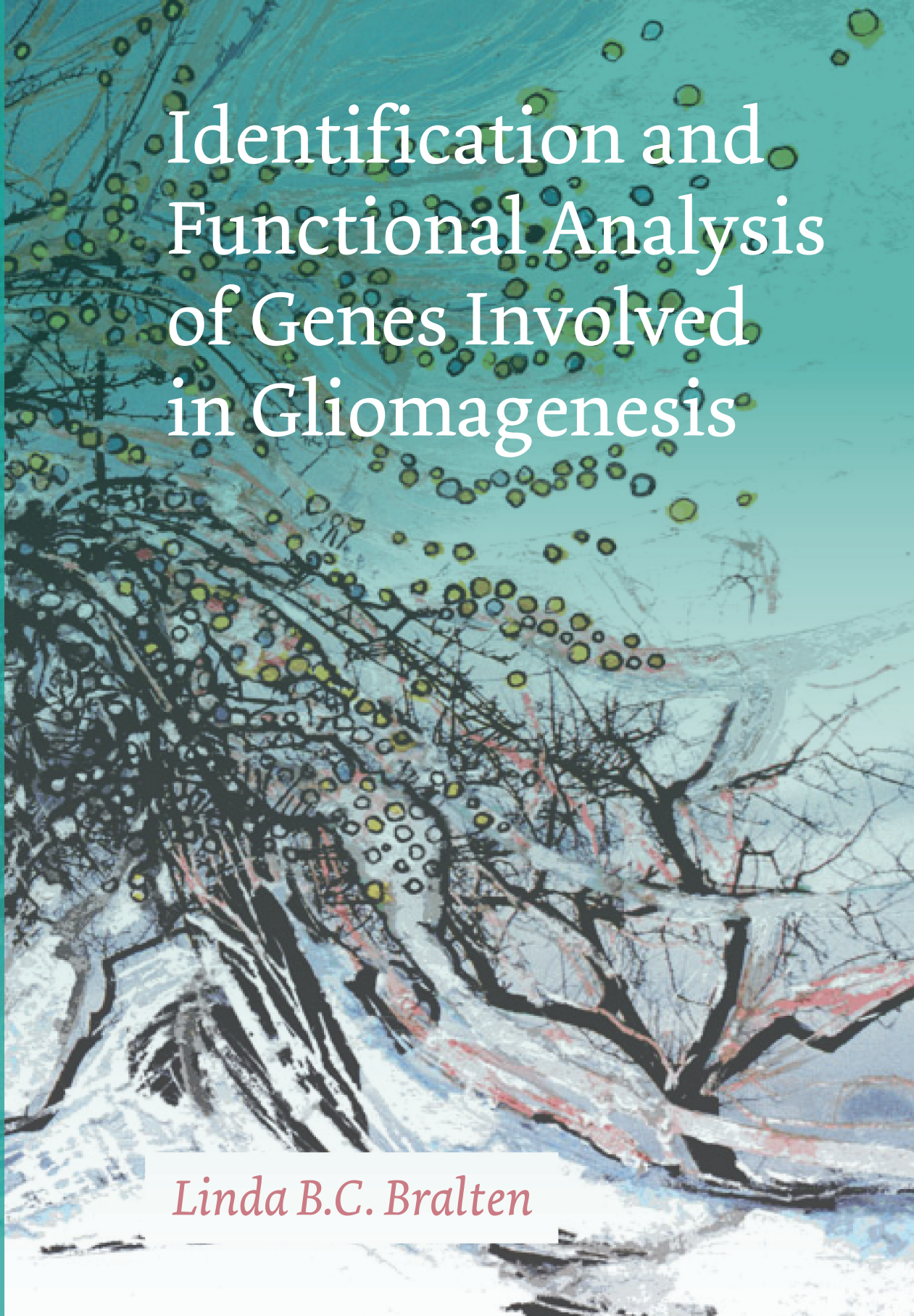

Identification and Functional Analysis of Genes Involved in Gliomagenesis

Linda B.C. Bralten

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Content

Aim and scope of the thesis

The median survival of glioma patient is still very poor and new therapeutic targets have to be discovered. The aim of this thesis is to identify genetic alterations in glioma and functionally characterize the most promising candidates. We describe several novel methods used to identify these mutations.

In chapter 1 an overview is given of the most common genetic alterations found in the different glioma subtypes. In most glioblastomas 3 main pathways are found to be affected. In lower grade gliomas much less was known about common genetic alterations until the recent discovery of mutations in the *IDH1* gene. In chapter 2 a review is given on mutations in the *IDH1* and the closely related *IDH2* genes in gliomas. It tells us that IDH1 is frequently mutated in low grade glioma, in both oligodendroglioma, which often have loss of 1p and 19q, and astrocytoma, which are more likely to have P53 mutations. Besides that it shows that *IDH1* is very specifically mutated in the same spot (R132), but that the amino acid change is not always the same; the most common change is R132H, but a mix of other changes occurs, we call these non-p.R132H mutations.
The distribution of specific types of *IDH1* mutations is listed in chapter 5. We were interested to find out how these non-p.R132H mutations relate to other main genetic alterations, like P53 mutations and loss of 1p and 19q or to molecular subgroups in glioma on the basis of RNA expression profiles. If so this could suggest functional differences between the *IDH1* R132H and *IDH1* non-p.R132H mutations. A non random distribution would also tell us something about the importance of *IDH1* mutations in gliomagenesis. Chapter 2 shows us that although the mutations found in these genes are very specific, they (all) cause a loss of normal enzymatic function of the gene. This would make us think that *IDH1* functions as a tumor suppressor gene. However, tumor measurements of metabolites show that *IDH* mutated tumors have a higher concentration of 2-hydroxyglutarate, and enzyme measurements show that mutated IDH1 produces 2-hydroxyglutarate, suggesting that mutated *IDH1* has oncogenic potential by means of this 2-hydroxyglutarate. It is still unclear if 2-hydroxyglutarate is the oncogenic effector or a simple byproduct. We wanted to know what the effect of the *IDH1* R132H mutation was on the growth and migration of cell lines, both in vivo and in vitro. Because no glioma cell lines harboring the *IDH1* R132H mutation were available, we stably over expressed *IDH1* R132H in several (glioma) cell lines. In chapter 6 a functional analysis of these cell lines is given.
In chapter 4 we describe the functional analysis of the *CASPR2* gene. This gene was mutated in at least 3 gliomas with decreased expression of the CASPR2 protein in these samples. We wanted to know if the *CASPR2* gene functioned as a tumor suppressor gene

and therefore investigated the effect of expression of the *CASPR2* gene on cell proliferation and migration.

The technical revolution that first has taken place in the field of RNA and DNA level detection and later in high throughput sequencing gave us the opportunity to use exon arrays, SNP arrays and next generation sequencing techniques to detect fusion genes and to systematically screen for other mutations. Before the array era especially translocations were very hard to screen for and to screen one gene for point mutations or small insertions or deletions was a long term project. Exon arrays and SNP arrays provide enormous amounts of data and besides the use of this information for tumor classification or the detection of big chromosomal gains and losses, we wanted to integrate this data and in this way zoom in on individual genes in a systematic way. In chapter 3 we describe a method to detect fusion genes by means of copy number alteration data integrated with exon array expression data. We also thought of a way to use exon arrays to screen for translocation sites. Chapter 4 describes the detection of a translocation of the *CASPR2* gene detected by means of exon array expression data.

In chapter 7, we describe the use of next generation sequencing to systematically sequence all exons on the 1p and 19q chromosomal arms in oligodendrogliomas. Because loss of 1p and 19q is common in oligodendrogliomas mutation of a gene on the remaining alleles may be a “second hit” on a putative tumor suppressor gene.

In summary, this thesis describes the search for new genetic alterations in glioma by means of novel techniques like exon arrays and next generation sequencing. One candidate gene from this screen, the *CASPR2* gene, is functionally analyzed, as well as the recently discovered R132H mutation in the *IDH1* gene.

Chapter 1

Genetic Alterations in Glioma

Linda B. C. Bralten and Pim J. French

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Abstract

Gliomas are the most common type of primary brain tumor and have a dismal prognosis. Understanding the genetic alterations that drive glioma formation and progression may help improve patient prognosis by identification of novel treatment targets. Recently, two major studies have performed in-depth mutation analysis of glioblastomas (the most common and aggressive subtype of glioma). This systematic approach revealed three major pathways that are affected in glioblastomas: The receptor tyrosine kinase signaling pathway, the TP53 pathway and the pRB pathway. Apart from frequent mutations in the *IDH1/2* gene, much less is known about the causal genetic changes of grade II and III (anaplastic) gliomas. Exceptions include *TP53* mutations and fusion genes involving the *BRAF* gene in astrocytic and pilocytic glioma subtypes, respectively. In this review, we provide an update on all common events involved in the initiation and/or progression across the different subtypes of glioma and provide future directions for research into the genetic changes.

1. Introduction

Gliomas are the most common primary brain tumor in adults (incidence 5.97 per 100,000, CBTRUS statistical report 2010, http://www.cbtrus.org/) and can be subdivided based on their histological appearance into an astrocytic (A), oligodendroglial (OD), or oligoastrocytic (OA) lineage. They can be further subclassified into grades: I (pilocytic astrocytomas, PA), II (low grade), III (anaplastic) and IV (glioblastoma, GBM), depending on the number of malignant features present (high cellularity, nuclear atypia, mitosis, necrosis, and endothelial proliferation). Secondary GBMs are those that have progressed from lower grade gliomas, whereas primary GBMs arise *de novo.*

The prognosis of glioma patients varies between the different histological subtypes and grades; for example, patients with grade II OD have the longest survival (median survival 11.5 years) whereas patients with a glioblastoma (the most common subtype of glioma) have a median survival of only 4.9 months [1]. Pilocytic astrocytomas are the only subtype of glioma with a favorable prognosis (94–96% five year survival) (CBTRUS statistical report 2010; [1]).

Current treatment options involve a combination of surgical resection, radiotherapy and chemotherapy. Complete surgical resection of gliomas is virtually impossible due to their invasive growth pattern. Radiotherapy is effective. However, the infiltrating cells can only be reached by whole brain irradiation where the benefits of radiotherapy may not outweigh the side effects caused by radiation damage. Temozolomide has thusfar proven effective in improving the median overall survival in glioblastoma by three months [2], and is most effective in those in which the *MGMT* promoter is hypermethylated [3]. Adjuvant procarbazine, CCNU (lomustine) and vincristine (PCV) improved progression free survival but not overall survival in anaplastic oligodendrogliomas and oligoastrocytomas [4,5]. In these studies, tumors harboring loss of 1p and 19q chromosomal arms comprise a favorable subgroup. In spite of these advances, the prognosis of gliomas remains poor and therefore other strategies have to be developed. A better understanding of the genes (and their associated molecular pathways) involved in gliomagenesis and/or progression may reveal new options for targeted therapy.

2. Glioblastoma

The Cancer Genome Atlas project has published mRNA expression data and DNA copy number alteration data of 206 GBMs and has sequenced >600 genes in 91 GBMs [6]. The project is still ongoing and ultimately aims to include data from 500 gliomas, but already has shown the importance of a systematical approach and high sample numbers. Combining all detected homozygous deletions, focal amplifications and validated somatic nucleotide substitutions, they found three major pathways affected in a high percen-

tage of glioblastomas: receptor tyrosine kinase signaling (altered in 88% of the GBMs), TP53 signaling (altered in 87%) and the pRB tumor suppressor pathway (altered in 78%). Novel genes in those pathways include the NF1 tumor suppressor gene and PIK3R1. Another large study on 22 GBMs sequenced all protein coding genes and performed copy number analysis and expression data analysis on these tumors [7]. All genes affected in more than two tumors were validated in a set of 80 GBMs. Besides confirmation of affected oncogenes and tumor suppressor genes in the formerly mentioned three pathways (50%, 64% and 68%, respectively) (see Scheme 1 and Table 1), they found the *IDH1* gene to be mutated in 12% of the GBMs [7]. These mutations occurred in younger patients with mostly secondary GBMs and such tumors had a relatively favorable prognosis [7,8]. (For a more elaborate discussion on *IDH1* see below).

Scheme 1. The three major pathways affected in a high percentage of glioblastomas and the most common genes affected in those pathways. Following the gene names, the percentages of genetic alterations found in glioblastoma are depicted.

Pathways

RTK	50 88%	TP53	64 87%	RB	68 78%
EGFR	37 45%	TP53	35 40%	RB	11 12%
PDGFRA	13%	CDKN2A-p14ARF	49 50%	CDK4	14 18%
ERBB2	8%				
cMET	4%	MDM2	14%	CDK6	1%
PTEN	30 36%	MDM4	7%	CCND2	2%
PIK3CA	5 10%			CDKN2A-P16INK	50 52%
PIK3R1	8 10%				
AKT	2%			CDKN2B	47%
FOXO	1%			CDKN2C	2%
RAS	2%				
NF1	15 18%				

2.1. Copy Number Alterations

Copy number amplifications are more frequent in GBMs than in lower grade gliomas [9,10]. A distinction can be made between focal, high copy number amplifications (e.g., ≥7n) and larger, intermediate copy number amplifications (e.g., 3n). High copy amplicons often occur in regions with known oncogenes (*EGFR*, *MDM2* and *CDK4*), but may also occur in other regions [11] (for a summary see [12]). Frequent intermediate copy number gains or losses include trisomy of chromosome 7 (42%) and monosomy of chromosome 10 (58%) [6,13,14]. The genes involved in gliomagenesis and/or progression on these chromosomes (except for *PTEN*) remain to be determined. GBMs with trisomy of

chromosome 7and loss of chromosome 10 have a poor prognosis [6,13,14]. In general, aCGH data can identify three genetic GBM subgroups; one with gain of chromosome 7 and loss of chromosome 10, one with only chromosome10 loss and one without gain of chromosome 7 or loss of chromosome 10 [15].

2.2. Receptor Tyrosine Kinase Signaling

The receptor tyrosine kinase (RTK) signaling pathway is involved in the translation of growth factor signals into increased proliferation and survival. The most frequently altered gene in the RTK pathway is *EGFR*. It is amplified in up to 45% of glioblastomas and results in an increase in mRNA and protein expression [6,16,17]. In addition to amplification, *EGFR* is often constitutively activated by variants including *EGFRVIII* in which exons 2–7 are deleted [18]. 3' truncations are also frequently observed [18]. Both variants are due to intragenic deletions, are thought to occur following *EGFR* amplification and result in constitutively active proteins [19-22]. Although a number of point mutations are also observed in the *EGFR* gene [6,7], the activating mutations in the ATP binding domain observed in lung cancer (NSCLC) are not observed in gliomas [23]. Other RTKs may also be affected in GBM and include *PDGFRA* (amplified in 13%), *ERBB2* (mutated in 8%) and *cMET* (amplified in 4%) [6].

RTKs signal (a.o.) through phosphoinositide 3 kinases (PI3 kinases) that phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This reaction is reversed by PTEN. Of the PI3 kinases, *PIK3CA* and its adaptor protein *PIK3R1* are most frequently mutated in GBM (15-27%) [6,7,24,25]. *PTEN* is homozygously deleted in 36% of GBMs and infrequent mutations in downstream PIP3 targets have been identified in *AKT* and *FOXO* (2% AKT amplification, 1% inactivating FOXO mutation) [6].

Ras is another important protein activated by receptor tyrosine kinases and a key regulator of tumorigenesis. *Ras* mutations (*N-Ras, H-Ras, KRas*) occur infrequently in GBM (2% activating *Ras* mutations) [6]. However, the *NF1* gene, which encodes for the Ras inhibiting protein neurofibromin 1 (a RasGAP), is frequently inactivated in GBM (15-18% inactivating mutations or homozygous deletions) [6,7].

2.3. TP53 Signaling

TP53 signaling is important in apoptosis, cellular senescence and cell cycle arrest in response to DNA damage. Most tumor types need to circumvent or shut down the TP53 pathway. *TP53* heterozygous dominant negative point mutations and homozygous deletions are common in GBM (35–40%) [6,7]. Two TP53 inhibitors, *MDM2* and *MDM4*, that are involved in the ubiquitinylation and degradation of TP53, are amplified in 14% and 7% of the glioblastomas, respectively [6]. The *CDKN2A* locus is also part of the TP53

pathway and is frequently deleted or inactivated in glioblastomas (49–50%) [6,7]. One of the two genes that can be expressed from the *CDKN2A* locus is *P14ARF*, which is an inhibitor of MDM2. The *CDKN2A* locus also encodes for p16INK4A which is part of the pRB signaling pathway (see below)

2.4. RB Signaling

The retinoblastoma protein (pRB) is a major protein involved in cell cycle progression from G1 to S phase. In the hypophosphorylated state pRB binds to the transcription factor E2F, thereby preventing cell cycle progression. Phosphorylated pRB does not associate with E2F, which results in cell cycle progression. The *pRB* gene is homozygously deleted or mutated in 11–12% of the GBMs [6,7]. Interestingly, *CDK4*, *CDK6* and *CCND2* phosphorylate pRB and are amplified in 14–18%, 1% and 2% of glioblastomas, respectively [6,7]. Conversely, *CDKN2A*/p16INK4A, *CDKN2B* and *CDKN2C*, inhibit the different CDKs and are frequently inactivated in GBM (homozygously deleted or mutated in 50–52%, homozygously deleted in 47%, homozygously deleted in 2%, respectively) [6,7].

3. Grade II and III Glioma

In general, only few frequent genetic changes have been identified in lower grade gliomas (Table 1). Larger chromosomal aberrations include combined loss of 1p19q in grade II and III oligodendroglioma (40–69%) and oligoastrocytoma (44–48%). Such losses are far less common in astrocytoma (0–11%) [1,26,27]. The remarkably high frequency of LOH of 1p and 19q suggests the remaining arms harbor yet to be identified tumor suppressor genes (Knudson two-hit hypothesis [28]). Frequent mutations have been identified in *IDH1* (all grade II and III gliomas, see below) and *TP53* genes (predominantly astrocytic). *TP53* mutations and LOH of 1p 19q are mutually exclusive [1], thereby distinguishing two different pathways of glioma development. Complete hemizygous losses of 1p are tightly associated with 19q loss and oligodendroglial phenotype and predict longer overall and progression free survival [29]. However, partial 1p deletions are mainly observed in astrocytic tumors, are not associated with 19q loss and have a negative prognostic value [29].

