high-resolution melting curve analysis.[14-19] While DNA sequencing is regarded as "gold standard" for diagnosing IDH1 mutational status, and pyrosequencing[20] and melting curve analysis[21] actually have higher sensitivity than Sanger sequencing, all of these methods are limited in terms of

sensitivity and ease of use. Therefore, IDH1mut IHC and allele-specific PCR were recently introduced as highly sensitive and specific alternative techniques.[22] In addition, quantitative PCR (qPCR) and IHC render a quantitative analysis of IDH1mut possible which was not feasible beforehand. In regard to the potential role of downstream products of mutated IDH1 in gliomagenesis and glioma progression, in this study, we aimed at (1) establishing qPCR as a tool in analysis of IDH1mut status and (2) analyzing the correlation between level of IDH1mut expression and course of the disease.

Materials and Methods

Patients

Expression of IDH1mut was analyzed in 113 samples from 84 patients with diagnosis of World Health Organization (WHO) grade II glioma (n = 21; 11 males/10 females; mean age = 37 years), grade III glioma (n = 22; 20 males/2 females; mean age = 47 years), secondary glioblastoma (n = 22; 13 males/9 females; mean age = 40.5 years), and primary glioblastoma (n = 10; 7 males/3 females; mean age = 51 years).

Additionally, 19 samples derived from 9 patients were included, which were collected at different time points during disease progression. Samples of peritumoral tissue histologically declared tumor free were used as control brain tissue (n = 9, 8 males/ 1 female; mean age = 48 years). Written informed consent was obtained preoperatively (see Table 1).

Tissue Sampling and Isolation of Nucleic Acids

Tissue samples were obtained during surgery and immediately snap frozen in liquid nitrogen. Sections of 10 μm were cut on a cryostat and stained with hematoxylin and eosin (HE) to ensure representative tissue was used for further analysis. Histology and grade of each sample were independently reviewed by 2 neuropathologists according WHO classification criteria.[23]

Using RNeasy Mini Kit (Quiagen, Hilden, Germany), total RNA was isolated from frozen tissue. Purity of RNA was measured spectrophotometrically and afterward reverse transcription was performed using QuantiTect Reverse Transcription Kit (Quiagen).

DNA Sequencing

IDH1 mutations were detected by PCR and direct sequencing of amplified complementary DNA (cDNA). The PCR primers were designed with the free Internet tool Primer3 v.0.4.0

Table 1. Patient and Sample Overview.

	Patients	Samples
n		
Total	84	113
M	59	
F	25	
Age	44	
Localization		
Left	41	
Right	42	
Frontal	41	
Temporal	22	
Parietal	14	
Occipital	5	
Other	2	
WHO grade		
II	21	21
III	22	30
>Primary	10	10
>Recurrent	12	20
sGBM	22	32
−Cx	14	20
+Cx	8	12
pGBM	10	20
−Cx	10	10
+Cx	10	10
Controls	9	10

Abbreviations: Cx, chemotherapy; F, female; M, male; pGBM, primary glioblastoma; sGBM, secondary glioblastoma; WHO, World Health Organization.

(http://bioinfo.ut.ee/primer3-0.4.0/). The PCR products were purified by Exo/SAP digestion with Exonuclease I (New England Biolabs, Beverly, Massachusetts) and Shrimp Alkaline Phosphatase (Promega, San Diego, California) and directly sequenced using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI 3730 sequencing instrument (Applied Biosystems, Foster City, California) following the manufacturer's instructions. All primer sequences and PCR conditions can be obtained on request from the authors.

Western Blot

In Western Blot analysis, for each sample 50 μg of protein were used for gel electrophoresis and then transferred to a nitrocellulose membrane. Precast gels (NuPage 4%-12% Bis-Tris 1 mm gel; Life Technologies (Carlsbad, California, USA) were chosen to ensure maximum reproducibility. Protein concentration was determined using the spectroscopic protein assay after Bradford. For blocking 5% dry non-fat milk dissolved in Tris-buffered saline with 0.05% Tween 20 as detergent (TBST) was used. Overnight incubation with the primary anti-IDH (mut./wt; IDH R132 H; Dianova#DIA-H09) antibody in 3% dry non-fat milk and TBST took place at 4°C as well as incubation with the secondary horseradish peroxidase-conjugated antibody (dilution 1:10 000 in TBST; Life technologies).

After determining the amount of protein for each band by detecting horseradish signals using chemiluminescence and densitometric analysis (Imagelab; Biorad, Munich, Germany),

the same membranes were incubated overnight at 4°C with an primary anti-β-actine antibody (Cell Signaling; dilution 1:10 000) and then incubated with the secondary horseradish peroxidase-conjugated anti-mouse antibody (dilution 1:10 000 in TBST) in order to verify the protein load for each sample and to normalize IDH1 values.

Real-Time PCR

The PCR samples had a final volume of 20 μL containing 1X SYBR Green Supermix (BioRad), 1× primer, and 5 μL cDNA (1:200). Primers were used as previously published IDH1wt-F(5'-GGTAAAACCTATCATCATAGGTCG-3'), IDH1mut-F (5'-GGGTAAAACCTATCATCATAGGTCA-3'), and IDH1-R (5'-CACATACAAGTTGGAAATTTCTGG-3').[22] Amplification was performed on a Rotor Gene Q cycler (Quiagen) using the following conditions (2-step cycling program): initial denaturation for 3 minutes at 95°C followed by 45 cycles of 95°C for 5 seconds and 60°C for 10 seconds. For each run, a melting curve analysis was performed to confirm specificity of the amplification. The results were normalized to succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA) as a reference gene.

Statistical Analysis

All statistical analysis was performed using SPSS and Prism Graph Pad (version 6). Outliers were identified (>2 standard deviation) and removed. The level of significance was set at 0.05. All results are depicted with standard error of the mean.