Based on these genetic changes, three large groups can be genetically characterized in low grade and anaplastic gliomas: tumors with *TP53* mutations and *IDH1/2* mutations (32%), tumors with LOH 1p19q and *IDH1/2* mutations (37%) and tumors with only *IDH1/2* mutations (17%) [30]. These molecular changes segregate with the distinct histological subgroups of glioma: for example, most of the diffuse astrocytoma have *TP53* and *IDH1/2* mutation and most of the oligodendrogliomas have LOH 1p19q and *IDH1/2* mutation. The oligoastrocytomas were more equally distributed among the three different groups (33%, 34% and 19% respectively) [30].

IDH1/IDH2

IDH1 mutations were initially discovered in GBMs by Parsons *et al.* [7]. However, *IDH1* mutations were detected at much higher frequencies (over 70%) in grade II and II gliomas [31-35].; Mutations in the homologous *IDH2* gene were also identified (around 5%), predominantly in oligodendroglial tumors [32,35]. *IDH1* mutations are an early event in tumorigenesis [34], are an independent favorable prognostic marker in gliomas and are closely associated with1p19q codeletion and *MGMT* methylation status [36]. *IDH1/2* mutations are heterozygous missense mutations affecting highly conserved arginines that are involved in substrate binding. Wildtype IDH converts isocitrate into alpha-ketoglutarate, whereas mutant IDH enzymes have reduced ability to catalyze this reaction [35]. Instead, mutant IDH enzymes have gained the ability to convert alpha-ketoglutarate into D-2-hydroxyglutarate [37]. The oncogenic function and the molecular pathway of IDH1/2 and D-2-hydroxyglutarate are not fully understood yet. However, a recent study suggests that *IDH1/2* mutations result in an increase in global methylation [38] and IDH1/2 mutations are associated with a more hypermethylated DNA methylation profile [39,40]. It is therefore possible that IDH1/2 mutations are involved in oncogenesis by the inactivation of tumor suppressor genes following promoter hypermethylation.

4. Pilocytic Astrocytoma

The majority of pilocytic astrocytomas are cytogenetically normal [41] except for a small tandem dulication of 2 Mb at 7q34 (66%) [42]. This duplication results in a fusion gene incorporating the kinase domain of *BRAF* to *KIAA1549* (exon 1-16/15) [42]. This fusion gene produces a constitutively active BRAF, which is able to transform NIH3T3 cells [42]. Activating *BRAF* point mutations (V600E/ins 3 bp at 598) can also occur in pilocytic astrocytoma [42,43].

5. Genetic Alterations in Molecular Subtypes

Current classification of gliomas is largely based on histological appearance. However, histological classification of gliomas is troublesome and subject to interobserver-variation [44-47]. Gliomas may also be classified based on their similarities in gene expression profiles [48-50]. Such classification correlates better with survival than histological classification of gliomas [48]. Distinct genetic changes also segregate into the different molecular subtypes of glioma [48,50]. For example, EGFR amplifications are predominantly found in classical and neural subtypes [50] or in cluster 18 gliomas [48]. *IDH1* mutations segregate in proneural type GBMs [50] or in gliomas containing + predominantly secondary GBMs (Cluster 22) as well as in gliomas with more favorable prognosis (Clusters 9 and 17) [48].

6. Epigenetic Changes

Methylation in cancer often occurs in the promoter regions of tumor suppressor genes [51,52]. Inactivation of gene expression by promoter methylation thus contributes to tumor formation as the second "hit" in tumor suppressor genes (Knudson's two-hit hypothesis [28]). Several groups have therefore performed genome wide methylation profiling in GBMs and have identified a subset of tumors that have more favorable prognosis [40,53]. These tumors show an overall increase in DNA methylation at CpG sites (CIMP; CpG island methylator phenotype) [40]. It remains to be determined whether reversal of CIMP status can be used as a treatment for gliomas.

7. Future Perspectives

Large scale sequencing efforts, such as those described in this review, have revealed an unprecedented insight into the biology of gliomas. The rapid development of novel sequencing techniques will lead to even more genetic data in the next decade. It can therefore be expected that virtually all genetic changes in all glioma subtypes will be identified in the near future. However, not all genetic changes in gliomas (or other tumors) are causal for the disease; mutations can arise during cell division and are then found throughout the tumor due to clonal expansion [54]. In fact, the majority of somatic mutations in cancer may be such "passenger" mutations [54]. Distinguishing such "passenger" mutations from the causal "driver" mutations is therefore required.

One way to distinguish driver from passenger mutations is by frequency analysis: causal genetic changes are thought to occur at a higher incidence than predicted by chance. However, few genetic changes are recurrent events (so-called "mountains" in the genomic landscape of [55]); only a handful of genes are mutated at frequencies >10% not only in gliomas [6,7,54] but also in many other cancer types [56,57]. These studies show that many more genes are mutated at low frequencies (so-called "hills"). Such infrequent candidates have been demonstrated to contribute to tumor formation and/or progression (see e.g., [58]). Future research will thus require distinguishing "drivers" from "passengers" on infrequently mutated genes.

Eventually, the knowledge of these genetic alterations can be used for the development of targeted therapy. However, many infrequent "hills" could indicate that each tumor has its own unique spectrum of causal genetic changes. Treatments aimed at targeting these individual genetic changes may therefore be difficult. Nevertheless, it is likely that different genetic changes are part of a select set of molecular pathways. Therefore, pathway inhibition or reactivation can be used to target a broader range of tumors. In the future, it is likely that individual cancer genomes will therefore be sequenced to direct targeted therapies. Such practice does require a further increase in sequencing capacity and speed and dedicated data analysis pipelines.

Table 1. Genes or regions with the most characteristic genetic alterations in glioma subtypes.
* % are calculated on a mixture of primary and secondary glioblastoma. LOH: loss of heterozygosity; PA: Pilocytic Astrocytoma.

Tumor Type	Ref.	[7]*	[6]*	[43]	[31]	[32]	[27]	[1]*
PA	BRAF			66%				
Oligodendroglioma	IDH1/2				69%	74.7–86.7%		
	LOH 1p19q						40%	69%
Oligoastrocytoma	IDH1/2				78%	72.3–82.9%		
	LOH 1p19q						48%	45%
	TP53							44%
Astrocytoma	IDH1/2				68%	64.9–73.6%		
	TP53							53-88%
Sec Glioblastoma	IDH1/2	12%			88%			
	TP53	40%	35%					31%
Prim Glioblastoma	EGFR	37%	45%					34%
	CDKN2A	50%	53%					31%
	PTEN	30%	36%					24%

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Chapter 2

Isocitrate dehydrogenase-1 mutations: a fundamentally new understanding of diffuse glioma?

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Abstract

The discovery of somatic mutations in the gene encoding isocitrate dehydrogenase-1 (IDH1) in glioblastomas was remarkable because the enzyme was not previously identified with any known oncogenic pathway. *IDH1* is mutated in up to 75% of grade II and grade III diffuse gliomas. Apart from acute myeloid leukaemia, other tumour types do not carry *IDH1* mutations. Mutations in a homologous gene, *IDH2***,** have also been identified, although they are much rarer. Although *TP53* mutations and 1p/19q codeletions are mutually exclusive in gliomas, in both of these genotypes *IDH1* mutations are common. *IDH1* and *IDH2* mutations are early events in the development of gliomas. Moreover, *IDH1* and *IDH2* mutations are a major prognostic marker for overall and progression-free survival in grade II–IV gliomas. Mutated IDH1has an altered catalytic activity that results in the accumulation of 2-hydroxyglutarate. Molecularly, *IDH1* and *IDH2* mutations are heterozygous, affect only a single codon, and rarely occur together. Because *IDH1* does not belong to a traditional oncogenic pathway and is specifically and commonly mutated in gliomas, the altered enzymatic activity of IDH1 may provide a fundamentally new understanding of diffuse glioma.

Introduction

In 2008, a genome-wide sequencing study identified mutations in a gene encoding isocitrate dehydrogenase (IDH1) in 18 (12%) of 149 samples of glioblastoma multiforme.[1] The mutations were most common in young patients with glioblastomas that had progressed from lowgrade gliomas to secondary glioblastoma multiforme. Patients with *IDH1* mutations were also associated with a longer survival than patients with glioblastomas without *IDH1* mutations. Several papers have since identified the *IDH1* mutations in other glioma subtypes and in various tumour types (table 1). Functional studies[2–17] on *IDH1* found that *IDH1* mutations in the gene are highly glioma specific. Investigations of large numbers of other tumour types showed that acute myeloid leukaemia (AML) was the only cancer other with a high incidence of *IDH1* mutations (table 1). *IDH1* is the most commonly mutated gene in many types of glioma, with incidences of up to 75% in grade II and grade III glioma (table 1). *IDH1* mutations are always heterozygous missense mutations that affect aminoacid residue 132 (figure 1) and are associated with longer survival in patients with glioma than patients with glioma without *IDH1* mutations (table 2, figure 2). *IDH1* mutations result in a reduced enzymatic activity towards the native substrate, isocitrate, and mutant IDH1 catalyses the formation of 2-hydroxyglutarate from α-ketoglutarate.

In addition to *IDH1*, mutations in a homologous gene, *IDH2,* with a different subcellular distribution have been identified in gliomas. *IDH2* mutation is much less common than *IDH1* mutation; the two rarely occur together, but both seem to be specific to gliomas.[5,6,11,24] Inactivating mutations in another gene, *IDH3B,* have been identified in patients with retinitis pigmentosa, but they do not seem to be associated with tumour formation.[25]

Structure and function

In human beings, five genes encode for isocitrate dehydrogenase: *IDH1*, *IDH2*, *IDH3A*, *IDH3B*, and *IDH3G*. These genes encode three catalytic isozymes: IDH1, IDH2, and IDH3. Both IDH1 and IDH2 function as homodimers whereas IDH3 functions as a heterotetramer composed of two α, one β, and one γ subunit.[26] The function of all isocitrate dehydrogenases is to catalyse the oxidative decarboxylation of isocitrate into α-ketoglutarate, with oxalosuccinate formed as an intermediate product (figure 1).[27] In mitochondria this reaction is part of the tricarboxicylic-acid cycle (also know as the Krebs cycle). During the enzymatic reaction, either nicotinamide adenine dinucleotide (NAD^+) in the case of IDH3 or nicotinamide adenine dinucleotide phosphate ($NADP^+$) for IDH1 and IDH2 function as the electron acceptor. Distinct isocitrate dehydrogenase (IDH) isozymes also differ in subcellular localisation; IDH1 is located in the cytoplasm and peroxisomes whereas IDH2

Table 1: Incidence of IDH1 and IDH2 mutations

	IDH1		IDH2	
	Mutations (%) and median (range)	Number of mutations/total number of investigated tumours	Mutations (%) and median (range)	Number of mutations/total number of investigated tumours
Astrocytic tumours				
Pilocytic astrocytoma[2–5]	1% (0–10%)	4/130	0%	0/21
Pleomorphic xanthoastrocytoma[2,5]	7% (0–14%)	1/14	..	..
Subependymal giant-cell astrocytoma[2,5]	0%	0/14	..	..
Diffuse astrocytoma[2–8]	74% (0–88%)	307/407	4% (1–7%)	4/257
Anaplastic astrocytoma[2–9]	59% (0–78%)	294/457	4% (0·9–5%)	5/301
Glioblastoma				
Primary[2–5,7–11]	5% (3–12%)	61/1167	0%	0/139
Primary paediatric[2,5]	4% (0–7%)	1/29	0%	0/15
Secondary[2–5, 7–10]	83% (50–100%)	94/121	0%	0/11
Giant-cell[2] glioblastoma multiforme	25%	2/8	..	..
Gliosarcoma[2]	0%	0/5	..	..
Oligodendroglial tumours				
Oligodendroglioma[2–8]	76% (67–84%)	283/366	4% (0–5%)	8/188
Anaplastic oligodendroglioma[2–8]	67% (49–94%)	237/355	5% (0–8%)	12/224
Oligoastrocytoma[2–7]	80% (50–100%)	153/196	1%	1/76
Anaplastic oligoastrocytoma[2–8]	75% (63–94%)	245/345	6% (0–8%)	11/181
Ependymal tumours				
Subependymoma[2–8]	0%	0/8	..	..
Myxopapillary ependymoma[2,3]	0%	0/14	..	..
Ependymoma[2–5]	0%	0/92	0%	0/30
Anaplastic ependymoma[2,3]	0%	0/17	..	..
Neuronal and mixed neuronal-glial tumours				
Ganglioglioma[8]	37·5%	3/8	0%	0/8

Anaplastic ganglioglioma[7]	60%	3/5	0%	0/5
Dysembryoplastic neuroepithelial tumour[3]	0%	0/4	..	..
Familial paraganglioma[12]	0%	0/65	0%	0/65
Sporadic paraganglioma[12]	2%	1/66	0%	0/66
Embryonal tumours				
Type IV medulloblastoma[2,3,5]	0%	0/149	0%	0/55
Primitive neuroectodermal tumour[2,3]	17% (0–33%)	3/17	..	..
Tumours of the cranial and paraspinal nerves				
Type I schwannoma[2]	0%	0/17	..	..
Vestibular schwannoma[3]	0%	0/48	..	..
Meningeal tumours				
Type I meningioma[2,3,11]	0%	0/86	0%	0/14
Type II atypical meningioma[2]	0%	0/17	..	..
Type III anaplastic meningioma[2]	0%	0/17	..	..
Tumours of the sellar region				
Type I pituitary adenoma[2]	0%	0/23	..	..
Haematological disorders				
Acute leukaemia[11]	..	..	0%	0/166
Acute myelogenous leukaemia[5,13,14]	1% (0–9%)	21/389	..	..

Listed are the median and range of the percentages of *IDH1* mutations from individual studies. The total number of mutations is a sum of all samples from all relevant studies. The study by Parsons and colleagues[1] is excluded from this table due to partly overlapping sample sets with Yan and colleagues.[5]

and IDH3 are found in mitochondria. Neither IDH1 nor IDH2 function as monomers, and both require a divalent metal ion, preferably Mn^{2+}, for enzymatic activity.[28–34]

Mutation frequency

Diffuse gliomas are the most common primary brain cancer in adults and typically have a poor prognosis.[35,36] On the basis of histology, gliomas are classified into astrocytic tumours (75%) and oligodendroglial tumours (25%). Oligodendroglial tumours comprise both classic oligodendrogliomas and mixed oligoastrocytic tumours.[37] Diffuse gliomas are further classified into grade II (or low grade), grade III (anaplastic), and grade IV (gliobastoma multiforme) depending on the presence of malignant features. Grade IV tumours, are the most common and aggressive type of glioma and are distinguished between those that occur de novo (primary gliobastoma multiforme) and those that have progressed from lowgrade gliomas (secondary).[38]

IDH1 mutation is common in almost all glioma subtypes, ranging from 50% to 80%, except in primary gliobastoma multiforme (table 1). *IDH1* is among the few genes that are commonly mutated in grade II and grade III gliomas.[39,40] The prevalence of *IDH1* mutation is much lower in primary (about 5%) than in secondary gliomas than in anaplastic gliomas.[7,41] *IDH2* mutations (about 75%) gliobastoma multiforme. Furthermore, are much less common than *IDH1* mutations and are *IDH1* mutation seems to be more common in grade II associated with oligodendrogliomas (table 1).[6] *IDH1* mutations are rarely seen in pilocytic astrocytomas, which are circumscript (non-diffuse) grade I gliomas.[42] Molecularly, *IDH1* mutations are associated with MGMT (O^6-methylguanine-DNA methyltransferase) methylation status, *TP53* mutations, and deletions of both 1p and 19q.[3,4,7,22] *TP53* mutations and deletions of both 1p and 19q are mutually exclusive and are thought to be early events in the development of astrocytomas and oligodendrogliomas respectively.

IDH1 mutations are almost exclusively detected in gliomas. However, of 224 patients with AML in one study,[14] 21 (9%) had *IDH1* mutations, which occurred predominantly (13 of 80 patients) in otherwise cytogenetically normal cells.[14] No other major subtype of leukaemia (chronic-lymphoid leukaemia, acute-lymphoid leukaemia, and chronic-myeloid leukaemia) was associated with *IDH1* mutation (table 1). *IDH1* mutations do not seem to occur in any of the other tumour types examined, although sporadic mutations have been found in paraganglioma, prostate carcinomas, and in a colon cancer metastasis.[12,13,43]

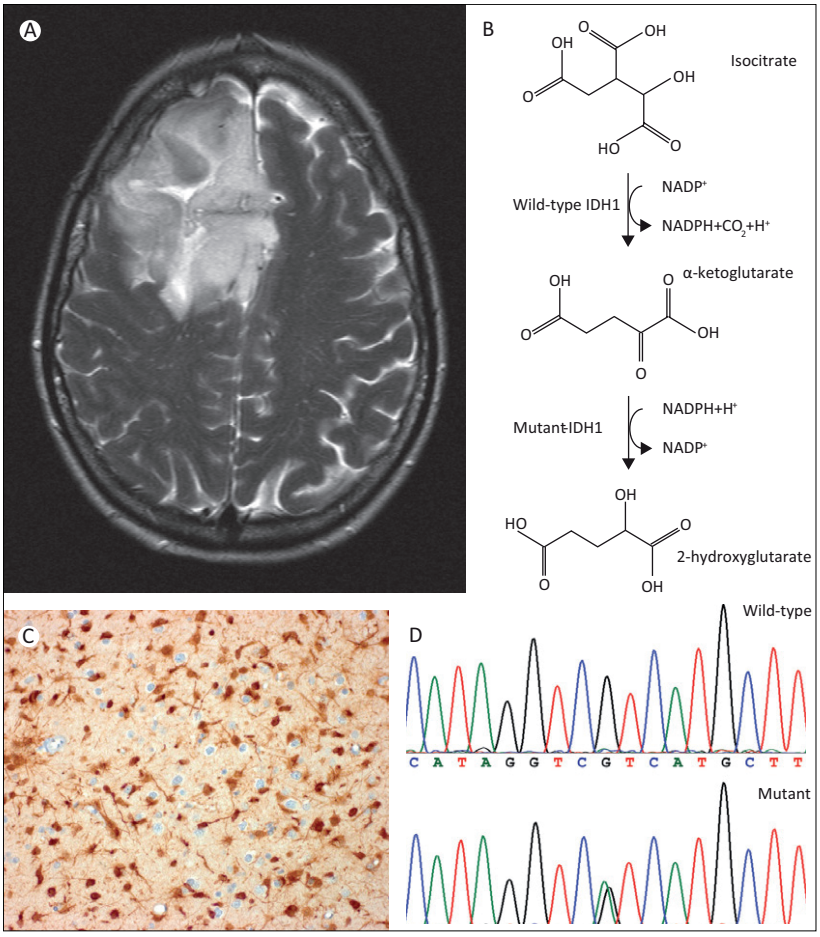

Figure 1: Isocitrate dehydrogenase 1 mutations in gliomas

An example of an MRI of a patient with a glial brain tumour (A). The enzymatic reactions catalysed by wild-type and mutant isocitrate dehydrogenase. Isocitrate is converted into α-ketoglutarate by wild-type isocitrate dehydrogenase 1 (IDH1). During the reaction, $NADP^+$ is converted into NADPH. Mutant IDH1 converts α-ketoglutarate and NADPH into 2-hydroxyglutarate and $NADP^+$(B). Immunohistochemistry on formalin-fi xed, paraffi n-embedded-glioma tissue using an antibody specific for R132H mutated IDH1. At the infi ltrating edge of the tumour into the cortex, only glioma cells stain positive[18,19] (C). A chromatogram of a tumour wild-type for IDH1 and a tumour that harbours a heterozygous mutation (395G→A) on position 395 of IDH1 (D).

Clinical implications

Histological diagnosis of gliomas is subject to substantial interobserver variation,[44] thus *IDH1* mutation status can provide an objective marker to identify the glioma subtype. *IDH1* mutations are rare in primary glioblastoma multiforme and are common in secondary glioblastoma multiforme; therefore, *IDH1* mutation status can distinguish between these two subtypes (sensitivity 71·0–73%, specificity 96%).[18] The low incidence of *IDH1* mutation in pilocytic astrocytomas also allows clinically relevant differentiation between diffuse and pilocytic astrocytomas (sensitivity 73–83%, specificity 100%).[18] Furthermore, *IDH1* mutations can have a role in distinguishing anaplastic astrocytomas from primary glioblastoma multiforme, or astrocytomas from ependymomas.[18] Several techniques have been developed to detect *IDH1* mutations (figure 1).[18,19,45,46] These techniques might aid diagnosis, even if samples contain little tumour tissue, and allow differentiation between tumour and reactive gliosis.

Extensive research has been done to determine the prognostic value of *IDH1* mutations (table 2). Patients with *IDH1* mutated glioblastoma multiforme have a better prognosis that those without *IDH1* mutations,[1] with respect to both overall survival[1,5,7,8,10,15,20–23] (figure 2) and progression-free survival.[7,20,22] Although patients with an IDH1 mutation are generally younger, and that age alone is an independent prognostic factor in gliomas, several studies have confirmed by multivariate analyses the independent prognostic value of IDH1 mutations.[7,10,15,20–22] The major prognostic importance of *IDH1* mutation status suggests that it should be used as a stratification factor in controlled clinical trials.

Two studies have examined whether *IDH1* mutation status can predict response to treatment in gliomas. In a group of patients with dedifferentiated low-grade astrocytomas progressive after radiotherapy response to temozolomide did not differ between *IDH1* mutant and wild-type tumours.[23] In patients with anaplasticoligodendroglial tumours treated with radiotherapy alone or radiotherapy with adjuvant PCV (procarbazine, lomustine, and vincristine), the presence of *IDH1* mutations had no predictive value for outcome to PCV.[22] Whether *IDH1* mutations have a predictive value in other subgroups or treatment regimens needs to be investigated; at present *IDH1* mutations seem to be mostly prognostic.

Are IDH1 and IDH2 mutations causal?

Despite the high mutation frequency, whether *IDH1* mutations cause disease is unknown. Several observations suggest a causal role for IDH1 and IDH2 in tumorigenesis. The prevalence and nature of somatically acquired mutations does not suggest a bystander mutation.[47] Moreover, *IDH1* and *IDH2* mutations rarely occur together.[6] Mutant IDH1 causes an accumulation of 2-hydroxyglutarate, high concentrations of which are

Table 2: Prognostic value of IDH1 and IDH2 mutations for progression-free survival and overall survival, according to tumour type

	n	HR (mutant *vs* wildtype)	HR (mutant *vs* PFS wildtype)	Median PFS OS (months)	Median OS (months)
Grade II–IV gliomas					
Multivariate[7]*	397	0·4	0·3	..	..
GBM					
Multivariate[10]†	407	..	..	..	11·3 *vs* 27·1
Multivariate[20]‡	301	0·4	0·4	6·5 *vs* 16·2	11·2 *vs* 30·2 (trend)
Multivariate[15]§	159	..	0·1	..	8·4 *vs* 24.0
Univariate[7]	183	..	..	8·8 *vs* 55	14·0 *vs* 27·4
Univariate[5]	129§§	..	..	..	15·0 *vs* 31.0
Univariate[1]¶	105§§	..	..	..	13·2 *vs* 45·6
Grade III gliomas					
Multivariate[21]||	318	0·4	0·5	..	..
Univariate[7]**	121	..	..	9·2 *vs* 37·0	19·4 *vs* 81·1
Anaplastic astrocytoma					
Univariate[5]	52	..	..	..	20·0 *vs* 65·0
Univariate[8]	21	..	..	..	22·0 *vs* 50·0
Anaplastic oligodendroglioma and anaplastic oligoastrocytoma					
Multivariate[22]††	159	0·3	0·2	7·8 *vs* 50·0	16·0 *vs* not reached
Grade II gliomas					
Univariate[7]	100	..	..	..	60·1 *vs* 150·9
Progressive low-grade astrocytoma					
Univariate[23]‡‡	49	..	..	..	48·0 *vs* 98·0

HR=hazard ratio. PFS=progression free survival. OS=overall survival. GBM=glioblastoma multiforme. KPS=Karnofsky performance score. *Tumour grade, age, *MGMT* methylation status, genomic profile (1p and 19q co-deletion, epidermal growth factor receptor [EGFR] amplification) and treatment were included in the multivariate analysis. †Age and gender were included in the multivariate analysis. ‡*MGMT* methylation status, age, KPS, extent of resection, and adjuvant therapy were included. §Molecular cluster, KPS, age, sex, extent of surgery, chemotherapy, epidermal growth factor receptor amplification, and 1p and 19q loss were included. ¶This was also significant in *TP53* mutated group younger than 35 years. ||Age, KPS, mini-mental status, resection, histology, *MGMT* methylation status, 1p and 19q codeletion, and *IDH1 R132H* mutation were included. **Significant in the non-1p–19q codeleted and non-EGFR amplified group (n=80). ††Necrosis, frontal localisation, and 1p and 19q co-deletion were included. ‡‡Age, presenting clinical symptoms, and treatment were the same in the two groups. §§Parsons and colleagues[1] and Yan and colleagues[5] have a partly overlapping sample set.

associated with brain tumours.[16,48,49] Specific mutation types segregate into distinct tumour types;[6,41] *IDH1* mutations are early events in glioma development and have been reported to precede both the loss of 1p and 19q and the acquisition of *TP53* mutations.[3,4] Furthermore, the incidence of *IDH1* mutations is higher in grade II than in grade III gliomas.[7,41] Thus, mutation type and incidence, as well as the timing of the mutation suggests a causal role for IDH1 and IDH2 in tumours.

Mutations of a single codon

All mutations in *IDH1* and *IDH2* are somatic, missense, heterozygous, and affect codon 132 (*IDH1*) or codon 172 (*IDH2*). Moreover, both codons have analogous positions in the isozymes.[50–52] Most mutations are single base transition substitutions: 395G→A for *IDH1* (about 90%), and 515G→A for *IDH2* (about 60%). These mutations result in an arginine to histidine (R132H) substitution in IDH1 or an arginine to lysine (R172K) substitution in IDH2. The remaining *IDH1* mutations are, with the exception of R132V (1 of 1630, 0·1%), single nucleotide substitutions on neighbouring bases that result in a substitution of arginine with cysteine (77 of 1630, 4·7%), glycine (34 of 1630, 2·1%), serine (27 of 1630, 1·7%), or leucine (13 of 1630, 0·8%).[2–10]

Although more than 90% of all mutations in *IDH1* are R132H substitutions, several studies reported an increased prevalence of other mutation types in specific histological subtypes. In gliomas, R132C mutations seem to be much more common in astrocytomas (32 of 591, 5·4%) than in oligodendrogliomas (2 of 662, 0·3%).[6,41] Additionally, all five *IDH1*-mutated astrocytomas in patients with Li-Fraumeni syndrome (ie, patients with a germline *TP53* mutation), had R132C substitutions.[53] In AML and in sporadic mutations of non-gliomas, only 11 of 26 mutations were R132H; R132C comprise most of the remaining mutations.[12–14,43] Genetic and epigenetic differences might explain differences in the incidence of mutation types in various tumour types and subtypes. However, functional differences between R132H and non-R132H mutated *IDH1* might also explain the segregation of distinct mutation subtypes.

Because only R132 in *IDH1* and R172 in *IDH2* are mutated in cancer, they probably confer unique properties on the enzyme. Both codons are highly conserved and have analogous positions in the isozymes.[50–52] In IDH, R132 and R172 are needed for the electrostatic attraction of isocitrate (both α-carboxylates and β-carboxylates)[52] and the actual enzymatic reaction; arginine (ie, R132 and R172) lowers the pK of nearby aspartic acids, which accept the cleaved proton of the hydroxyl group (OH^-) before transfer to $NADP^+$. Under high pH conditions, the enzyme function is conserved by site directed mutagenesis of porcine R133 (corresponding to R172 in human IDH2).[52] R132 seems to be a structurally important residue when changing between the different conformational states of the enzyme. Substitution of histidine for arginine is likely to shift the equilibrium to the closed (ligand and $NADP^+$ bound) conformation.[16] Therefore, R132 and R172 are involved in both the substrate binding and the enzymatic reaction.

Altered enzymatic activity

Results of studies examining the functional consequences of IDH1 and IDH2 mutant proteins have shown a strong decrease in the isocitrate-dependent production of reduced NADPH production.[3,5,17] Such a decrease in enzymatic activity suggests that both *IDH1* and *IDH2* are tumour suppressor genes; however, mutant IDH1 has altered substrate specificity and is more compatible with an oncogenic function.[16]

A tumour suppressor function of wild-type IDH1 is possible because the mutant protein exerts a dominant negative effect on its activity. Because *IDH1* mutations are heterozygous, both wild-type and mutant IDH1 are expressed in a single cell.[14] When dimers are formed between a mutant and a wild-type protein (heterodimers), mutant IDH1 substantially reduces the activity of the wild-type protein.[17] Similar findings have been reported for IDH2.[54] However, many other mutations generated in *IDH1* or *IDH2*, which are scattered across the various domains of the IDH1 and IDH2 proteins, also strongly reduce enzymatic activity.[27,52,55–58] Therefore, if *IDH1* and *IDH2* do function as tumour suppressor genes, then this enzymatic activity is only susceptible to mutation in R132 and R172, respectively.

Two observations contradict a dominant negative effect for *IDH1.* First, overexpression of recombinant mutant IDH1 or IDH2 in a cell line with two normal copies of the isozymes does not seem to reduce isocitrate dehydrogenase activity.[3,5] Zhao and colleagues[17] showed that the dominant negative effect of mutant IDH1 is dependent on the isocitrate concentration,[17] but the isocitrate concentration in enzymatic activity measurements (using lysates of cells overexpressing recombinant mutant IDH1) were too high for such a dominant negative effect to be seen. Additional experiments on cell lysates with lower isocitrate concentrations are needed to confirm the dominant negative effect of mutant IDH1. Second, a dominant negative tumour-suppressor function is difficult to reconcile with the reported segregation of R132C mutations in tumour subtypes;[6,14,41] however differences in mutation frequency might be explained by genetic and epigenetic differences. Nevertheless, the possibility of a dominant negative function of mutant IDH1 or IDH2 in vivo requires further analysis.

An oncogenic role of IDH1 is most compatible with the reported range of mutations; hotspot mutations, with the exception of *TP53*, are commonly identified in oncogenes (eg, H-Ras[59], PIK3CA[60]). This oncogenic role of IDH1 could result from altered substrate specificity. Site directed mutagenesis of the *Esherichia coli* $NADP^+$dependent isocitrate dehydrogenase has shown that point mutations can result in altered specificity for this enzyme.[61,62] Mutant IDH1 uses α-ketoglutarate as a substrate to produce 2-hydroxyglutarate (figure 1).[16] Concentrations of 2-hydroxylglutarate increase to over 50 times that of normal concentrations in IDH1 mutated tumours.[16] Mutated IDH1 generates the (R)-enantiomer of 2-hydroxyglutarate (R-2HG). Accumulation of R-2HG is associated with

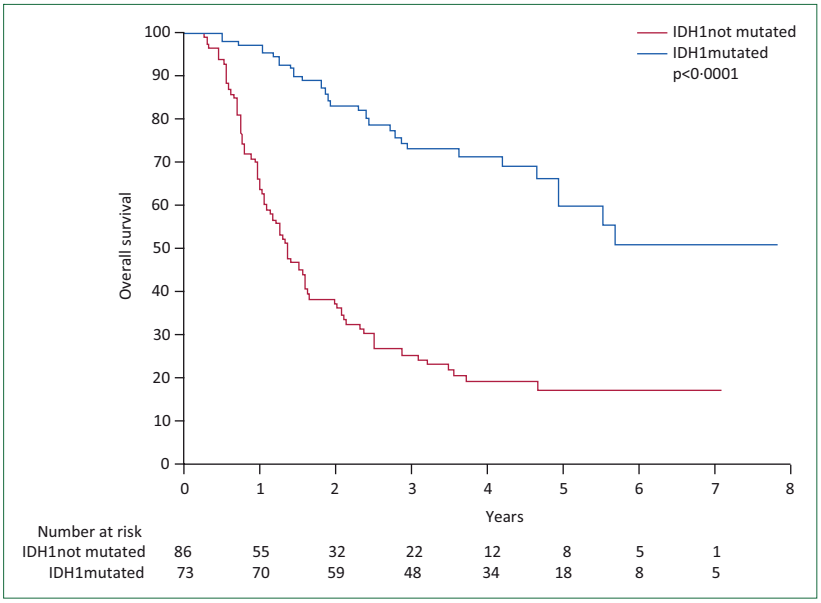

Figure 2: The prognostic value of isocitrate dehydrogenase 1 mutation status
Overall survival in 159 patients (univariate analysis) from a prospective randomised phase 3 study22 on procarbazine, lomustine, and vincristine chemotherapy for anaplastic oligodendroglioma (EORTC study 26951), in whom isocitrate dehydrogenase mutations could be determined.22 Observed events 70 in IDH1 not mutated, 26 events in IDH1 mutated.

D-2-hydroxyglutaric aciduria, a rare neurometabolic disorder with highly variable phenotypes, caused by a defect in D-2-hydroxyglutarate dehydrogenase. The S-enantiomer of 2-hydroxyglutarate (S-2HG) is dehydrated to α-ketoglutarate by the metabolic repair enzyme L-2-hydroxyglutarate dehydrogenase.[63] L-2hydroxyglutaric aciduria, a hereditary encephalopathy due to a defect in L-2-hydroxyglutarate dehydrogenase, is associated with different brain tumour types, some of which are not associated with *IDH1* mutations (ependymomas and medulloblastomas).[48,49] S-2HG is not a known intermediate in any mammalian pathway.[63] Moreover, S-2HG and R-2HG exert different biological effects.[64] Unlike L-2-hydroxyglutaric aciduria, D-2hydroxyglutaric aciduria is not associated with tumour formation. Thus, how an increase in R-2HG contributes to tumour formation needs to be investigated.