Results

Clinical Data

Our study population consisted of 113 samples derived from 84 patients. Of those, 21 patients were diagnosed with grade II glioma, 22 grade III glioma, and 32 glioblastoma consisting of 10 primary and 22 secondary glioblastoma. Secondary glioblastoma samples were subdivided into 2 groups, untreated patients (n = 14, 6 males/8 females; mean age = 43 years) and patients previously treated with chemotherapy (n = 8, 7 males/1 females, mean age = 37 years). Primary glioblastoma samples were taken at time point of primary diagnosis and recurrent disease, respectively. Twenty-seven grade II and III glioma samples were solely of astrocytic origin, whereas 16 samples were mixed oligoastrocytic tumors.

DNA Sequencing

DNA was amplified in all 113 (100%) samples. IDH1 mutation was found in 71 (62.8%) of 113 patient samples. In most of these samples, the G395A mutation was detected (61 of 71 patients, 85.9%), while other mutations were only found in 10 (14.1%) of 71 samples. Among those, C394T, C394A, and C394G mutations were found in 2, 4, and 4 samples, respectively. Diffuse glioma (n = 21) were positive for G395A
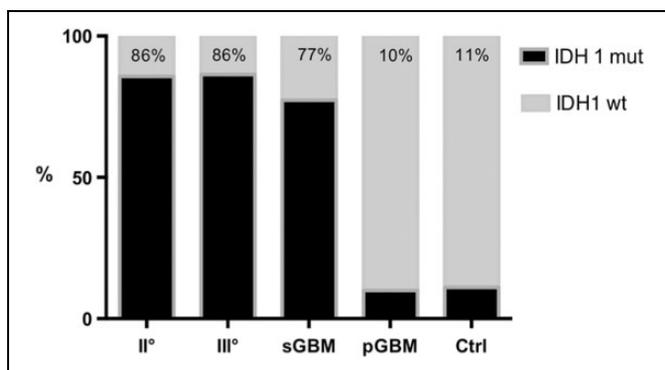
Figure 1. IDH1 mutation in different glioma groups based on DNA sequencing. A high percentage of LGG and secondary HGG bear the IDH1 mutation, whereas it is rare in pGBM and control tissue. sGBM indicates secondary glioblastoma; pGBM, primary glioblastoma; Ctrl, control brain tissue; LGG, low-grade glioma; HGG, high grade glioma.

Table 2. DNA Sequencing Results in Patients and Samples.[a]

	Patients	Samples
n	84	113
IDH1 mut	55	71
IDH1 mut, %	65.5	62.8
G395A	48	61
G395A, %	87.3	85.9
394 mut	7	10
394 mut, %	12.7	14.1
C394T	1	2
C394A	4	4
C394G	2	4

[a]IDH1 mutation was detected in 65.5% of patients. In most of those samples, G395A mutation was found (48/55; 87.3%).

Table 3. IDH1 Mutation in Glioma Subgroups Based on DNA Sequencing.[a]

	n	IDH1mut	IDH1mut, %	G395	G395, %
II	21	18	85.7	16	88.9
III	22	19	86.4	19	100.0
Primary III	10	8	80.0	8	100.0
Recurrent III	12	11	91.7	11	100.0
sGBMl	22	17	77.3	13	76.5
sGBM−Cx	14	12	85.7	8	66.7
sGBM+Cx	8	5	62.5	5	100.0
pGBM	10	1	10.0	0	0.0
Ctrl	9	1	11.1	0	0.0

Abbreviations: Ctrl, control; Cx, chemotherapy; HGG, high-grade glioma; LGG, low-grade glioma; pGBM, primary glioblastoma; sGBM, secondary glioblastoma.
[a]IDH1 mutation is common in LGG and secondary HGG and nearly abundant in primary glioblastoma. G395A is the most common mutation in all samples.

mutation in 18 (86%) patients, anaplastic gliomas (n = 22) in 19 (86%) patients, and secondary glioblastomas (n = 22) in 17 (77%) patients. No G395A IDH1mut was found in primary glioblastoma samples (see Figure 1 and Table 2). Except for

1 patient, IDH1mut status remained stable during progression of disease (Table 3).

Real-Time PCR

All patient and control brain tissue samples were analyzed by real-time PCR (n = 113). Results were normalized to SDHA expression, as reference gene, and evaluated as relative IDH1-mut expression. In relation to the respective sequencing results, a cutoff value of 0.50 relative IDH1mut expression was defined to identify the presence of IDH1 mut (see Table 4). All IDH expression values were sorted in a descending order, and the best cutoff value is obvious in Table 4. Hereby, qPCR identified IDH1mut in 62 (55%) samples, whereas in 51 samples (45%), no IDH1 mutation was found. Above this threshold, sensitivity and specificity of qPCR were 98% and 94%, respectively, in comparison to DNA sequencing as gold standard (see Table 5).

Based on qPCR results, 76% of patients with diffuse glioma, 86% of patients with anaplastic glioma, and 59% of patients with secondary glioblastoma were positive for the G395A IDH1mut. Similar to sequencing results, IDH1mut status remained unchanged in individual patients during progression of disease. As the IDH1mut primer only allows amplification of the G395A mutation, no other mutations of IDH1 were detected.

In one case, 2 tissue samples were analyzed from a single patient. Of those samples, one was characterized as GBM based on HE staining, whereas the other sample was diagnosed as tumor free (peritumoral tissue). In both samples, IDH1mut was detected by DNA sequencing as well as by qPCR. Hence, qPCR was able to detect IDH1mut even in samples bearing only a low tumor cell load (see Figure 2).