Cellular functions affected by mutant IDH1 and IDH2

Although IDH1 mediates a reaction identical to that which happens in the tricarboxylic-acid cycle, IDH1 does not play a direct part in the reaction because it is localised in the cytosol. Thus, IDH1 does not seem to mediate the Warburg effect (the observation that

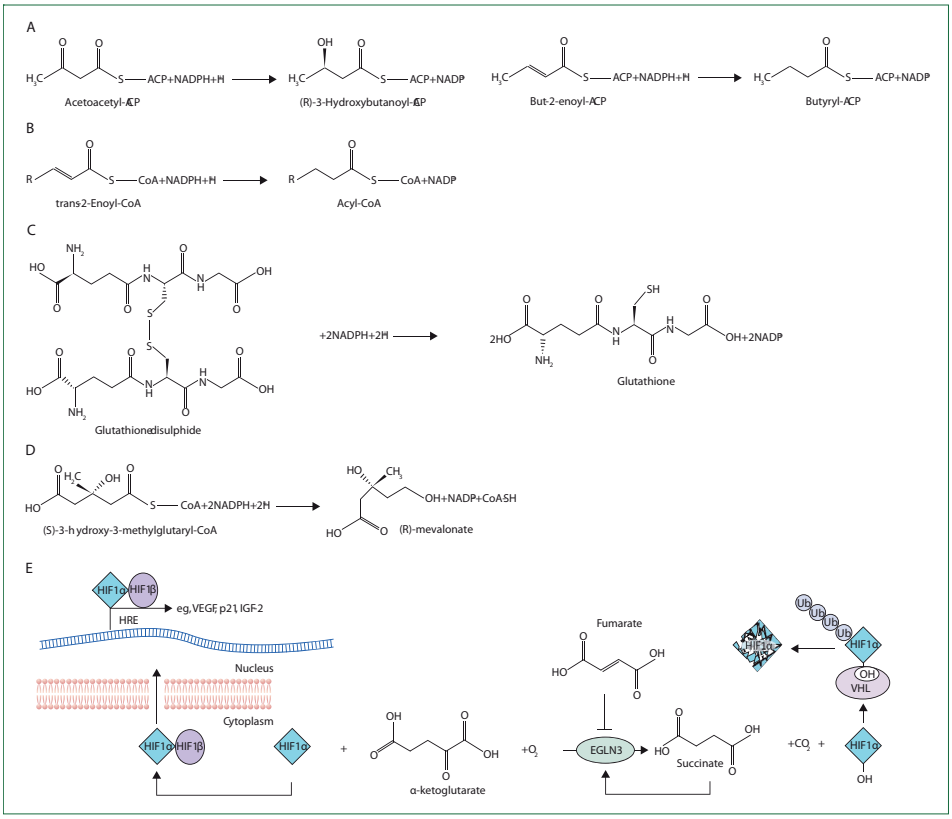

Figure 3: Pathways affected by products of isocitrate dehydrogenase 1

Products of isocitrate dehydrogenase 1 (IDH1) are used in various processes. NADPH is used as a cofactor in palmitate synthesis (A), fatty-acid chain elongation (B), and antioxidant formation (C). NADPH is also used as a cofactor in the rate limiting step ofcholesterol synthesis by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (D). Hypoxia-inducible factor 1α (HIF1α) degradation; under normoxic conditions HIF1α is hydroxylated by egl nine homologue 3 (EGLN3), in a reaction that requires α-ketoglutarate as a substrate. This reaction results in the generation of succinate and hydroxylated HIF1α, which allow the von Hippel-Lindau (VHL) tumour suppressor to bind HIF1α, resulting in the proteasomal degradation of HIF1α. The reaction catalysed by EGLN is inhibited by succinate and fumarate. Under hypoxic conditions the hydroxylation does not occur and allows HIF1α to form a complex with HIF1β. This complex binds to hypoxia-responsive elements (HRE) in the DNA to promote transcription of HIF responsive genes such as those encoding vascular endothelial growth factor (VEGF), p21, and insulin-like growth factor 2 (E; IGF2). ACP=acyl-carrier protein. Ub=ubiquitin.

even under normoxic conditions tumour cells preferentially use glycolysis over aerobic respiration).[65] Other tumour-associated mutations in metabolic enzymes have been described in hereditary phaeochromocytoma-paraganglioma syndrome, hereditary leio-

myomatosis, and renal-cell cancer. Hereditary phaeochromocytoma-paragangliomas have dysfunctional succinate-dehydrogenase complexes, and hereditary leiomyomatosis and renal-cell cancer have dysfunctional fumarate hydratase.[65,66] Because IDH1 is not part of the tricarboxylic-acid cycle, the *IDH1* mutations in gliomas are different from the inactivating mutations in the metabolic pathways of other tumour types.

Little is known about cytosolic IDH1 and its cellular function in tumours. Homozygous deletions are embryonically lethal in *Drosophila melanogaster*.[67] The biochemical role of IDH1 in α-ketoglutarate and NADPH production allow the cellular function of IDH1 to be inferred. Biochemical pathways might be affected by and fatty-acid biosynthetic pathways (figure 3).[68,69] In changes in cellular α-ketoglutarate and NADPH nucleic-acid synthesis, IDH1 competes with the pentose concentrations. The anabolic pathways of lipid synthesis, phosphate pathway for $NADP^+$ as an electron acceptor.[70] cholesterol synthesis, and fatty–acid-chain elongation Cytosolic IDH attenuates the glucose-induced increase depend on cytoplasmic NADPH. *IDH1* knock-in mice in pyruvate cycling in the tricarboxylic-acid cycle, show substantial changes in lipid metabolism perhaps by affecting the concentrations of organic acid,[71] (eg, hyperlipidaemia, fatty liver, and obesity). and is involved in glucose-stimulated insulin secretion Furthermore, IDH1 activity is regulated by cholesterol in pancreatic islet cells.[71] α-Ketoglutarate might affect

the activity of IDH isozymes by end product inhibition.[72] Finally, 2-hydroxyglutarate metabolism might be affected as mutant IDH1 converts α-ketoglutarate to 2-hydroxyglutarate.[16]

Several biochemical pathways might be affected by changes in α-ketoglutarate and NADPH concentrations (figure 3). Overexpression of *IDH1* results in increased protection against reactive-oxygen species as well as protection against radiation, whereas inhibition of *IDH1* results in increased sensitivity.[73–77] Similarly, overexpression of *IDH2* result in a decrease of intracellular reactiveoxygen species.[75] Although cellular response to oxidative stress might be altered in tumours with mutated IDH, this is not reflected in an increase in the number of mutations.[1,47,77] *IDH1* expression also changes in response to other kinds of stressors, such as protein excretion by *Helicobacter pylori* and heat.[78,79] IDH mutations might also play a part in the cellular migration and cellular response to hypoxia. For example, inhibition of cytosolic IDH1 has been reported to increase lactate production, which promotes migration of glioma cells.[71,80]

α-ketoglutarate promotes the degradation of hypoxiainducible factor (HIF) 1 α, through EGLN (egl nine homologue) prolyl hydroxylases (figure 3). Activation of the HIF pathway results in the induction of vasucular endothelial growth factor (VEGF).[81] However, the most vascularised gliomas (primary glioblastoma multiforme) have the lowest incidence of *IDH1* mutation among glioma. This finding suggests that decreased α-ketoglutarate concentrations might affect other HIF dependent (energy metabolism) or independent pathways (EGLN-related apoptosis).[17,65,81–83] Nonetheless, the HIF path-

way is likely to be affected in tumours with mutations in metabolic enzymes, such as in phaeochromocytomaparaganglioma syndrome.[84] By contrast with glioma, *IDH1* mutated AML does not show an increased expression of *HIF1*α downstream target genes.[14,17] Moreover, other biochemical and cellular pathways are affected by mutations in *IDH1* and *IDH2*, and the alternative products generated by mutated IDH1 or IDH2 might affect entirely different pathways. Further investigation is needed to establish whether IDH1 and IDH2 are functionally important to tumour formation and tumour progression.

Conclusion

The incidence of *IDH1* mutation is very high in most diffuse gliomas, but not in primary glioblastomas, and to a lesser extent in AML. Mutations in the homologous *IDH2* gene also occur albeit with a much lower incidence than *IDH1*. These mutations are early events in gliomagenesis, and arise both in *TP53* mutated astrocytomas and in 1p and 19q codeleted oligodendrogliomas. IDH1 is a strong prognostic marker for overall survival and progression-free survival in gliomas, suggesting that future clinical trials might need stratification by *IDH1* mutation status. Molecularly, *IDH1* and *IDH2* mutations are inversely correlated, heterozygous, affect a single codon, and result in different catalytic properties. Because of the high mutation frequency, mutated IDH1 could potentially form a good target for new treatments. IDH1 is expressed at high levels and seems to be expressed in all tumour cells. The three-dimensional structure of the native protein has also been identified. However, for effective pharmacological intervention, research into tumour-cell biology and biochemical defects associated with *IDH1* and *IDH2* mutations is needed. Further clinical research will identify the diagnostic relevance of these mutations.

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Chapter 3

Integrated Genomic Profiling Identifies Candidate Genes Implicated in Glioma-Genesis and a Novel *LEO1-SLC12A1* Fusion Gene

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Abstract

We performed genotyping and exon-level expression profiling on 21 glioblastomas (GBMs) and 19 oligodendrogliomas (ODs) to identify genes involved in glioma initiation and/or progression. Low-copy number amplifications ($2.5 < n < 7$) and high-copy number amplifications ($n > 7$) were more frequently observed in GBMs; ODs generally have more heterozygous deletions per tumor. Four high-copy amplicons were identified in more than one sample and resulted in overexpression of the known oncogenes *EGFR*, *MDM2*, and *CDK4*. In the fourth amplicon, *RBBP5*, a member of the RB pathway, may act as a novel oncogene in GBMs. Not all hCNAs contain known genes, which may suggest that other transcriptional and/or regulatory elements are the target for amplification. Regions with most frequent allelic loss, both in ODs and GBMs, resulted in a reduced expression of known tumor suppressor genes. We identified a homozygous deletion spanning the *Pragmin* gene in one sample, but direct sequencing of all coding exons in 20 other glioma samples failed to detect additional genetic changes. Finally, we screened for fusion genes by identifying aberrant 5'-3' expression of genes that lie over regions of a copy number change. A fusion gene between exon 11 of *LEO1* and exon 10 of *SLC12A1* was identified. Our data show that integrated genomic profiling can identify genes involved in tumor initiation, and/or progression and can be used as an approach to identify novel fusion genes.

Introduction

In the Western world, more than 40,000 people are diagnosed with a malignant primary brain tumor each year (CBTRUS 2004-2005 statistical report; www.cbtrus.org). Diffuse gliomas are the most common histological subtype in adults with an often dismal prognosis and invariably fatal outcome. Based on their histological appearance, gliomas can be divided into astrocytic tumors (75%), pure oligodendroglial tumors, and mixed oligoastrocytic tumors (25%) according to standard WHO classification (Kleihues and Cavenee, 2000). Glioblastomas (GBM) is the most malignant and most common of the astrocytic tumors and can be divided into those that have developed from lower grade gliomas (secondary or progressive GBM) and those that have formed de novo (primary or de novo GBM). Despite advances in neurosurgery, chemotherapy and radiotherapy, the prognosis for most glioma patients remains dismal. For example, the median survival for GBM is 9–12 months (Ohgaki and Kleihues, 2005). This dismal prognosis is reflected in the disproportionally high-mortality rate of gliomas compared to most other types of cancer. The treatment of these tumors therefore urgently requires improvement.

Genetic changes involved in glioma-genesis or progression are potential therapeutic targets, and a number of such changes have been identified in gliomas. In GBMs, causal genes include the EGFR, MDM2, CDK4, and MDM4 oncogenes and the PTEN and CDKN2A tumor suppressor genes (Louis, 2006; Cancer Genome Atlas Research 2008; Parsons et al., 2008). In lower grade oligodendrogliomas (ODs), very few genes have thus far been identified with the notable exception of the frequently mutated IDH1 gene (Balss et al., 2008; Parsons et al., 2008; Bleeker et al., 2009; Watanabe et al., 2009; Yan et al., 2009). Causal genetic changes therefore remain to be identified in the various histological subtypes of glioma.

Recent data on glial brain tumors have indicated that integrated genomic profiling (overlaying copy number aberrations [CNAs] onto gene expression profiles) can further narrow down on candidate causal genes (Bredel et al., 2005; Nigro et al., 2005; Liu et al., 2006; Ruano et al., 2006; Beroukhim et al., 2007; Lo et al., 2007; de Tayrac et al., 2009). However, integrated genomic profiling has mainly focused on GBMs and not on other histological subtypes. We therefore performed integrated genomic profiling both on GBMs and ODs using high-density SNP arrays and exon-level expression profiles.

Materials and methods

Tumor Samples

All glioma samples were derived from patients treated within the Erasmus MC. Patient data and histological diagnosis are listed in Supporting In-formation Table I. Samples were collected immediately after surgical resection, snap frozen, and stored at -80˚C as described (French et al., 2005, 2007). All samples were visually inspected on 5-lm H&E-stained frozen sections by the neuropathologist (J.M.K.) and revealed at least 70% tumor nuclei for each specimen. Tissue adjacent to the inspected sections was subsequently used for nucleic acid isolation. Forty glioma samples of the following histologies were selected for this study: 17 anaplastic oligodendrogliomas (ODIIIs), 2 low-grade oligodendrogliomas (ODIIs), and 21 GBMs. Four control brain samples from patients with no history of neurological disease were also included (ctrs).

Nucleic Acid Isolation, cDNA Synthesis, and Array Hybridization

High-quality genomic DNA was isolated from 20 to 40 cryostat sections of 40-lm thickness using the QIAmp DNA mini kit (Qiagen) according to the manufacturer's instruction. Sample labeling and array hybridization on 250k NspI arrays was performed using high-quality genomic DNA according to the Genechip Mapping 500k Assay Manual. A combination of four nondiseased brain samples run in addition to the tumor samples and six publicly available reference samples (www.affymetrix.com) were used as genotyping reference. Average SNP call rate was 93.6% (range, 87.9-97.4).

Total RNA was isolated from 20 to 40 (40-lm) cryostat sections adjacent to those used for copy number analysis using Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Exon-level expression profiles have been reported previously (French et al., 2007; Schutte et al., 2008).

Data Integration

Cel files from genotyping arrays were imported into Partek Genomics Suite 6.08 (Partek, St. Louis, Missouri). Baseline allele intensities were defined using all control samples. Regions with copy number variations (CNVs) were identified and exported to Excel. Genomic positions of genes were then overlayed onto regions of CNV. Genes were defined as being in a region of CNV when the regional CNV average was <0.80 for a homozygous deletion (homDel), between 0.8 and 1.65 for a heterozygous deletion (hetDel), between 2.5 and 7 for low-copy number amplification (lCNA), and >7 for high-copy amplification (hCNA). The lower limit of amplification detection (2.5) was chosen to allow for the presence of non-neoplastic tissue and to detect focal copy number gains. As can be expected, increasing the lower limit of detection from 2.5 to 2.7 or 2.9 will decrease

the number of detected regions (81% and 67%, respectively). However, the regions that have copy number changes between 2.5 and 2.9 are identified across the genome and not restricted to defined ''hotspots.'' Therefore, increasing the lower limit of detection would not affect the results (data not shown).

The ratio of 7.0 to distinguish between lowand high-copy number gain is based on Supporting Information Figure 2. This figure shows that large amplicons (>6 Mb) never exceed copy number gains of seven. Changing the cutoff value between hCNA and lCNA from 7.0 to 6.0 would result in the identification of seven additional hCNAs. However, all seven regions are unique and show no overlap with hCNAs of other tumors (data not shown)

The chromosomal location of genes on HuEx_1.0_st arrays was then used to determine whether a gene resided in a region of CNV. If more than one tumor showed CNV at the same locus/gene, the average expression level was taken. We also identified all genes that only partly overlapped the region with CNV. These genes were used to identify fusion genes as is outlined in the Results and Discussion section. Copy number changes were visualized using CNAG 3.0 software (Nannya et al., 2005; Yamamoto et al., 2007). All statistical analysis was performed using t tests, unless indicated otherwise.

5⁰ Rapid Amplification of cDNA End-Polymerized Chain Reaction

5' Rapid amplification of cDNA ends (RACE) polymerized chain reaction (PCR) was performed on cDNA of sample 96 using the Marathon RACE kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol (French et al., 2001). Primers used for 5' RACE and RT-PCR confirmation were designed using primer 3 (frodo.wi. mit.edu) and stated in Supporting Information Table II.

Results and discussion

CNAs in ODs and GBMs

We first screened for differences between ODs (grades II [n = 2] and III [n = 17]) and GBMs (n = 21) in copy number aberration (CNA) amplitude and frequency. In general, homozygous deletions were significantly smaller than heterozygous deletions ($P < 0.001$) as were hCNAs compared to lCNAs ($P < 0.001$, see also Supporting Information Figs. 1 and 2). It is possible that the smaller size of hCNAs are a result of structural (high-copy ampifications require more energy to maintain and therefore are selected against) and/or functional (to avoid coamplification of genes that have growth inhibitory effects) size constraints. lCNAs may also be larger, because more tumor-promoting genes reside on the region.

All tumors showed CNAs, although the distribution between different types of CNAs

varies between histological subtypes. For example, both lCNAs and hCNAs were more frequently observed in GBMs than ODs; 21 of 21 versus 15 of 19 for lCNAs $P < 0.05$ and 16 of 21 versus 1 of 19 for hCNAs, $P < 0.001$, Fisher's exact test. Homozygous and heterozygous deletions were observed at roughly equal frequency in GBMs and ODs; homDels were observed in 7 of 21 GBMs and 4 of 19 ODs, hetDels in 20 of 21 GBMs and 19 of 19 ODs.

Apart from the number of ODs and GBMs that exhibit copy number changes, the average number of CNAs per tumor also differs between the histological subtypes (Supporting Information Fig. 3). For example, lCNAs and hCNAs were more prevalent in GBMs (7.0 ± 8.2 vs. 2.7 ± 3.2 $P < 0.05$ and 2.4 ± 3.1 vs. 0.2 ± 0.9, $P < 0.01$, respectively), and hetDels were more prevalent in ODs (3.3 ± 4.2 vs. 5.8 ± 3.1 $P < 0.05$). Our data thus indicate that histological subtypes differ in CNA type (more GBMs have copy number gains than ODs) as well as copy number frequency (copy number gains are more frequent in GBMs than ODs, whereas hetDels are more frequent in ODs).

CNA Amplitude, Frequency, and Gene Expression

All regions of CNA were overlaid onto the chromosomal location of genes present on the HuEx_1.0_st arrays. This gene-centric overlay allows direct comparison with the genes' expression level (see below). Fold changes in copy number gains for each histological subtype is shown in Figure 1A. Many regions show lCNAs, whereas four distinct regions with hCNAs were identified [residing on chromosomes 1, 7, and 12 (2x)]. The frequency of copy number gains is shown in Figures 1B and 1C for hCNAs and lCNAs, respectively. The frequency of deletions is shown in Figure 1D. Regions frequently amplified include chromosomes 1 and 7 in GBM and 7q in ODs. Regions with genomic loss observed include chromosomes/chromosome arms 9p, 10, 13, and 14 in GBMs and 1p, 4, 9p, and 19q in ODs. We then examined whether genes that reside on regions of chromosomal gain have a different expression level in amplified versus nonamplified samples. In general, hCNAs are associated with a markedly higher expression level in amplified versus nonamplified samples (Fig. 1E, Table I), whereas genes that reside in lCNA regions show more modest differential expression (Fig. 1F). Known oncogenes EGFR, CDK4, and MDM2 all show a greater than sixfold increase in gene expression level. Approximately one-third of all genes that reside in regions of lCNA show an increase in gene expression level >20% (both in GBMs and in ODs). Similarly, in regions of deletion, approximately one-third of all genes show a reduction in expression level >20%. Known tumor suppressor genes PTEN and CDKN2A show a >50% reduction in expression levels (GEO accession number GSE 9385, analyzed data available on request).