The presence of IDH1mut remained unchanged in most glioma samples over the whole course of the disease. However, in 1 particular patient, DNA sequencing revealed a change in IDH1mut status from an IDH1 mutated low-grade glioma (LGG) to a consecutive higher grade glioma (IDH1-wt) and in a later sample back to IDH1mut again. Yet, in the same patient, qPCR showed an IDH1mut expression in all 3 samples that were above the defined threshold of 0.5. Therefore, based on qPCR results, IDH1mut status remained unchanged in this patient suggesting a diagnostic mistake in DNA sequencing.

IDH1 Mutation Quantification by qPCR

In contrast to DNA sequencing, qPCR allows the quantification of gene expression. Thus, the level of IDH1 expression was analyzed in all samples (patients n = 48; samples n = 61) regarded as IDH1mut based on qPCR findings (relative IDH1-mut expression ≥0.50).

IDH1 mutation expression was analyzed based on tumor grading (WHO II and III, secondary GBM). Hereby, a significantly higher expression of IDH1mut was found in secondary glioblastoma (relative IDH1mut expression = 3.52 ± 0.55) compared to diffuse (IDH1mut = 1.54 ± 0.22; $P = .01$) and

Table 4. rtPCR Detects IDH1 Mutation With High Sensitivity and Specificity.[a]

Grade	Seq	rtPCR	Grade	Seq	rtPCR	Grade	Seq	rtPCR
II	G/A	39.46	Recurrent III	G/A	1.41	pGBM+Cx	G/G	0.31
Primary III	G/A	23.22	Recurrent III	G/A	1.22	ctrl	G/G	0.30
sGBM−Cx	G/A	11.11	Recurrent III	G/A	1.19	sGBM−Cx	G/G	0.26
II	G/A	7.01	II	G/A	1.18	sGBM+Cx	G/A	0.26
sGBM−Cx	G/A	6.79	II	G/A	1.13	pGBM+Cx	G/G	0.25
II	G/A	6.27	sGBM+Cx	G/A	1.11	sGBM+Cx	G/G	0.25
sGBM−Cx	G/A	6.08	II	G/A	1.06	pGBM−Cx	G/G	0.24
sGBM−Cx	G/A	4.51	Primary III	G/A	1.06	sGBM−Cx	G/G	0.22
sGBM+Cx	G/A	4.43	sGBM−Cx	G/A	0.97	sGBM+Cx	G/G	0.22
sGBM−Cx	G/A	4.37	Recurrent III	G/G	0.95	pGBM−Cx	G/G	0.21
sGBM−Cx	G/A	4.26	Recurrent III	G/A	0.90	pGBM−Cx	G/G	0.21
Recurrent III	G/A	4.25	sGBM+Cx	G/A	0.90	pGBM−Cx	G/G	0.21
Primary III	G/A	4.19	II	G/A	0.88	pGBM−Cx	G/G	0.19
sGBM−Cx	G/A	3.72	Primary III	G/A	0.83	pGBM+Cx	G/G	0.18
sGBM+Cx	G/A	3.50	Primary III	G/A	0.75	Recurrent III	G/G	0.18
sGBM+Cx	G/A	3.38	Recurrent III	G/A	0.73	pGBM+Cx	G/G	0.18
sGBM−Cx	G/A	3.25	Recurrent III	G/A	0.73	pGBM+Cx	G/G	0.17
Recurrent III	G/A	3.13	Recurrent III	G/A	0.72	II	G/G	0.17
Recurrent III	G/A	2.98	ctrl	G/A	0.64	pGBM+Cx	G/G	0.16
II	G/A	2.90	Recurrent III	G/A	0.62	ctrl	G/G	0.13
II	G/A	2.87	pGBM+Cx	G/G	0.59	pGBM−Cx	G/G	0.12
sGBM−Cx	G/A	2.86	Primary III	G/A	0.51	sGBM−Cx	G/G	0.12
sGBM+Cx	G/A	2.67	II	G/A	0.51	ctrl	G/G	0.12
sGBM+Cx	G/A	2.51	II	G/A	0.50	pGBM+Cx	G/G	0.11
Primary III	G/A	2.29	sGBM−Cx	G/G	0.50	Recurrent III°	G/G	0.11
II	G/A	2.16	II	G/G	0.48	sGBM−Cx	G/G	0.10
Recurrent III	G/A	2.08	sGBM−Cx	G/G	0.44	pGBM−Cx	G/G	0.08
Recurrent III	G/A	1.98	Primary III	G/G	0.44	pGBM+Cx	G/G	0.08
Primary III	G/A	1.94	pGBM−Cx	G/G	0.42	ctrl	G/G	0.08
sGBM−Cx	G/A	1.93	II	G/G	0.40	ctrl	G/G	0.07
Recurrent III	G/A	1.92	Primary III	G/G	0.40	ctrl	G/G	0.07
II	G/A	1.86	sGBM−Cx	G/G	0.38	II	G/G	0.07
II	G/A	1.72	sGBM+Cx	G/G	0.38	Recurrent III	G/G	0.06
sGBM−Cx	G/A	1.72	II	G/G	0.36	ctrl	G/G	0.05
II	G/A	1.66	pGBM−Cx	G/G	0.36	pGBM+Cx	G/G	0.03
II	G/A	1.59	sGBM−Cx	G/G	0.34	ctrl	G/G	0.03
Recurrent III	G/A	1.54	sGBM+Cx	G/G	0.33	ctrl	G/G	0.02
Recurrent III	G/A	1.42	pGBM−Cx	G/G	0.31			

Abbreviations: Ctrl, control; Cx, chemotherapy; pGBM, primary glioblastoma; rtPCR, real-time polymerase chain reaction; sGBM, secondary glioblastoma.
[a]After sorting rtPCR results based on IDH1 mutation expression level, a cutoff value was set at a relative expression of 0.5.