It should be noted that not all regions of CNA harbor genes. In 32 regions with CNA (16 amplifications and 16 deletions), genes were absent on the HuEx_1.0_st arrays. Ten

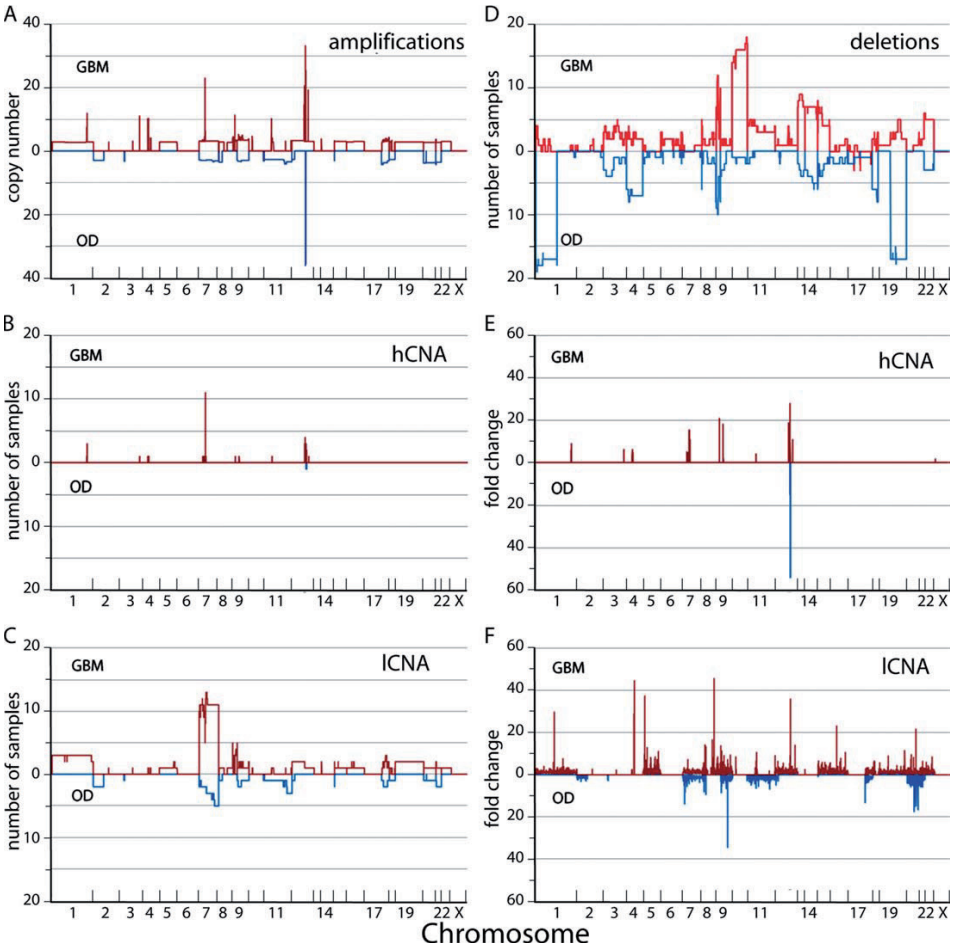

Figure 1. Integrated genomic profiling in GBMs and ODs. A: Fold changes in copy number gains in GBMs (red) and ODs (blue). Four distinct regions of high-copy number amplifications are identified on chromosomes 1, 7, and 12 (23), whereas many regions show lowcopy number gains. The frequency of high-copy number gains (B), low-copy number gains (C), and deletions (D) plotted at each chromosomal position. Regions frequently amplified in GBMs include chromosomes/chromosomal arms 1, and 7 in GBM and 7q in ODs.

of these regions are in high-copy number amplicons. In four of these hCNA regions, we observed clear overexpression of several probesets that lie outside the annotated genes (''full'' probesets, Supporting information Fig. 4). This suggests that copy number-dependent, transcriptionally active regions reside on at least a subset of these CNAs, and it is possible that such regions contribute to tumor formation.

Table 1. Genes on Frequent hCNAs Often Show a Strong Increase in Expression

INTEGRATED GENOMIC PROFILING IN GLIOMAS

Chr 1	*n*	Fc	Chr 12	*n*	Fc	Chr 12	*n*	Fc	Chr 7	*n*	Fc
LRRN2	3	4.9	*GLI1*	3	6.2	*MDM2*	4	12.1	*VSTM2A*	10	2.6
NFASC	3	5.2	*ARHGAP9*	3	1.4	*CPM*	4	10.5	*SEC61G*	11	14.7
CNTN2	3	1.7	*MARS*	3	9.8	*FRS2*	3	5.1	*EGFR*	15	6.9
TMEM81	3	4.9	*CENTG1*	4	4.2	*CCT2*	3	8.4	*LANCL2*	8	5.9
RBBP5	3	8.9	*TSPAN31*	4	9.8	*LRRC10*	3	1.5	*ECOP*	7	2.4
RIPK5	3	2.4	*CDK4*	4	11.4	*BEST3*	3	2.8			
			Mar-09	4	15.7	*RAB3IP*	3	1.2			
			CYP27B1	4	9.0						
			METTL1	4	21.3						
			FAM119B	4	15.0						
			TSFM	4	9.6						
			AVIL	4	9.2						
			XRCC6BP1	3	20.4						

Fc, fold change amplified versus nonamplified; *n*, number of samples showing hCNA.

Regions with genomic loss observed include chromosomes/chromosomal 9p, 10, 13, and 14 in GBMs and 1p, 19q, 4, and 9p in ODs. E: Genes that reside on regions of hCNA show markedly higher expression levels in amplified versus nonamplified samples whereas genes that reside on regions of lCNA (F) show more modest differential expression levels. Not all chromosomes are labeled on the *X* axis due to space constraints. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Integrated Genomic Profiling Identifies RBBP5 as a Candidate Oncogene in GBM

We next screened for candidate oncogenes based on our genomic and transcriptomic data. Genes that are involved in gliomagenesis and/or tumor progression often reside on frequently amplified hCNAs and show strong mRNA overexpression. Examples include the EGFR, CDK4, and MDM2 oncogenes (Bredel et al., 2005; Nigro et al., 2005; Louis, 2006; Ruano et al., 2006; Cancer Genome Atlas Research, 2008; Parsons et al., 2008). We therefore identified all genes that reside on recurrent hCNAs. Most of the genes on these amplicons indeed show strong overexpression (Table I). The candidates include the oncogenes EGFR, CDK4, and MDM2. However, the amplicon in chromosome band 1q32 does not contain any of the known glioma oncogenes. It has been suggested that the MDM4 gene is the main amplification target in this amplicon (Riemenschneider et al., 2003) although it is not always coamplified in gliomas with hCNA of 1q32 [this

study and see, e.g., (Rickman et al., 2001)]. Because single amplicons may contain several causal genes (Holtkamp et al., 2007) and the observation that MDM4 is not always coamplified, the 1q32 amplicon may contain additional oncogenes.

RBBP5 is one of the genes that reside on the 1q32 amplicon and shows strong differential expression. RBBP5 was identified as an RB-binding protein (Saijo et al., 1995) and members of the RB pathway are required for G1 ! S-phase transition (Sherr, 1993; Medema et al., 1995; Yamanaka et al., 2008). Our data indicate that RBBP5 amplification (3 of 21) appears to be mutually exclusive from CDK4 amplification (4 of 21), another member of the RB pathway. Similarly, in both the Rembrandt (Madhavan et al., 2009) and Cancer genome (Cancer Genome Atlas Research, 2008; Freire et al., 2008) datasets, overexpression of RBBP5 (3 of 139 and 5 of 236, respectively) does not occur in samples with CDK4 overexpression (21 of 129 and 32 of 236). These data may indicate that RBBP5 is a novel oncogene in GBM although the frequency of amplification is too low to reach statistical significance.

Candidate Tumor Suppressor Genes

Tumor suppressor genes commonly reside in regions with (bi-) allelic loss within the minimal region of overlap. As a result, these genes show a reduction in expression levels. We therefore screened for tumor suppressor genes in our dataset by screening for regions of homozygous chromosomal loss. Genes within these regions include established tumor suppressor genes CDKN2A and PTEN. Few other regions with homozygous deletions were observed. However, in GBM142, a small homozygous deletion was identified that spans the entire Pragmin gene (Fig. 2A). No other genes were present in this region. As a result, the gene is expressed at lower levels on HuEx_1.0_st arrays (more than twofold reduction, Fig. 2B) and on HU133 plus 2.0 arrays [more than fourfold reduction (Gravendeel et al., 2009)].

We were unable to determine whether the Pragmin deletion is somatic as no control DNA was available for this patient. It is therefore possible that the genomic deletion represents a rare structural variant. However, such variants have not been identified in other control samples (see projects.tcag.ca/variation). Thus far, only one intraintronic deletion that is unlikely to disrupt the Pragmin open reading frame has been identified. Based on the reduced expression and single homozygous deletion, we subjected Pragmin to further mutational analysis.

We screened for genetic changes in the Pragmin gene by direct sequencing of all coding exons in an additional 10 GBMs and 10 ODs. No mutations were found in any of the coding exons. In addition, we screened for genomic changes in the Pragmin gene in a large external dataset (Cancer Genome Atlas Research, 2008; Freire et al., 2008). In this dataset, no tumors were identified with genomic changes in the Pragmin gene (data not shown). We also screened for aberrant

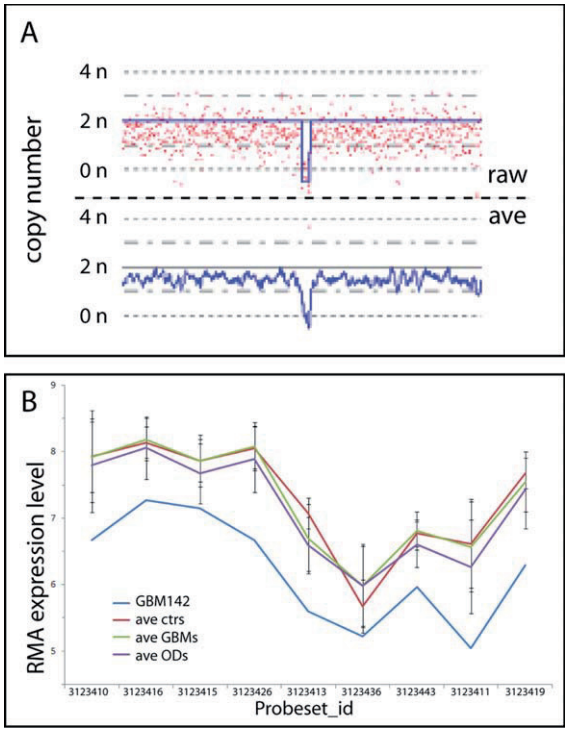

Figure 2. Identification of *Pragmin* as a candidate tumor suppressor gene. A: In GBM142, a homozygous deletion was identified on 8p23.1 (genomic position 8.2–8.3 Mb) that spanned the entire *Pragmin* gene. No other gene resides in the deleted region. B: Pragmin expression is markedly reduced following deletion in GBM142. Values are ±SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Pragmin expression in three large external datasets: GSE16011 (Gravendeel et al., 2009), GSE7696 (Murat et al., 2008), and the Rembrandt dataset (caintegrator.nci.nih.gov/Rembrandt). Only three samples showed a more than fourfold-reduced Pragmin expression: Samples GBM142 (the identical sample as used for this study) and GBM432 in GSE16011 and E08021 in the Rembrandt dataset (Supporting Information Fig. 5). Whether the reduced Pragmin expression in these samples is due to a genomic change remains to be determined.

Pragmin has thus far not been implicated in glioma initiation and/or progression. Functionally, Pragmin affects actin cytoskeletal rearrangement and cell contraction through RhoA activation (Tanaka et al., 2006). The cytoskeletal rearrangements render it is possible that Pragmin plays a role in the motility of gliomas cells. However, the low frequency of Pragmin mutations suggests that this gene is not often involved in glioma initiation and/or progression.

Integrated Genomic Analysis Identifies Translocations

A genomic breakpoint occurs at each site of a copy number change. Such breakpoints are a potential source of fusion genes when the coding region of one gene is inserted into the transcribed region of a different gene. We therefore hypothesized that screening for genes that reside over chromosomal breakpoints may identify fusion genes. In our

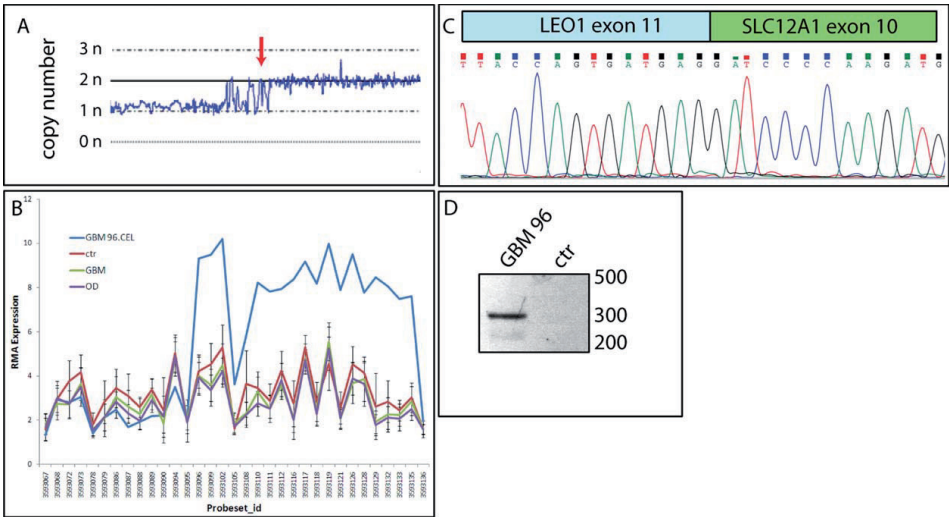

Figure 3. Identification of a LEO1-SLC12A1 fusion gene. A: In GBM96 on chromosome 15q21.1 (genomic location 46.3 Mb), the 5' end of SLC12A*1* was identified to lie in a region of chromosomal loss whereas the 3' end does not. The arrow denotes the location of *SLC12A1*.B: SLC12A*1* in GBM96 shows high-3' expression, whereas the 5' end is not expressed. The region in which 3' expression occurs matches the change in copy number on SNP arrays. SLC12A*1* is not expressed in any of the other samples; values are ±SD. C: 5^{0} RACE PCR identified an in-frame fusion gene between exon 11 of LEO1 and exon 10 of SLC12A1. D: The *LEO1-SLC12A1* fusion gene is confirmed by an independent RT-PCR reaction. None of the other tumor samples investigated showed a similar translocation (data not shown). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

dataset, 244 genes reside over changes in copy number (26, 82, and 136 for hCNA, lCNA, and deletions, respectively). We then focused our analysis on those genes of which the 5' end resides in a deleted region; high expression of the 3'-end would suggest that the 3' end is driven either by a promoter within the same gene (alternative promoter) or by a promoter from a different gene (fusion gene).

Forty-three candidates have a deletion of the 5' end of the gene. We then visually inspected all candidates for differential expression between the 3' and 5' ends of the transcript. In one of the candidates (SLC12A1/BSC1/NKCC2), a high-3' expression was observed, whereas the 5' end was not expressed. The region in which 3' expression occurs matches the change in copy number on SNP arrays (Figs. 3A and 3B). 5' RACE-PCR was performed using primers located in the expressed region and identified that LEO1 was fused at the 5' end of the SLC12A1 gene (Fig. 3C). The breakpoints occurred at intron–exon boundaries and resulted in an in-frame fusion between exon 11 of LEO1 and exon 10 of SLC12A1. The fusion gene was confirmed by three independent RTPCR reactions

(Fig. 3D and data not shown). In our dataset, no other tumor appeared to have a similar translocation (data not shown).

To determine whether the LEO1/SLC12A1 fusion is a recurrent genetic aberration in gliomas, we screened for genomic changes in a large external dataset (Cancer Genome Atlas Research, 2008; Freire et al., 2008). No tumors were detected that have chromosomal aberrations in SLC12A1 and/or LEO1 in this dataset (data not shown). In addition, as SLC12A1 expression is restricted to adult kidney (Supporting Information Fig. 6), we screened for aberrantly high-SLC12A1 expression in four large external datasets: GSE16011 (Gravendeel et al., 2009), GSE7696 (Murat et al., 2008), and the Rembrandt and TCGA datasets (Supporting Information Fig. 5). Of the 869 glioma samples analyzed, two samples show a strong increase in SLC12A1 expression:

GBM62 in GSE16011 (identical to the sample used in this study) and GSM187196 in GSE7696. A further two samples show a more modest expression: Grade II astrocytoma 134 in GSE16011 and HF1708 in the Rembrandt dataset. It remains to be determined whether the increased SLC12A1 expression in these samples is due to genomic changes.

SLC12A1 is a Na–K–Cl cotransporter that mediates active reabsorption of sodium-chloride in the thick ascending limb of the loop of Henle in the kidney (Quaggin et al., 1995). It is interesting to note that the coiled-coil dimerization domain of SLC12A1 is preserved in the fusion protein. Such dimerization domains have been observed in several oncogenic fusion proteins (Houtman et al., 2007; Soda et al., 2007) and can cause a constitutive activation of the fusion partners. LEO1 is part of the PAF1 protein complex that associates with the RNA polymerase II subunit POLR2A (Rozenblatt-Rosen et al., 2005). LEO1 interacts with b-catenin in the Wnt pathway (Mosimann et al., 2006; Chaudhary et al., 2007), a known oncogenic pathway in colon carcinomas. Although the functional consequences of the LEO1-SLC12A1 fusion gene remains to be determined, it is interesting to note that delayed senescence, increased proliferation, and life span extension is observed in cells expressing LEO1, lacking the COOH-terminal domain (Zhao et al., 2005). Interestingly, a LEO1 COOH-terminal truncation is one of the consequences of the LEO1-SLC12A1 fusion product. However, the observation that no additional samples were identified with genomic changes in SLC12A1 or LEO1 and that only few samples show aberrant SLC12A1 expression indicates that the LEO1/SLC12A1 fusion gene is not a recurrent event in gliomas.

In summary, we have performed integrated genomic profiling on 21 GBMs and 19 ODs. We have identified RBBP5, a member of the RB pathway, as a novel candidate oncogene in GBMs. In addition, by screening for aberrant exon expression levels in genes that reside over chromosomal breakpoints, we identified a fusion gene between LEO1 and SLC12A1. Our data therefore show that integrated genomic profiling can identify novel genes involved in tumor initiation and/or progression and can be used as an approach to identify novel fusion genes.