Table 5. rtPCR Detects IDH1 Mutation With High Sensitivity and Specificity.[a,b]

	Sequencing +	Sequencing −	Total
rtPCr +	60	3	63
rtPCR −	1	49	50
Total	61	52	113
rtPCR sensitivity, %		98.4	
rtPCR specificity, %		94.2	

Abbreviation: rtPCR, real-time polymerase chain reaction
[a]IDH1 mutation status remains unchanged during disease progression. Nevertheless, in 1 patient IDH1 mutation status based on DNA sequencing changes from one time point to the next (underlined samples), whereas IDH1 mutation status remains unaltered in rtPCR results.
[b]Thus, a good correlation between DNA sequencing and rtPCR was detected (sens = 98%; spec = 94%).
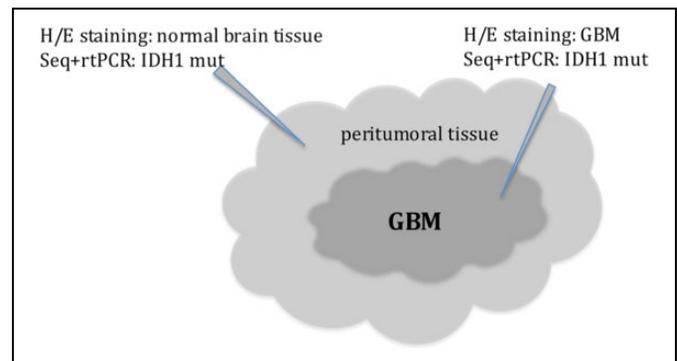

Figure 2. Real-time polymerase chain reaction (rtPCR) and DNA sequencing are able to detect IDH1 mutation even in peritumoral tissue characterized as tumor free. GBM indicates glioblastoma.

Table 6. Higher Expression of IDH1 Mutation in sGBM Compared to Diffuse and Anaplastic Glioma.[a]

WHO Grade	n	Mean IDH1 Expression	SEM
II	13	1.54	0.22
III	23	1.67	0.23
Primary	7	1.65	0.49
Recurrent	16	1.68	0.26
sGBM	20	3.52	0.55
−Cx	12	4.30	0.79
+Cx	8	2.35	0.52

Abbreviations: Ctrl, control; Cx, chemotherapy; sGBM, secondary glioblastoma; SEM, standard error of mean; WHO, World Health Organization.
[a]Included were all results from samples diagnosed as IDH1 mutation by DNA sequencing. Outliers were identified and removed.
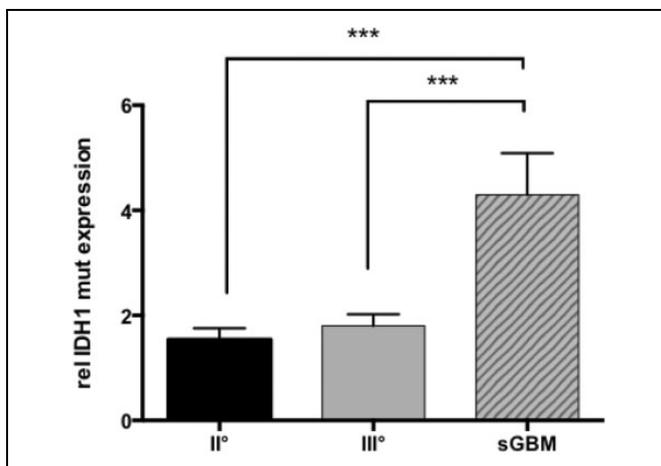
Figure 3. High expression of IDH1 mutation in secondary glioblastoma (sGBM). A significantly higher expression was found in sGBM compared to diffuse and anaplastic glioma (for statistics see Table 5). Anaplastic astrocytoma showed a higher IDH1 mutation expression than diffuse glioma which failed to reach statistical significance.

Figure 4. IDH1 mutation expression is lower in patients after prior chemotherapy. A, In anaplastic glioma, no difference was found in IDH1 mutation expression between primary (n = 7; relative expression = 1.65 ± 0.49) and recurrent tumors (n = 16; relative expression = 1.68 ± 0.26). B, IDH1 mutation level did not differ between mixed oligoastrocytoma (n = 15; relative expression = 1.6 ± 0.27) and astrocytoma (n = 18; relative expression = 1.8710.38). C, However, IDH1 mutation expression was lower in samples derived from patients with secondary glioblastoma who had received prior chemotherapy (n = 12; relative expression = 4.3 ± 0.79) than in untreated patients (n = 8; relative expression = 2.35 ± 0.51).

anaplastic glioma (IDH1mut = 1.67 ± 0.23; P = .001). Moreover, IDH1mut was slightly higher expressed in grade III glioma than in grade II glioma without reaching statistical significance. However, our results hint at an increasing IDH1mut expression with higher glioma grade (see Table 6 and Figure 3).

In a subgroup analysis, no difference was found in IDH1mut expression between primary grade III and recurrent grade III gliomas (primary III n = 7, IDH1mut = 1.65 ± 0.49; recurrent III n = 16, IDH1mut = 1.68 ± 0.26; P = .8). Furthermore, IDH1mut expression level was independent of glioma subtype (astrocytic vs oligoastrocytic; see Figure 4).