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Chapter 4

The CASPR2 cell adhesion molecule functions as a tumor suppressor gene in glioma

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Abstract

Genomic translocations have been implicated in cancer. In this study, we performed a screen for genetic translocations in gliomas based on exon-level expression profiles. We identified a translocation in the contactinassociated protein-like 2 *(CASPR2)* gene, encoding a cell adhesion molecule. *CASPR2* mRNA was fused to an expressed sequence tag that likely is part of the nuclear receptor coactivator 1 gene. Despite high mRNA expression levels, no CASPR2 fusion protein was detected. In a set of 25 glioblastomas and 22 oligodendrogliomas, mutation analysis identified two additional samples with genetic alterations in the *CASPR2* gene and all three identified genetic alterations are likely to reduce CASPR2 protein expression levels. Methylation of the *CASPR2* gene was also observed in gliomas and glioma cell lines. CASPR2-overexpressing cells showed decreased proliferation rates, likely because of an increase in apoptosis. Moreover, high *CASPR2* mRNA expression level is positively correlated with survival and is an independent prognostic factor. These results indicate that *CASPR2* acts as a tumor suppressor gene in glioma.

Introduction

Gliomas are the most frequently diagnosed type of primary brain tumor in adults, with an incidence of E3.5/100 000 (http://www.cbtrus.org). Depending on the cell type they resemble, gliomas can be subdivided histologically into astrocytomas (As), oligodendrogliomas (ODs) and oligoastrocytomas (OAs), the latter exhibiting features of both astrocytes and ODs. Gliomas can be further subdivided into various grades of malignancy (II–IV), with grade IV tumors (glioblastomas (GBMs)) being the most common and malignant. GBMs can occur *de novo* (primary GBM) or evolve from lower-grade gliomas (secondary GBM). Despite extensive therapy (a combination of surgery, radiation and/or chemotherapy), all grade II–IV gliomas are eventually fatal, with a median survival time ranging from over 10 years (grade II ODs) to less than 1 year in GBM (Ohgaki and Kleihues, 2005; Louis *et al.*, 2007).

Causal genetic changes in cancer cells are direct targets for treatment. In GBM, the most frequently affected genes include *CDKN2A* (50%), *EGFR* (37%), *TP53* (40%) and *PTEN* (30%) (Parsons *et al.*, 2008). In lower-grade gliomas, frequent alterations are a loss of heterozygosity on 1p and 19q (Kraus *et al.*, 1995), and the recently discovered *IDH1* mutations (Balss *et al.*, 2008; Gravendeel *et al.*, 2009; Yan *et al.*, 2009). Although frequently mutated causal genes have been found, treatments aimed at the molecular changes have thus far had limited success in glioma. To provide new targets for therapy, tumorigenesis has to be understood at the molecular level, and new causal genes and pathways have to be identified.

Genomic translocations that result in the expression of a fusion gene have been implicated in cancer. As such translocations often show aberrant expression of the fusion partners, screening for aberrant expression can identify novel fusion genes (Tomlins *et al.*, 2005). Exonlevel expression profiling is a method that can identify differentially expressed splice variants (Clark *et al.*, 2007; French *et al.*, 2007). We have recently shown that genomic mutations in cancer that lead to skipping of one or more exons are also detected by such arrays (Schutte *et al.*, 2008). Aberrant expression of exons also occurs when translocations lead to a gene fusion and exons from one gene are driven by a promoter from another gene. We therefore screened for such translocations based on exon array data.

Our screen identified the contactin-associated proteinlike 2 (*CASPR2*) gene, encoding a cell adhesion molecule, as the 3'fusion partner of a translocation. In a set of 25 GBMs and 22 ODs, we additionally identified one intragenic deletion and one larger deletion that includes the entire *CASPR2* gene. All three identified genetic alterations are likely to reduce CASPR2 protein expression levels. *CASPR2* methylation was also observed in gliomas and glioma cell lines. Interestingly, a significant decrease in proliferation rate was observed in cells overexpressing CASPR2-green fluorescence protein (GFP). In addi-

tion, high *CASPR2* expression is associated with longer survival, and multivariate analysis indicated that CASPR2 mRNA expression level is an independent prognostic factor. The genetic alterations identified, the reduced proliferation rate of CASPR2-GFP-expressing cells and the positive correlation between CASPR2 expression and survival indicate that CASPR2 acts as a tumor suppressor gene in glioma.

Results

Identifying a novel translocation involving EST AI364529 and CASPR2

We have recently demonstrated that exon-level profiling can detect exon-skipping mutants and splice variants (French *et al.*, 2007; Schutte *et al.*, 2008). In this study, we used these expression profiles to screen for fusion transcripts. We first identified transcripts with aberrant expression ratios between 5' and 3' exons in 25 GBMs and 22 ODs (see Materials and methods). Eleven genes were found in which the 5'/3' ratios differed 416-fold from the mean (see for example Figure 1a and Supplementary Table 3). Visual inspection reduced the number of candidates by eliminating those with, for example, one aberrant probeset expression or those with a highly skewed 5'/3'expression ratio in all samples. In several of the identified candidates, the altered 5' expression was likely caused by transcripts derived from an alternative promoter (data not shown). Transcripts driven from an alternative promoter will also have an altered 5'/3' expression ratio, and the identification of such transcripts validates our approach.

In GBM142, 5' RACE-PCR identified a candidate fusion gene between exon 9 of *CASPR2* and an expressed sequence tag (EST AI364529) located on chromosome 2. The EST AI364529–*CASPR2* fusion gene was confirmed by reverse transcriptase–PCR using a forward primer in EST AI364529 and a reverse primer in *CASPR2* (Figure 1b). EST AI364529 is located upstream of the nuclear receptor coactivator 1 (*NCOA1*) gene. Sequence alignment suggests that, similar to mouse Ncoa1, this EST forms part of the human *NCOA1* gene (Figure 1c). The translocation does not appear to affect expression of the *NCOA1* gene in GBM142 (Figure 1d). Apparently, the remaining allele is sufficient to drive normal NCOA1 expression levels. The observation that NCOA1 expression is not altered led us to focus our further experiments on the *CASPR2* gene.

Genomic translocations in cancer can result in expression of fusion proteins that act as oncogenes (Heisterkamp *et al.*, 1985; Tomlins *et al.*, 2005). However, western blot failed to detect expression of the AI364529–CASPR2 fusion protein (Figure 2). Thus, in spite of high transcript expression levels (Figure 1a); no protein product appears to be expressed. The absence of protein expression may be explained by the absence of a clear translation initiation codon with a consensus Kozak sequence in the fusion transcript. The absence of an EST AI364529–CASPR2 protein expression in GBM142 suggests that the fusion protein does not act as an oncogene.

Mutation screen in CASPR2

Causative genetic changes in cancer often have a significant mutation frequency. We therefore screened for additional mutations in the *CASPR2* gene. We first determined whether the specific EST AI364529– *CASPR2* translocation occurs in other tumor samples. Using a forward primer in EST AI364529 and a reverse primer in *CASPR2*, reverse transcriptase–PCR failed to identify this specific translocation in 25 GBMs (Figure 3a). We then analyzed all exon-expression profiles in detail (25 GBMs and 22 ODs) to identify other candidate breakpoints within the *CASPR2* gene. Such candidates may have escaped detection in our initial screen because of focusing on top candidates. In another sample (GBM96), the 5'/3' exon expression also shows an aberrant ratio (Figure 3b). However, such profile can be expected when *CASPR2* expression is derived from the alternative promoter present between exons 21 and 22 (CR933671). CASPR2 protein expressed from this alternative promoter contains only the last three coding exons and, on the protein level, lacks the extracellular domain. This isoform of CASPR2 apparently does not function as a cell adhesion molecule and may compete with expression of full-length CASPR2 (for protein expression in GBM96, see Figure 2b).

We then performed mutational analysis of all 24 coding exons of *CASPR2* in 23 GBMs and 18 ODs (Supplementary Table 4). One heterozygous missense mutation in GBM139 (73 G-A) was identified (Figure 3c). This base change causes an alanine amino acid to be replaced by a threonine (A24T). The mutation lies in exon 1, within the conserved region coding for the signaling peptide of CASPR2. The base change is not present in single-nucleotide polymorphism databases, but was present in DNA isolated from the patients' blood sample, indicating that the mutation is not somatic. Western blot detected very low protein expression in GBM139 (Figure 2b).

We next screened for changes in CASPR2 copy number in the same sample cohort. Copy number changes were inferred from genotyping arrays (Bralten *et al.*, 2010). As reported previously (Bredel *et al.*, 2005; Freire *et al.*, 2008), trisomy of the entire chromosome 7 was frequently observed in gliomas. However, in GBM254, genotyping identified a heterozygous deletion of a large region including the entire *CASPR2* gene (chr7:102106439–end, Figure 3d). Interestingly, in OD53, genotyping also identified an intragenic deletion (chr7:146208141–147249840, Figure 3d) that spans exons 4–14 and will result in a premature termination codon in exon 15. No mutations were found in the coding exons of the remaining *CASPR2* allele. However, both GBM254 and OD53 had low CASPR2 mRNA expression (Supplementary Figures 1 and 2) and no CASPR2 protein expression (Figure 2c).

As intronic *CASPR2* deletions have been associated with schizophrenia and epilepsy (Friedman *et al.*, 2008), intronic deletions may affect CASPR2 function. We therefore screened for small intronic deletions in four samples (in which no base change or large

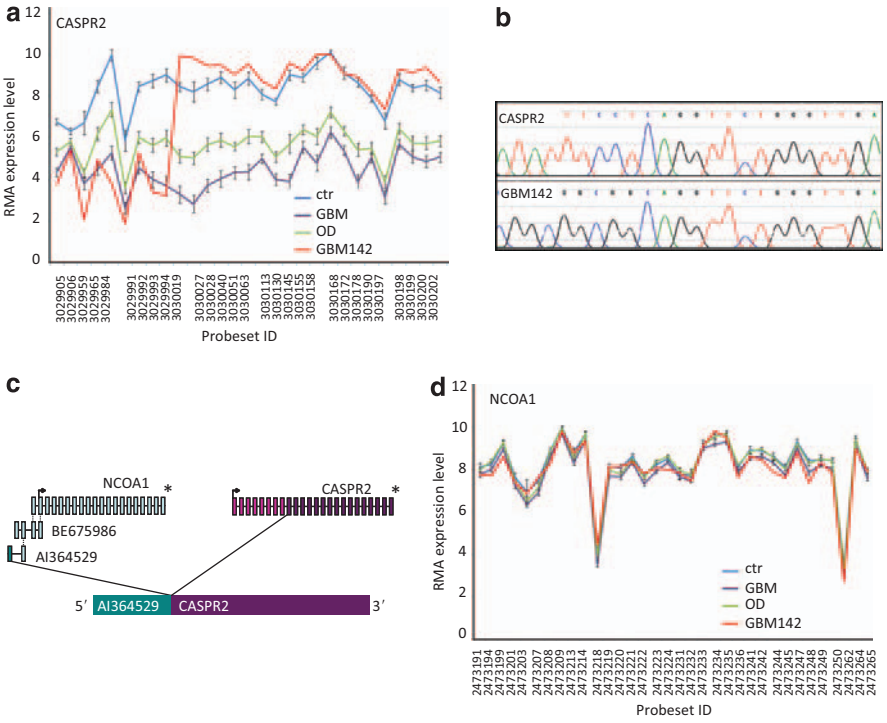

Figure 1 Identification of an EST AI364529–CASPR2 translocation. (a) The RMA expression levels (y axis) of all probesets of the CASPR2 gene (x axis) in GBM142 (red line) and mean CASPR2 expression of controls (ctr; n =6, blue), glioblastomas (n =24, purple) and oligodendrogliomas (n =22, green). In GBM142, the expression of exons 1–8 is B100-fold lower than the expression of exons 9–24. Values are shown as ±s.e. (b) Sequence of the translocation region. Upper panel: the CASPR2 reference sequence. Lower panel: sequence data of the EST AI364529–CASPR2 fusion gene. (c) Graphical representation of the EST AI364529–CASPR2 fusion gene. (d) NCOA1 expression levels in GBM142 (red line) does not appear to be affected by the EST AI364529–CASPR2 gene fusion. GBM142 expression is similar to the mean expression of controls (n =6, blue), glioblastomas (n =24, purple) and/or oligodendrogliomas (n =22, green).

deletion was identified), using custom-made genotyping arrays that span the entire *CASPR2* gene. Custom arrays confirmed the trisomy on chromosome 7 in GBM157 in the CASPR2 region. No additional genetic changes were identified using these arrays (data not shown).

As *CASPR2* is methylated in pancreatic carcinoma (Omura *et al.*, 2008), we determined the CASPR2 methylation status of six cell lines (U87, U118, U138, U373, T98 and human embryonic kidney (HEK)) and seven tumor samples. In all glioma cell lines, the CASPR2 gene was methylated and all these cell lines have low CASPR2 mRNA expression. The CASPR2 gene is not methylated in HEK cells, which may explain CASPR2 mRNA

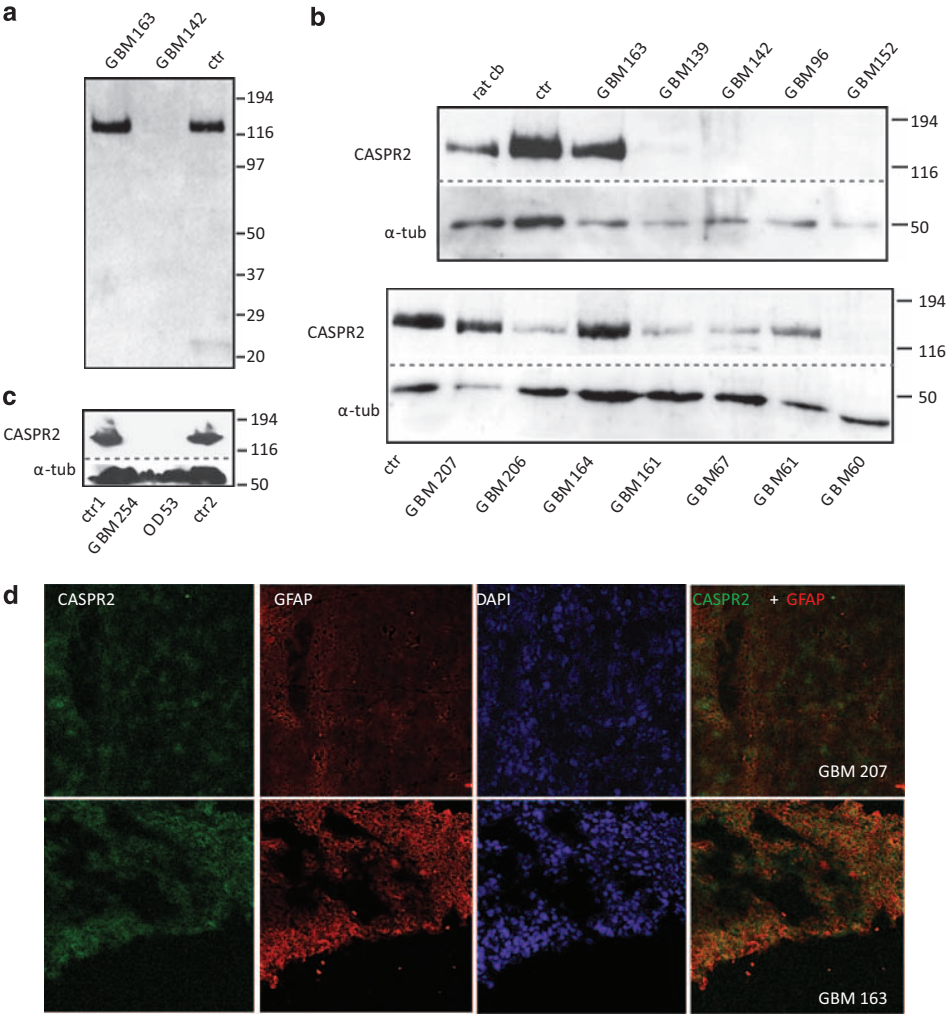

Figure 2 CASPR2 protein expression. (a) Western blot of protein lysates derived from GBM163, GBM142 and a control brain sample. Note that GBM142 does not express any alternative-sized CASPR2 protein. In this western blot, an equal amount of protein was loaded as used for (b). (b) Western blot of CASPR2. Top panel: CASPR2, expected size 148 kDa. Lower panel: Tubulin-a (a-tub), expected size 50 kDa. Numbers indicate individual samples. Non-neoplastic brain tissue (control) and rat cerebellum (rat cb) were used as controls. The EST AI364529–CASPR2 fusion gene therefore apparently does not result in fusion protein expression. (c) Western blot of CASPR2 of the two samples with heterozygous deletions (GBM254 and OD53) (see also Figure 3d) and two control brain samples. Both samples do not express CASPR2. (d) Immunocytochemical staining of GBM163 and GBM207 demonstrates that CASPR2 and GFAP are expressed by the tumor.

expression in these cells (GSE1309). However, western blot analysis failed to detect CASPR2 protein expression in HEK cells (data not shown). Four out of seven tumor samples had CASPR2 methylation, and methylation status correlated with mRNA expression in five out of seven samples. Methylation of the CASPR2 gene, therefore, may be an epigenetic mechanism that downregulates CASPR2 expression in gliomas.

In summary, our comprehensive mutational analysis identified at least three genetic changes in the CASPR2 gene in gliomas: one translocation, one intragenic deletion and one larger deletion in a set of 47 glioma samples. In addition, CASPR2 functioning may also be compromised in GBM96, where expression might be driven from an alternative promoter. The CASPR2 gene is also methylated in a subset of gliomas. At least several of the identified mutations appear to disrupt normal CASPR2 functioning.

CASPR2 overexpression decreases the proliferation rate

The relatively high frequency in which we identified genetic changes in the CASPR2 gene led us to perform functional experiments to determine the role of CASPR2 in glioma formation and/or progression. We first created a CASPR2 construct with a hemagglutinin (HA) epitope inserted directly c-terminal of the signaling peptide domain and enhanced-GFP inserted intracellularly, following the transmembrane domain (Supplementary Figure 3). The final HA-CASPR2-GFP construct allowed us to use the extracellular HA tag to confirm transmembrane localization using immunocytochemistry (Supplementary Figure 4). The intracellular GFP tag allowed visualization of the construct in live cells while preserving functioning of the intracellular CASPR2 domain (Horresh *et al.*, 2008). The construct was then used to create stable HA-CASPR2-GFPexpressing U87MG, U373 and HEK cell lines.