Expression of IDH1-wt was analyzed in 10 samples of each group (II, III, secondary glioblastoma [sGBM] ± Cx, primary glioblastoma [pGBM], and control). IDH1-wt expression was upregulated in all glioma groups compared to control brain tissue; however, no difference was found between different glioma grades (see Figure 5). IDH1-wt expression correlated with IDH1-mut expression levels in
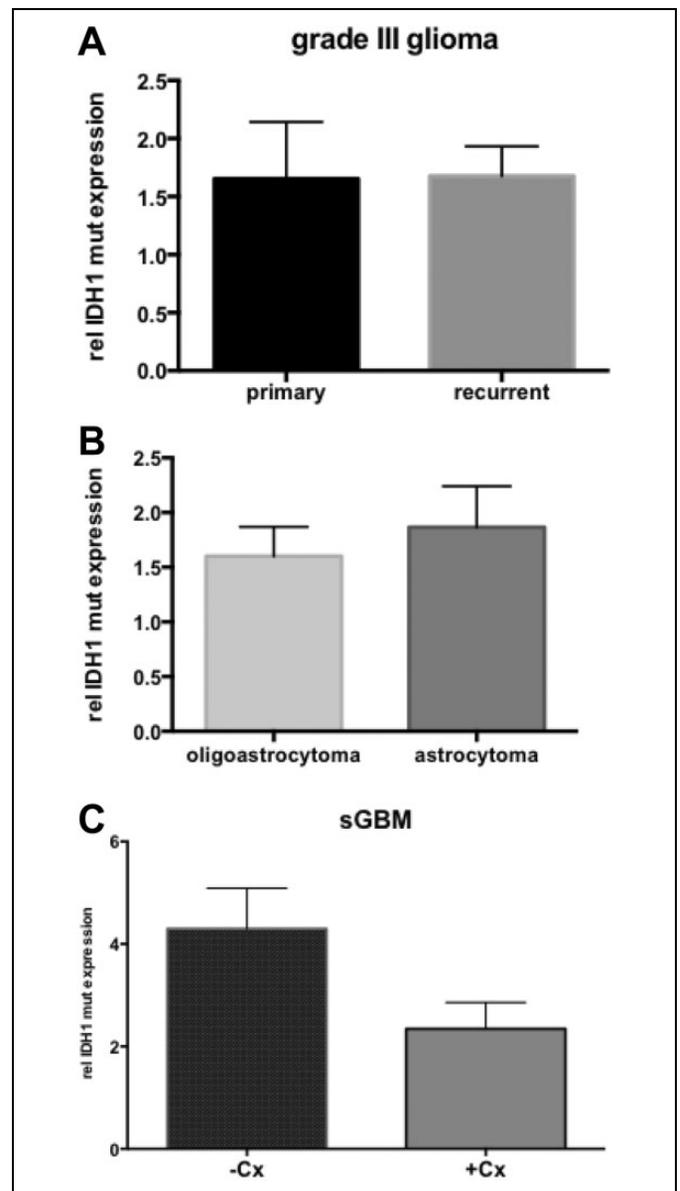
grade II and grade III glioma but not in secondary glioblastoma (see Figure 6). Thus, in secondary glioblastoma, IDH1mut seems to be upregulated independent of general IDH1 activation.

Among the patients who were characterized as IDH1mut by DNA sequencing, 9 were patients who underwent repeated surgery over the time of disease progression. Samples derived
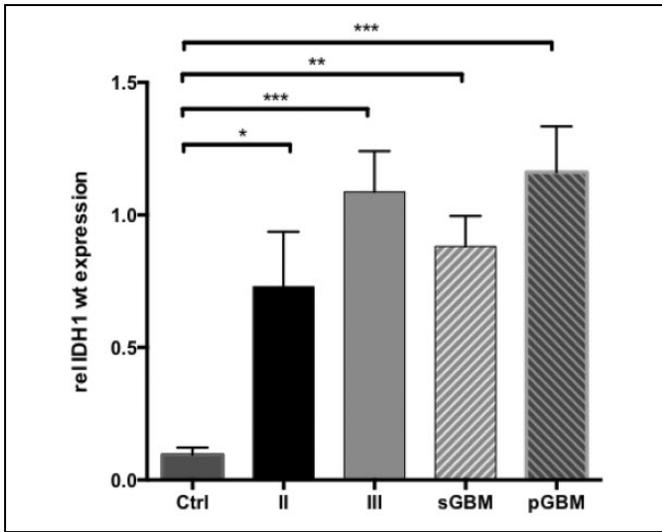
Figure 5. IDH1-wt expression is upregulated gliomas of all grades. IDH-wt expression level was analyzed in 10 samples of each group. While IDH1-wt was upregulated, all glioma grades compared to control tissue, no significant difference was observed between glioma grades. Ctr1 = 0.09 ± 0.03; II = 0.73 ± 0.21; III = 1.09 ± 0.15; sGBM = 0.88 ± 0.11; pGBM = 1.16 ± 0.17). sGBM indicates secondary glioblastoma; pGBM, primary glioblastoma; Ctr1, control.

from these patients enabled us to analyze the level of IDH1mut expression during individual disease progression. The cumulative data analysis of 19 samples derived from those longitudinally observed patients showed an increase in IDH1mut expression over disease progression (n = 19; II relative expression = 1.15 ± 0.23, n = 7; III relative expression = 2.07 ± 0.46, n = 5; sGBM relative expression = 3.35 ± 0.46, n = 7; see Figure 7A). In the individual analysis of longitudinal data, most patients showed an increase of IDH1mut expression over their course of disease (see Figure 7B and C). However, in 2 patients, IDH1mut expression fell from the initial level, when they were diagnosed with anaplastic astrocytoma, to their second operation (secondary GBM). In contrast to the other patients, these 2 patients had received prior chemotherapy (sGBM+Cx; see Figure 7D).

To further elucidate the effect of prior chemotherapeutic treatment on IDH1mut expression level, the sGBM patient group was analyzed based on whether they had received prior chemotherapeutic (Cx) treatment (sGBM-Cx: n = 11; sGBM + Cx: n = 7). In this analysis, a trend toward lower expression of IDH1mut was found in patients who had received prior Cx treatment (relative IDH1mut expression: sGBM-Cx = 3.7 ± 0.5 vs sGBM+Cx = 2.7 ± 0.5; see Figure 8).
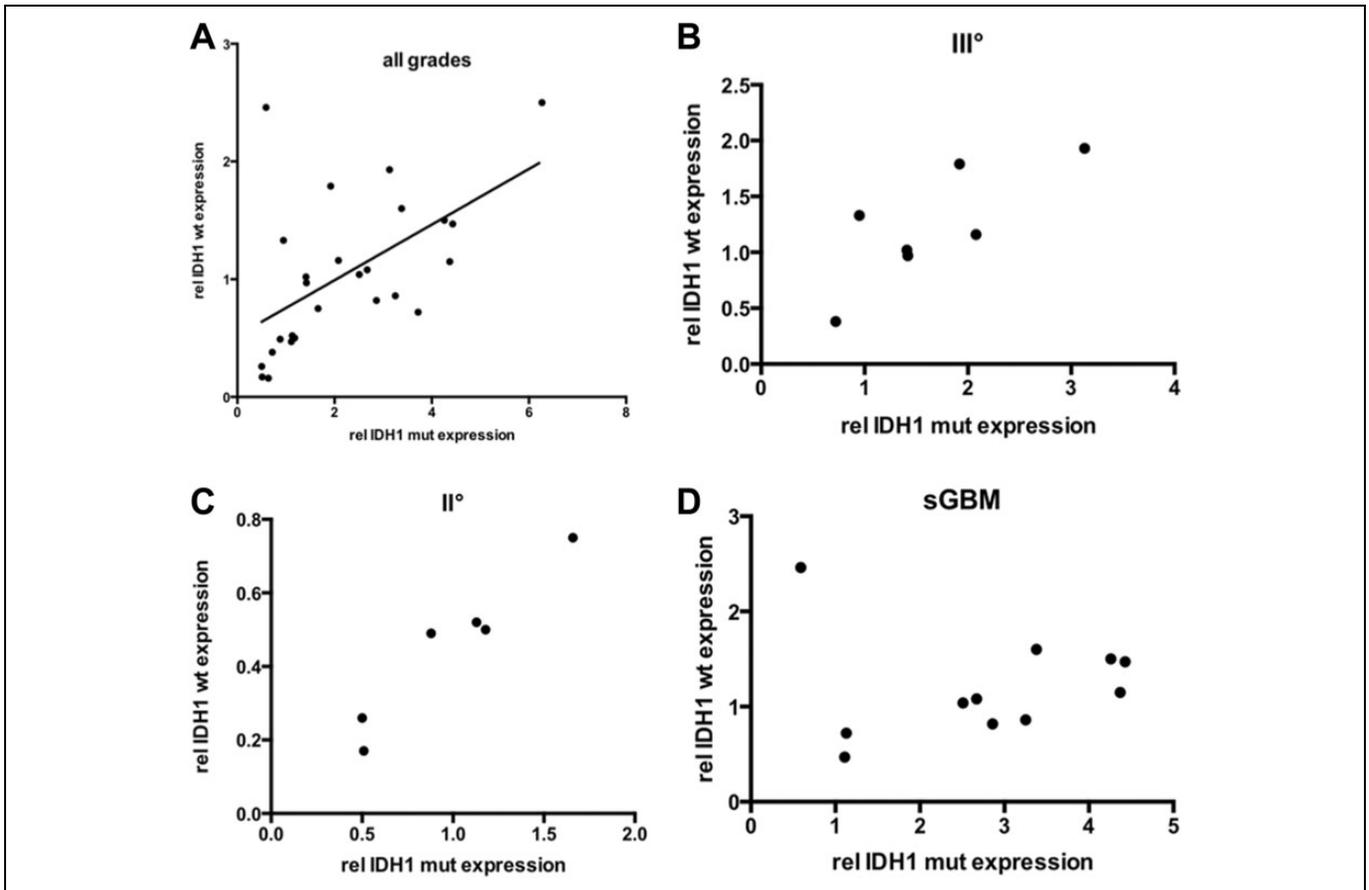