U87MG and HEK cells stably expressing HACASPR2-GFP showed a decrease in proliferation rate compared with control (DsRed2) expressing cells in a WST-1 cell proliferation assay. The mean reduction in proliferation rate was 13% (range 7.3–22.2%, P =0.007). This reduction appears to be specific for CASPR2 as other overexpressed enhanced green fluorescent protein fusion genes, in our laboratory do not show a decreased proliferation (LBC Bralten and PJ French, data not shown). We also analyzed co-cultures of U87MG cells that initially contained a mixture of HA-CASPR2-GFP-and DsRed2-expressing cells (see Materials and methods). Cell cycle analysis of the mixture shows a much lower G2 peak in the HACASPR2-GFP-expressing cells compared with the DsRed2-expressing cells (Figure 4a). After 9–10 cell culture passages, the number of HA-CASPR2-GFPexpressing cells in three independent experiments was reduced to 0.3, 0.3 and 4.5%, respectively, of the total number of cells (Figure 4b). Fluorescence activated cell sorting (FACS) analysis on U87MG mixed cell cultures therefore confirmed the reduced proliferation as observed in our WST-1 cell proliferation assays.

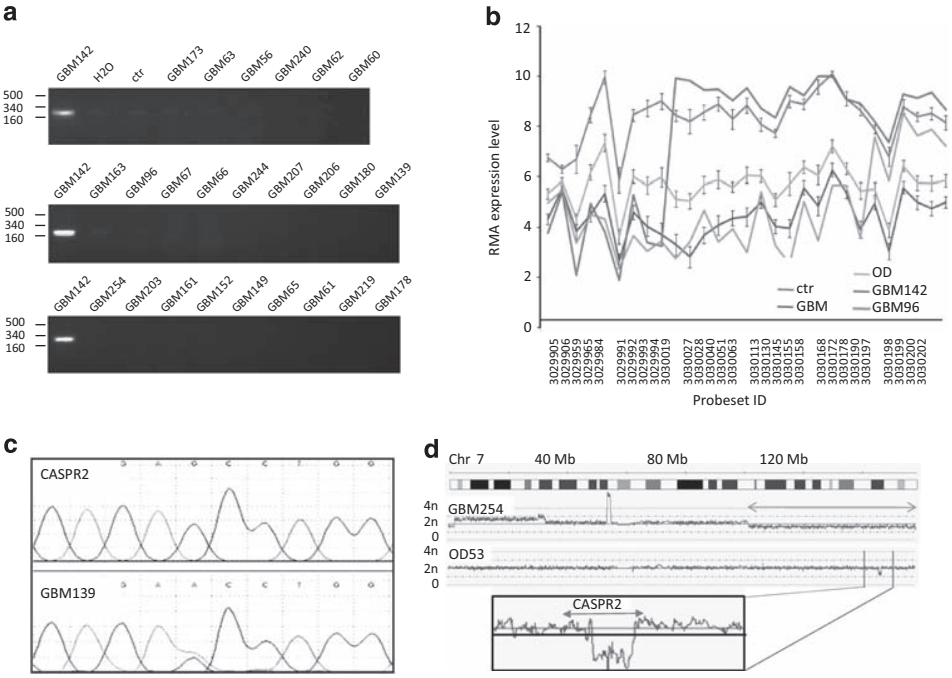

Figure 3 CASPR2 analysis. (a) The EST AI364529(exon 1)–CASPR2(exon 9) translocation is unique in a cohort of 25 glioblastomas. RT–PCR only detects a specific fusion gene in GBM142 and not in other samples. Control (Ctr): non-neoplastic brain tissue. (b) A high expression of the last five probesets in GBM96 was observed compared with control brain samples (n =6, blue), glioblastomas (n =24, purple) and oligodendrogliomas (n =22, green). GBM142 is shown in red. The high expression of 3'exons of GBM96 could be because of expression by an alternative promoter. (c) A single base change 73G4A, resulting in an alanine to be replaced by threonine (A24T), was identified in GBM139 (lower panel). The mutation lies in exon 1, within the conserved region coding for the signaling peptide of CASPR2 (upper panel: reference sequence). (d) Genotyping identified 2/40 samples with allelic loss of the CASPR2 gene. In GBM254, a large heterozygous loss including the CASPR2 gene was identified (chr7:102106439–end). In OD53, a heterozygous intragenic deletion was identified corresponding to a deletion of exons 4–14 (chr7:146208141–147249840). A full colour version of this figure is available at the Oncogene journal online.

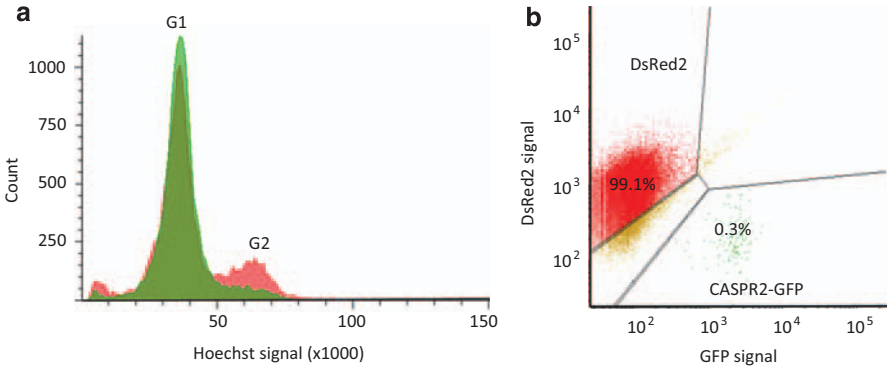

Figure 4 FACS analysis of a mixed population (equal amounts) of U87MG cells stably expressing DsRed2 or HA-CASPR2-GFP. (a) Cell cycle analysis (passage 1). The red graph depicts the DsRed2-expressing cells, the green graph the HA-CASPR2-GFPexpressing cells, dark green represents the overlap of the two graphs. Note the much lower G2 peak in the HA-CASPR2-GFPexpressing cells. (b) After nine passages, the number of HA-CASPR2-GFP cells was reduced to 0.3% of the total cell population, indicating that HA-CASPR2-GFP-expressing U87MG cells have a reduced proliferation rate.

To determine the effects of CASPR2 overexpression HA-CASPR2-GFP-expressing cells (U118: 94.9 vs on cell proliferation in other cell lines, U118, U138, 1.5%; U138: 57.6 vs 0.7%; U373: 51.8 vs 5.4%; T98: U373, T98 and HEK cells were transfected with HA-9.9 vs 1.3%; HEK: 94.9% vs 18.1%; P =0.007). These CASPR2-GFP or DsRed2. After 3 weeks of culture on differences are in line with the hypothesis that selection medium, the percentages of DsRed2-expressing HA-CASPR2-GFP-expressing cells have a growth cells were significantly higher than those of the disadvantage.

FACS analysis showed a lower percentage of cells that have entered the cell cycle (S-, G2-and M-phase) in the HA-CASPR2-GFP-expressing U87MG, U373 and HEK cells compared with DsRed2-expressing cells (20.4 vs 29.0% (P =0.0044), 18.8 vs 21.9% (P =0.077) and 20.5 vs 28.6% (P =0.014), respectively; other cell lines not determined).

The absence of CASPR2 expression in established cell lines (U87MG, U118, U138, U251, U373, U387, SF126, SF268, SF539, A172, SNB19, SNB75, LN229 and T98), according to the GEO datasets (GSE10547, GSE5720 and GSE4536), prohibited knockdown experiments.

We next aimed to determine the mechanism of the CASPR2-associated decrease in proliferation rate. b-Galactosidase assays did not reveal a difference in cellular senescence between U87MG cells expressing HA-CASPR2-GFP and DsRed2 (1.26% and 1.48%, respectively, P =0.63). The HA-CASPR2-GFP-expressing cells do not show a marked increase in cell volume compared with the DsRed2-expressing cells, arguing against increased CASPR2-associated cellular senescence (Supplementary Figure 5). However, a 46% in-

crease in apoptosis was observed in HA-CASPR2GFP-compared with DsRed2-expressing U87MG cells (P =0.0019). This increase in apoptosis in CASPR2-overexpressing cells may therefore explain the CASPR2-associated reduction in proliferation.

CASPR2 overexpression does not affect migration and invasion

Cell adhesion molecules, like neural cell adhesion molecule, are thought to have a role in invasiveness and progression in cancer (Zecchini and Cavallaro, 2010). As CASPR2 also functions as a cell adhesion molecule, we determined whether CASPR2 expression may affect cell migration and invasion. No difference in migration and invasion of U87MG cell lines was observed both by Millipore migration and invasion assays (Millipore, Billerica, MA, USA; P =0.25 and 0.38, respectively) and in the Incucyte (migration only) (P =0.91).

CASPR2 expression is positively correlated with patient survival

As CASPR2-overexpressing tumor cells in vitro have a decreased cell proliferation, we looked at survival and CASPR2 mRNA expression in a set of 268 glial brain tumors. CASPR2 mRNA expression was positively correlated with overall survival (Figures 5a and b). Patients with low CASPR2 expression (less than the median) had a median survival of 0.79 years compared with 2.83 years in the group with high CASPR2 expression (Po0.0001). Even within defined histological subgroups (AIII, AOIII and ODIII), patients with high CASPR2 expression (greater than the median) had a significantly better survival than patients with low CASPR2 expression (less than the median) (P =0.0197, 0.0371 and 0.0001, respectively). Similarly, the survival of GBM patients with high CASPR2 expression (top 25th percentile) was significantly longer than that of other GBM patients (P =0.00451) (Supplementary Figure 6 and Supplementary Table 5). No difference in survival was observed in tumors with high expression of glial (GFAP) or neuronal (NEFH

Table 1 Multivariate analysis including age, Karnofsky performance score, diagnosis and CASPR2 mRNA expression

	HR	*s.e.m.*	*z*	*P>z*	*95%*	*CI*
Age	1.03045	0.00568	5.44	<0.001	1.019378	1.041642
KPS	0.992927	0.002582	-2.73	0.006	0.98788	0.998
PA	0.719409	0.030271	-7.83	<0.001	0.662461	0.781253
CASPR2	0.713202	0.091988	-2.62	0.009	0.553892	0.918333

CASPR2 expression

Abbreviations: CASPR2, contactin-associated protein-like 2; CI, confidence interval; HR, hazard ratio; KPS, Karnofsky performance score; PA, diagnosis.

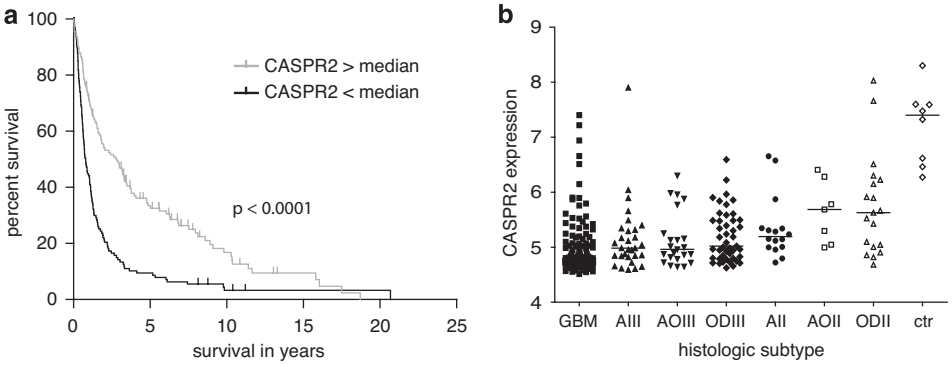

Figure 5 CASPR2 expression is positively correlated with patient survival. (a) Kaplan–Meier survival plots of patients with high (greater than the median, gray line) and low CASPR2 expression (lesser than the median, black line) in a cohort of 268 glioma patients. CASPR2 expression is positively correlated with overall survival (2.83 vs 0.79 years, Po0.0001). (b) CASPR2 mRNA expression in distinct histological glioma subgroups and eight control samples. Horizontal bars represent the median of each subgroup. Nonneoplastic brain tissue has the highest CASPR2 mRNA expression levels.

or NEFM) markers excluding the possibility of low tumor content in gliomas with high CASPR2 expression (Supplementary Figure 7 and Supplementary Table 6). Furthermore, CASPR2 immunocytochemistry on GBM163 and GBM207 confirmed that CASPR2 is expressed by tumor cells and is not due to a contamination of non-neoplastic brain tissue (Figure 2d).

Multivariate analysis demonstrated that CASPR2 expression is a prognostic factor that is independent of histological diagnosis (including subtype and grade), age and Karnofsky performance score (P =0.009) (Table 1).

Discussion

In this paper, we describe a novel translocation involving the CASPR2 gene using exon-expression arrays. CASPR2 was fused with an EST (EST AI364529) that is likely to be part of the NCOA1 gene. The fusion of this EST and CASPR2 resulted in loss of CASPR2 protein expression, despite high mRNA expression. Mutation analysis of CASPR2 revealed at least two other glioma samples with inactivating mutations. Methylation of the CASPR2 gene was also observed in gliomas and glioma cell lines. Furthermore, lower proliferation rates were observed after CASPR2 overexpression in glioma cell lines, most likely due to an increase in apoptosis in these cells. Finally, multivariate analysis showed that high CASPR2 expression is positively correlated with patient survival. Our results are consistent with CASPR2 functioning as a tumor suppressor gene in gliomas.

The CASPR2 gene spans a region of 2.3 Mb on chromosome 7. It encodes CASPR2, a transmembrane cell adhesion molecule and member of the neurexin superfamily. Extracellularly, CASPR2 binds to contactin 2 (TAG-1), a neural recognition protein of the immunoglobulin cell adhesion molecule superfamily (Poliak *et al.*, 2003). The CASPR2–contactin 2 complex is involved in clustering of K^+ channels in the juxtaparanodal region of the nodes of Ranvier (Poliak *et al.*, 2003) and in neurite outgrowth (Lieberoth *et al.*, 2009). Germline genetic alterations in the CASPR2 gene have been found in several neurological and psychiatric diseases, including recessive symptomatic focal epilepsy and autism (Strauss *et al.*, 2006; Bakkaloglu *et al.*, 2008; Jackman *et al.*, 2009), combined schizophrenia and epilepsy (Friedman *et al.*, 2008) and Gilles de la Tourette's syndrome with obsessive–compulsive disorder (Verkerk *et al.*, 2003). All genetic alterations found were inactivating. Furthermore, linkage studies have identified single-nucleotide polymorphisms in CASPR2 that predispose for autism (Alarcon *et al.*, 2008; Arking *et al.*, 2008) and language impairments (Vernes *et al.*, 2008). These studies emphasize the importance of CASPR2 in the central nervous system.

Several other studies have highlighted a potential role of CASPR2 in cancer formation and/or progression. First, Thorell *et al.* suggest CASPR2 to be a tumor suppressor gene; they show a fivefold lower CASPR2 expression in unfavorable neuroblastomas compared with favorable neuroblastomas (Thorell *et al.*, 2009). CASPR2 was the gene with the largest change in expression found between these two groups (Thorell *et al.*, 2009). Second, CASPR2 is a common integration site of the sleeping-beauty transposon in sarcomas from Arf-/-mice (Collier *et al.*, 2005). In three independent sarcomas, the transposon disrupted the coding sequence of CASPR2 (Collier *et al.*, 2005). Finally, CASPR2 is methylated in 82% (47/57) of the pancreatic cancers, compared with 3% (1/34) of the cases of normal pancreas tissue (Omura *et al.*, 2008). In our group of 268 gliomas, CASPR2 expression was heterogeneous (Figure 5b) and methylation of the CASPR2 gene could in part be an explanation for the remaining lowexpressing samples. More genera-

lly, the CASPR2 gene is part of a common fragile site (McAvoy *et al.*, 2007). Such fragile sites are frequently disrupted in cancer and are thought to contain tumor suppressor genes (Smith *et al.*, 2007). In summary, disruption of the CASPR2 gene has been reported in several cancer subtypes, as well as in various neurological and psychiatric disorders. Disruptive genetic changes were also identified in the present study.

With its intracellular domain, CASPR2 binds to the 4.1B protein (Denisenko-Nehrbass *et al.*, 2003), which is also known as DAL-1 (Differentially expressed in Adenocarcinoma of the Lung). DAL-1 is a member of the protein 4.1 family and acts as a tumor suppressor in meningiomas (Gutmann *et al.*, 2000). Another wellknown member of the protein 4.1 family in brain cancer is schwannomin (merlin), the product of the NF2 tumor suppressor gene. Loss of heterozygosity of the DAL-1 region was found in 39% of gliomas (Tran *et al.*, 1998). DAL-1 forms a functional complex with CASPR2. This complex is thought to be involved in cell signaling through the activation of c-Jun amino-terminal kinase, a regulator of the retinoblastoma protein (Gerber *et al.*, 2006). The mechanism of action of CASPR2 as a tumor suppressor gene may be similar to that of DAL-1. Apparently, CASPR2 is part of a complex that, when inactivated, is involved in tumorigenesis.

CASPR2 is predominantly expressed in cells of neuronal origin (Poliak *et al.*, 1999; Lieberoth *et al.*, 2009). As gliomas are thought to be of non-neuronal origin, we examined whether the CASPR2 protein is also expressed in glial cells. Immunocytochemistry, using antibodies against CASPR2 and the glial marker GFAP or Vimentin on primary cultured rat hippocampus, indeed confirmed that a subpopulation of glial cells (7 and 4%, respectively) express CASPR2 (Supplementary Figure 8). In whole rat brain, however, CASPR2 and GFAP expression are seldomly colocalized (Supplementary Figure 9). As CASPR2 is expressed in a subset of cultured rat hippocampal glial cells and in certain gliomas, it is possible that certain gliomas originate from or dedifferentiate in the direction of this subpopulation of glial cells.