Figure 6. IDH1 mutation expression is independent of IDH1-wt expression in sGBM. (A) IDH1 mutation expression correlates with IDH1-wt expression in all glioma grades ($R^2 = .31$; $P = .003$). In subgroup analysis, IDH1 mutation expression correlates with IDH1 wt expression in (B) grade H glioma ($R^2 = .93$; $P = .002$) and (C) grade III glioma ($R^2 = .61$; $P = .04$) but not in (D) secondary glioblastoma ($R^2 < 0.001$; $P = .95$).
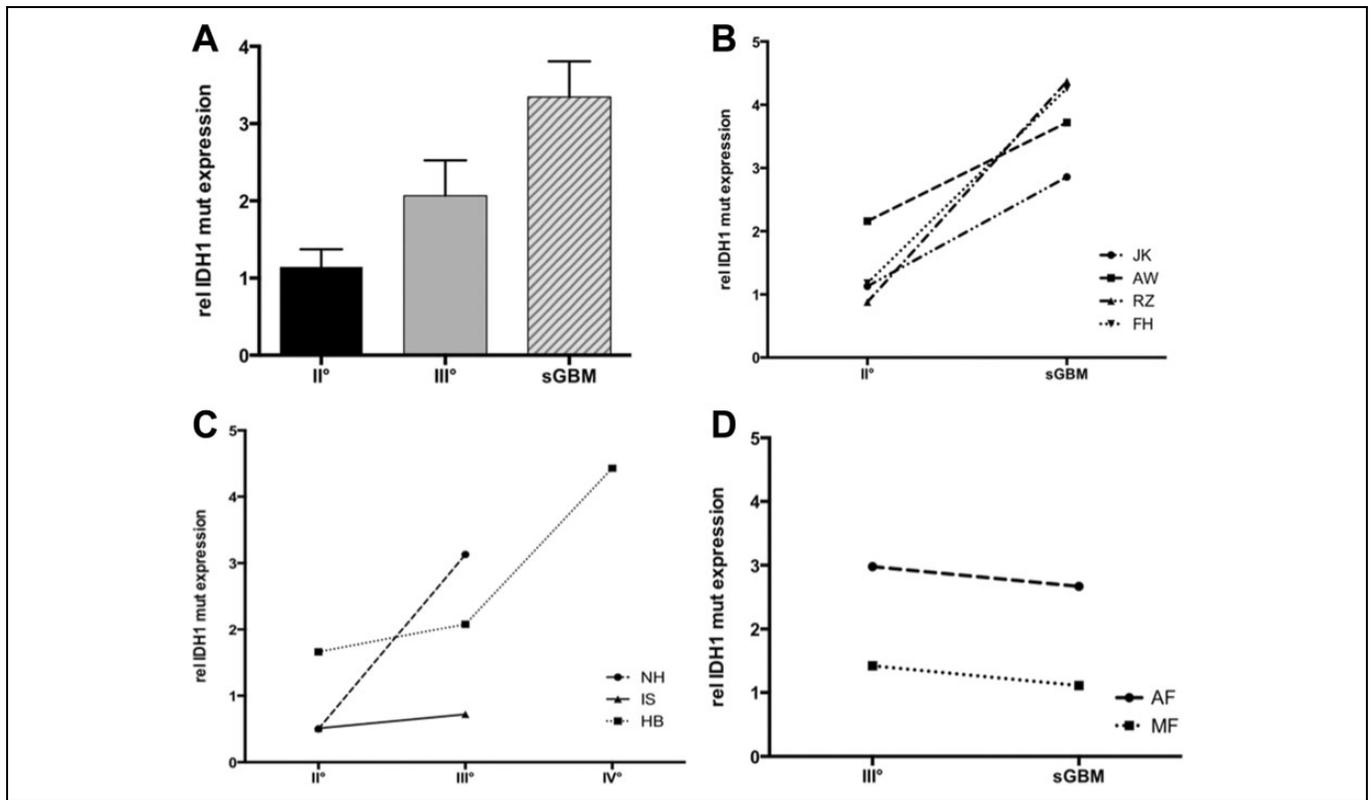
Figure 7. IDH1 mutation expression level increases with progression of the disease. In most samples of individual patients, IDH1 mutation expression level rose over the course of disease. (A) Cumulative IDH1 mutation expression in all samples analyzed over disease progression (patients n = 9; samples II n = 7; III n = 5; sGBM n = 7). (B) Patients who progressed from diffuse to anaplastic glioma and in 1 case to sGBM (n = 3). (C) Patients who progressed from diffuse glioma and were than diagnosed with sGBM (n = 4). (D) Patients with anaplastic glioma who progressed to sGBM (n = 2). Just in those 2 samples IDH1 mutation level decreased with disease progression. However, only those 2 patients had received prior chemotherapy before they were diagnosed secondary glioblastoma (sGBM+Cx; lines represent single patients, patient initials are given below). sGBM indicates secondary glioblastoma.
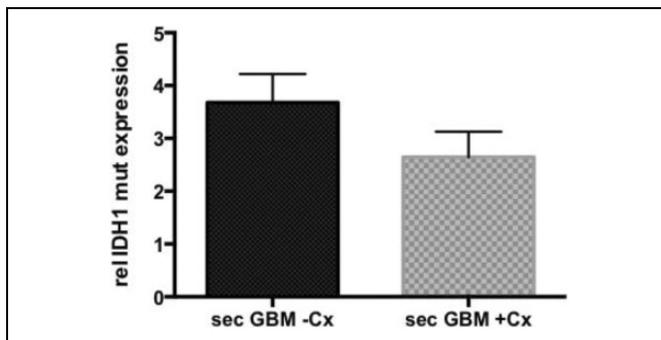

Figure 8. In sGBM, IDH1 mutation expression is lower after prior chemotherapy. sGBM indicates secondary glioblastoma.

Western Blot Analysis

IDH1 mutation positive samples of patients from all subgroups (II, III, sGBM, and pGBM [±Cx]) were analyzed by Western Blot analysis. IDH1mut / wt protein expression was detected in all samples (see Figure 9). Thereby, Western blot analysis had a very high sensitivity and specificity in determining the IDH1mut status (sensitivity 100%; specificity 100%).

Discussion

In our study, we detected IDH1mut in 85.7% of grade II gliomas, 86.4% of grade III gliomas, and 77.3% of secondary glioblastomas by DNA sequencing. These results are in line with previous publications.[3,24] In the vast majority of our samples (87.3%), the most common G395A mutation was found.

Using allele-specific qPCR, as previously published, we were able to define a threshold above which qPCR results are highly sensitive (98.4%) and specific (94.2%) in determining the IDH1mut status.

One of the 4 samples, in which the qPCR result did not match the corresponding sequencing results, was derived from a patient who progressed from a grade II glioma to a grade III glioma. In total, tumor tissue was taken from this particular patient at 3 time points. DNA sequencing detected the IDH1-mut in his first tumor (grade II glioma) and his last tumor (recurrent grade III tumor); however, in the tumor sample in between, no IDH1mut was found. As the IDH1mut status usually remains unchanged during progression of disease,[25] this result appears at least questionable. Unlike the sequencing result, qPCR detected IDH1mut in this particular sample, making a mistake in DNA sequencing very likely. While qPCR
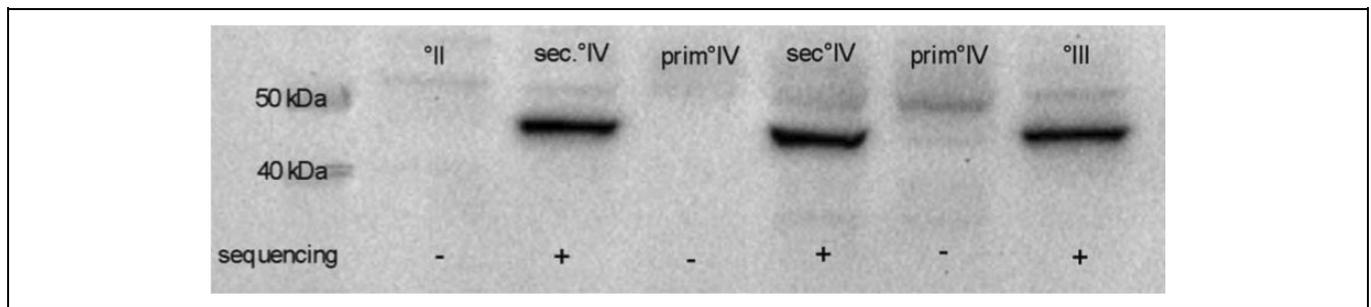
Figure 9. Western blot analysis detects IDH1 mutation with high sensitivity and specificity. Western blot analysis was performed in 6 samples. The results show a high concordance to DNA sequencing results.

might be even more sensitive in detecting IDH1mut than DNA sequencing in some cases, a drawback of this technique is that it only detects the most common point mutation (G395A). Hence, in our study, approximately 13% of IDH1mut were not detected by qPCR. Nevertheless, qPCR might be an additional tool in IDH1mut analysis in case of inconsistent results by IHC and sequencing.

Control tissue used in our study was derived from peritumoral tissue that was characterized as tumor free based on HE staining by an experienced neuropathologist. However, in 1 control sample, which was derived from peritumoral tissue of a secondary glioblastoma patient, IDH1mut was detected based on DNA sequencing and qPCR. Thus, qPCR seems to be a sensitive method in detecting IDH1mut even in case of small amounts of tumor cells. Immunohistochemistry with an IDH1mut-specific antibody has been described as a potential method to differentiate diffuse astrocytoma from astrocytosis.[26] As qPCR seems to be able to detect IDH1mut even in a zone of low tumor load, it could be an additional tool to identify glioma infiltration zone or even help to differentiate diffuse glioma from gliosis.

In addition to qualitative analysis of IDH1mut status, qPCR allows a quantification of IDH1mut expression. Particularly, with regard to the potential role of IDH1mut and its downstream product 2-hydroxyglutarate in gliomagenesis, level of IDH1mut expression might be a putative biomarker for the course of the disease. To our knowledge, no investigation on the level of IDH1mut expression has been published yet. As the primer used in our study specifically amplifies the most common G395A IDH1mut, only the IDH1mut expression levels of those samples that were characterized as IDH1mut by DNA sequencing were further analyzed. Thus, IDH1mut expression level analysis was performed in 61 samples from 48 patients. In these samples, we found an increasing level of IDH1mut expression from low-grade to high-grade glioma in accordance with the potential role of IDH1mut in glioma progression. This also held true for IDH1mut expression in 7 individuals whose IDH1mut expression was analyzed in consecutive tumor samples during their progression of the disease.

Additionally, prior Cx treatment seems to influence IDH1mut expression level, as the IDH1 mut expression level was lower not only in the previously treated sGBM group but also in

2 further individuals who had received prior chemotherapy. An effect of chemotherapy on IDH1mut expression has not been shown yet. However, in a recent publication, mutant IDH1 was found to sensitize glioma cells to oxidative stress as induced by chemotherapy.[27] Hence, the reduced expression of IDH1mut in previously treated secondary glioblastomas may be caused by a loss of cells bearing high levels of mutated IDH1 due to Cx treatment. The potential influence of prior Cx on IDH1 expression levels could suggest that IDH1 mutant cells may be more sensitive to DNA damage, as suggested in the published literature.[28] Mutant IDH1 drives a unique set of transformative events that indirectly enhance homologous recombination and facilitate repair of temozolomide-induced DNA damage and temozolomide resistance.[28]

Our results suggest not only a certain correlation between IDH1mut expression and course of disease but also an effect of chemotherapy on IDH1mut expression level. Thus, IDH1mut expression level might be a potential biomarker for disease progression and treatment effect. However, some questions remain unsolved, as our study has certain limitations. First, we did not analyze the correlation of IDH1mut expression level with time to progression or overall survival. Therefore, we are unable to answer conclusively how clinically relevant our observation is.

Moreover, our study did not include an analysis of 2-hydroxyglutarate levels as a downstream product of mutated IDH1. However, in a recent study, 2-hydroxyglutarate levels were found not to correlate with progression in IDH1-mutated LGG.[25] Therefore, it is possible that, due to a yet unknown mechanism, the translation from IDH1mut messenger RNA to mutated IDH1 is inhibited, leading to a mismatch between IDH1mut expression level and 2-hydroxyglutarate levels during progression of glioma.

The clinical uses in establishing qPCR for the detection of IDH1 mutational status could be cases with small amounts of tumors cells or longitudinal cases, where the mutational status seems to change. In these cases, qPCR would be optimal and probably superior to the other methods. Moreover, the benefits of qPCR as an additional tool could be to identify glioma infiltration zones or even help to differentiate diffuse glioma from gliosis. Quantitative PCR could be added to the traditional used methods in a step-wise process: (1) IHC for screening,

(2) sequencing in unclear cases, and (3) qPCR in questionable cases with low tumor load or within the infiltration zone.

Our results corroborate previously published results concerning the high sensitivity and specificity of qPCR in detecting IDH1mut in gliomas. Even if there are limitations due to the use of mutation-specific primers, it seems to be a valuable additional tool in detecting IDH1mut. In our laboratory, we were able to establish a threshold of relative IDH1mut expression above which IDH1mut can be identified with high sensitivity.

Moreover, we found in our quantitative analysis of IDH1 expression in LGG and secondary high-grade glioma, a certain correlation between IDH1mut expression level and tumor grade as well as an influence of prior chemotherapy. However, due to the limitations of our study, it is premature to regard IDH1mut as a potential biomarker for the course of the disease.

Authors' Note

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Declaration of Conflicting Interests

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