NCOA1, the 5' fusion partner of the translocation, has also been reported in cancer. NCOA1 is a transcriptional co activator for steroid and nuclear hormone receptors. In GBM NCOA1 is a frequent breakpoint (Freire *et al.*, 2008), and in alveolar rhabdomyosarcoma NCOA1 was reported as an alternative for FKHR as a translocation partner of PAX3 (Wachtel *et al.*, 2004). These data indicate that the NCOA1 region is a site with genomic instability in cancer. However, the translocation does not affect the expression of NCOA1 (Figure 1d), which suggests that NCOA1 does not have a role in our sample cohort.

Although our method of detecting translocations proved to be successful, rigorous testing remains to be done. However, compared with other ways of screening for gene rearrangements (Tomlins *et al.*, 2005; Maher *et al.*, 2009), our method may provide a cost-effective alternative that does not require large amounts of sample or depend on large-sample cohorts.

In summary, the absence of protein expression resulting from the translocation in GBM142, the intragenic deletion in OD53, the reduced proliferation rate of CASPR2-expressing cells and the positive correlation between CASPR2 expression and survival indicates that CASPR2 functions as a tumor suppressor gene in gliomas

Materials and methods

Sample collection

All glioma samples were derived from patients diagnosed between 1988 and 2004 and treated at the Erasmus Medical Center. All histological diagnoses were made on formalinfixed, paraffin-embedded hematoxylin and eosin-stained sections by an experienced neuropathologist (JMK) (Kleihues and Cavenee, 2000). Samples were collected immediately after surgical resection, snap-frozen and stored at -80 1C. All samples were visually inspected on 5-mm paraffin-embedded hematoxylin and eosin-stained frozen sections by the neuropathologist (JMK) to ensure 470% tumor content. Use of material was approved by the Institutional Review Board. Non-diseased adult cortical brain samples were obtained from the Erasmus University Medical Center (n =4) and the Dutch Brain Bank (n =3) or purchased (n =1; Qiagen, Venlo, The Netherlands) as described (Gravendeel *et al.*, 2009).

RNA, DNA and protein isolation

Tissues adjacent to the paraffin-embedded hematoxylin and eosin-stained frozen sections were used for nucleic-acid isolation as described (French *et al.*, 2007). For this study, we also isolated protein from the Trizol fraction according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). RNA quality was assessed on a Bioanalyser (Agilent Technologies, Amstelveen, The Netherlands). High-quality RNA (that is, RNA integrity number 47.0; Schroeder *et al.*, 2006) was used for our experiments. All genomic DNA was amplified using a REPLI-g mini kit (Qiagen).

Arrays

Identification of translocation candidates made use of exonlevel expression profiles reported in French *et al.* (2007) and Schutte *et al.* (2008). This dataset, GSE9385, consists of 26 GBMs, 22 ODs and 6 control samples. CASPR2 copy number was inferred from genotyping arrays performed on 21 GBMs and 19 ODs using 250k NspI arrays (Affymetrix, Santa Clara, CA, USA) (Bralten *et al.*, 2010). Survival analysis made use of clinical data and expression profiles from a cohort of 268 glioma samples (GSE16011: 117 GBM, 30 AIII, 23 AOIII, 45 ODIII, 16 AII, 7 AOII, 19 ODII and 11 AI; Supplementary Table 1) on U133 plus 2.0 arrays (Affymetrix) described in Gravendeel *et al.* (2009). Ultra-high resolution

copy number variation analysis of a 2.91-Mb genomic region containing the complete CASPR2 gene was performed on four samples (GBM56, GBM96, GBM142 and GBM157) using a custom NimbleGen microarray (Roche NimbleGen Systems, Madison, WI, USA). The average probe distance for this region was 23 bp.

Identification of fusion genes

The signal intensity estimate and the P-value for each probeset were extracted from the Human Exon 1.0 ST arrays in Affymetrix genotyping console software using RMA. We then screened for candidate fusion genes based on aberrant expression ratios between 5' and 3' exons. We first excluded exons with low expression levels in all samples (that is, expression levels o6.5). We then excluded exons that show poor correlation with the estimated transcript expression level (metaprobeset level), that is, those with a correlation o0.65. We then calculated the average ratio between the two 5' and two 3' probesets. Ratios deviating 416-fold from the median ratio were marked as strong candidate fusion partners. All remaining candidates were visually inspected for aberrant expression.

5' and 3' rapid amplification of cDNA ends (RACE)-PCR was performed using the Marathon RACE Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. All primers were from Invitrogen and were designed using Primer3 (biotools.umassmed.edu/bioapps/primer3_www. cgi) (Supplementary Table 2).

Western blotting

Approximately 2 mg protein was loaded onto 10% polyacrylamide gels, and blotted onto a Hybond-C super nitrocellulose membrane (Amersham Biosciences, Roosendaal, The Netherlands). Membranes were incubated in rabbit-anti-CASPR2 polyclonal antibody (Chemicon International, Temecula, CA, USA) (1:1000) or mouse-anti-tubulin monoclonal antibody (Sigma, St Louis, MO, USA) (1:3000). Membranes were then incubated with either horseradish peroxidase-conjugated polyclonal swine-anti-rabbit (Dako, Glostrup, Denmark) (1:2500) or polyclonal rabbit-anti-mouse (Dako) (1:2500). Detection was carried out using the Amersham ECL advance western blotting detection kit (GE Healthcare, Diegem, Belgium).

PCR amplification and bisulfite sequencing

All 24 coding exons of CASPR2 were amplified by PCR in 23 GBMs and 18 ODs. The sequence could be evaluated in a total of 527/552 and 318/432 exons in GBMs and ODs, respectively. In 25 GBMs a PCR was performed with a forward primer designed in EST AI364529 and a reverse primer designed in exon 9 of CASPR2. Primers and PCR conditions are listed in Supplementary Table 2. Genomic DNA of six cell lines (U87MG, U118, U138, U373, T98 and HEK) and seven glioma samples (four GBM, three ODIII), extrac-

ted using Qia-amp DNA mini kits (Qiagen), was bisulfite treated using the MethylEasy DNA Bisulphite Modification Kit (Human Genetic Signatures, Sydney, New South Wales, Australia) according to the manufacturer's protocol. Samples with 425% methylation on evaluable CpG island were considered methylated. PCR primers and conditions to determine CASPR2 methylation were performed as described (Omura *et al.*, 2008). Amplified products were sequenced using the Big Dye Terminator v3.1 (v1.1 for bisulfite-treated samples) Cycle Sequencing Kit (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Samples were analyzed using Mutation Surveyor V2.51 (SoftGenetics, State College, PA, USA).

Constructs

CASPR2 cDNA (Origene Technologies, Rockville, MD, USA) was fused to HA and GFP as outlined in Supplementary Figure 1. Our construct preserved the N-terminally localized signal peptide and the C-terminally located PDZ domain (see also Horresh *et al.*, 2008). Primers and conditions are stated in Supplementary Table 2. HA-CASPR2-GFP was cloned into the pcDNA3.3-TOPO vector (Invitrogen). The control construct consisted of the DsRed2 gene cloned into the pcDNA3.3-TOPO vector.

Cell lines

U87MG, U118, U138, U373, T98 and HEK cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (GIBCO, Breda, The Netherlands) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin) at 37 1C, in a 5% CO2 humidified chamber. For each cell line two 10-cm Petri dishes were transfected with 15 mg HA-CASPR2-GFP or DsRed2 using FuGENE 6 (Roche, Woerden, The Netherlands) or, in the case of HEK cells, Polyethylenimine 'Max' (Polysciences, Eppelheim, Germany). After 2 days cell cultures were supplemented with 600 mg/ml (U87MG) or 400 mg/ml (U118, U138, U373, T98 and HEK) Geneticin (Invitrogen). CASPR2 mRNA levels in the stable U87MG cell line were 1.7 times higher than the levels in control brain, as was determined by quantitative PCR using SYBR green PCR master mix (Applied Biosystems; French *et al.*, 2005). CASPR2 expression levels were evaluated relative to GAPDH expression. All primers had an amplification efficiency 480% (determined by serial dilution) and generated a single amplification product (determined by melting point analysis).

FACS sorting and cell cycle analysis

After 3 weeks in selection medium, HA-CASPR2-GFP and/or DsRed2 expressing U87MG (in triplicate), U373 and HEK cells were FACS sorted (FACSAria, BD Biosciences, San Jose, CA, USA) into wells containing 10 000 HACASPR2-GFP cells, 10 000 DsRed2 cells, or a

mixture of 5000 HA-CASPR2-GFP cells and 5000 DsRed2 cells (U87MG cells only). Parallel sorted cells were analyzed for sorting efficiency (496%) and stained with Hoechst 33258 (Sigma) to analyze viability (480%). FACS-sorted cells were either isolated alive and stained with 20 mg/ml Hoechst 33342 (Sigma) for 20 min at 37 1C (U87MG) or fixed with 4% PFA and stained with 20 mg/ml Hoechst 33258 (Sigma) for 1 h at 37 1C. The mixture of stable U87MG cells was fixed in 75% ethanol. Ki-67 expression was detected using rabbit-anti-Ki67 (1:100) (Abcam, Cambridge, UK) and an antigen-presenting cell-conjugated donkey-anti-rabbit antibody (1:200) (Jackson ImmunoResearch, Suffolk, UK).

Assays

U87MG cells expressing either HA-CASPR2-GFP or DsRed2 were plated in two 96-well plates in three different concentrations (200, 400 and 800 cells/well), in triplo, for all three independently sorted cell populations. HEK cells expressing either HA-CASPR2-GFP or DsRed2 were plated at 1000 cells/ well, in triplo, and the experiment was performed in duplo. The percentage of cells expressing HA-CASPR2-GFP or DsRed2 was determined using FACS (U87MG— HA-CASPR2-GFP: 26.1–69.5%, DsRed2: 69.6–99.1%; HEK—HA-CASPR2-GFP: 47.9%, DsRed2: 97.4%). The cell proliferation reagent WST-1 (Roche) was added (1:10) at either 4 h or 3 days after plating. Absorbance was measured in a microplate reader (Multiskan Ascent, Thermo Electron Corporation, Breda, The Netherlands) at 440 nm every 30 min for 3.5 h. The WST-1 reaction is linear in this timeframe; therefore, the slope of the trend line of the absorbance data was used as a measure of viability. The relative increase in this slope after 3 days was used as a measure of proliferation. 24-Well Cell Migration and Invasion Assay Kits (both from Millipore) were used according to the manufacturer's protocol on 0.5 x10^6 stable and sorted U87MG cells for 3 days. Results were quantified by colorimetric reading at 560 nm. Migration was also determined using an Incucyte (Essen Instruments, Hertfordshire, UK), for 72 h, at one frame per hour. MTrackJ, a plug-in of the ImageJ software, was used to quantify cell movement.

Apoptosis of stable and sorted U87MG cells was determined using the Cell Death Detection ELISA Kit (Roche) according to the manufacturer's protocol. Thousand cells per well were plated in a 96-well plate and left for 3 days before the start of the protocol. The experiment was performed two times in triplo with two independently sorted sets of stable cell lines. Senescence was detected in U87MG cells using the Senescence b-Galactosidase Staining Kit (Cell Signaling, Bioke, Leiden, The Netherlands) according to the manufacturer's protocol. Two independently sorted stable cell lines were grown on chamber glasses in duplo for 3 days and stained with 250 ml staining solution.

Immunocytochemistry

Primary rat hippocampal E18 cell cultures, prepared as described in Jaworski *et al.* (2005), were fixed after 26 days in culture with 4% paraformaldehyde. In addition, sections of normal rat brain and two gliomas (GBM163 and GBM 207) with high CASPR2 protein expression were fixed with ethanol. Cultures and sections were incubated with mouse-anti-GFAP monoclonal antibody (1:20 000) (Sigma) or mouse-anti-Vimentin monoclonal antibody (1:200) (Sigma) and rabbit-anti-CASPR2 polyclonal antibody, at dilutions of 1:100 for cultures and 1:2000 for sections (Chemicon International). To check for membrane localization of HA-CASPR-GFP, transfected U87MG cells were incubated with mouse-anti-HA (1:1000) (Abcam). Secondary antibodies used were Alexa Fluor 488 goat-anti-rabbit IgG or Alexa Fluor 568 goat-anti-mouse, at dilutions of 1:2500 for cultures and 1:400 for sections and extracellular staining (both from Invitrogen). Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA).

Survival analysis

Kaplan–Meier survival analysis was performed with Graph-Pad Prism 5.0 (GraphPad Software, San Diego, CA, USA), using a log-rank (Mantel–Cox) test. Multivariate analysis using Cox regression and the Breslow method for ties was performed using STATA 11.0 (StataCorp LP, College Station, TX, USA).

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Chapter 5

Segregation of Non-p.R132H Mutations in IDH1 in Distinct Molecular Subtypes of Glioma

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Abstract

Mutations in the gene encoding the isocitrate dehydrogenase 1 gene (IDH1) occur at a high frequency (up to 80%) in many different subtypes of glioma. In this study, we have screenedfor IDH1 mutations in a cohort of 496 gliomas. IDH1 mutations were most frequently observed in low grade gliomas with c.395G>A (p.R132H) representing >90% of all IDH1 mutations. Interestingly, non-p.R132H mutations segregate in distinct histological and molecular subtypes ofglioma. Histologically, they occur sporadically in classic oligodendrogliomas and at significantly higher frequency in other grade II and III gliomas. Genetically, non-p.R132H mutations occur intumors with TP53 mutation, are virtually absent in tumors with loss of heterozygosity on 1p and19q and accumulate in distinct (gene-expression profiling based) intrinsic molecular subtypes. TheIDH1 mutation type does not affect patient survival. Our results were validated on an independentsample cohort, indicating that the IDH1 mutation spectrum may aid glioma subtype classification.Functional differences between p.R132H and non-p.R132H mutated IDH1 may explain the segregation in distinct glioma subtypes.

Introduction

The most common type of primary brain tumor (~40%) are gliomas. Based on their histological appearance, gliomas can be divided into three distinct types of tumors according to the standard WHO classification (Kleihues and Cavenee 2000): Astrocytic tumors (75%), pure oligodendroglial tumors (ODs) and mixed oligoastrocytic tumors (MOAs) (25%). Tumors are further classified into grades II, III (anaplastic) and IV (Glioblastomas, GBMs) depending on the number of malignant features present. Despite advances in neurosurgery, chemotherapy and radiotherapy the prognosis for most glioma patients remains dismal (Stupp et al., 2005).

Recently, a genetic screen identified somatic mutations in the isocitrate dehydrogenase 1 gene (IDH1; MIM# 147700) in glioblastomas (Parsons et al., 2008). IDH1 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and uses NADP(+) as the electron acceptor (Geisbrecht and Gould 1999; Margittai and Banhegyi 2008). Additional analysis showed that IDH1 mutations occur at a very high frequency (up to 80%) in all grade II and III gliomas (astrocytic, oligodendrocytic and oligoastrocytic) and secondary GBMs (Balss et al., 2008; Parsons, et al., 2008; Yan et al., 2009; Dubbink et al., 2009; Watanabe et al., 2009a; Ichimura et al., 2009; Sanson et al., 2009; Sonoda et al., 2009). IDH1 mutations do not occur at significant frequencies in other tumor types, with the notable exception of acute myeloid leukemia (AML) (Bleeker et al., 2009; Yan, et al., 2009; Mardis et al., 2009). The reported mutations in IDH1 all result in a reduced enzymatic activity towards its native substrate, isocitrate (Yan, et al., 2009; Ichimura, et al., 2009; Zhao et al., 2009).

Mutations in the IDH1 gene are heterozygous and virtually always affect only a single residue (arginine 132) which is replaced by a histidine in approximately 90% of tumors (c.395G>A resulting in p.R132H (Balss, et al., 2008; Ichimura, et al., 2009; Yan, et al., 2009; Sanson, et al., 2009). However, non-p.R132H mutations in the IDH1 gene (e.g. p.R132C) have been reported to accumulate at higher frequencies in histological subtypes of glioma (Hartmann et al., 2009), in astrocytomas of Li-Fraumeni patients (Watanabe et al., 2009b) and in patients with AML (Mardis, et al., 2009). Distinct mutations within the same gene but affecting the same codon therefore appear to segregate in distinct tumor subtypes.

In this study, we demonstrate that non-p.R132H mutations specifically accumulate in distinct histological subtypes of glioma. Because histological classification of gliomas is subject to significant interobserver variability (Kros et al., 2007), we also used molecular markers (TP53; MIM# 191170), 1p/19q LOH and intrinsic molecular subtypes) to identify the glioma subtypes. Our results demonstrate that non-p.R132H mutations indeed segregate in distinct histological, genetic and molecular subtypes of gliomas

Materials and methods

Samples

Glioma samples were collected from five hospitals in the Netherlands (Erasmus MC, Rotterdam; UMCU, Utrecht; NCI and VUMC, Amsterdam; RUNMC, Nijmegen) from patients, operated from 1989-2009. Use of patient material was approved by the Institutional Review Board. All samples are listed in Supp. Table S1. Histological diagnoses were made on formalin-fixed, paraffin-embedded Haematoxylin & Eosin sections and were reviewed by the neuropathologist (J.M.K.). Genomic DNA from snap frozen or formalin fixed, paraffin embedded tissue samples was isolated as described (Gravendeel et al., 2009; Dubbink, et al., 2009). Of the 496 glioma samples reported in this study, 247 were derived from a series of gliomas of all histologies (set A) (Gravendeel, et al., 2009), 54 from a series of low grade astrocytomas (set B) (Dubbink, et al., 2009), 24 were derived from samples operated in Rotterdam that were included in EORTC 26951 (set C) (van den Bent et al., 2006) and 171 novel glioma samples (set D). The IDH1 status of 297 samples was described previously (Gravendeel, et al., 2009; Dubbink, et al., 2009). IDH1 (NM_005896.2, GI:28178824) mutational status of the remaining 199 samples was determined as described (Bleeker, et al., 2009), see also Supp. Table S2.

1p/19q status was determined in 317 samples. 1p19q LOH was determined by fluorescent in-situ hybridization (FISH) (Kouwenhoven et al., 2006), inferred from genotyping arrays or by microsatellite analysis. Microsatellites were amplified by PCR on 20 nanogram genomic DNA, using a fluorescently labeled forward primer and a reversed primer. Allelic losses were statistically determined as described and scored by two independent researchers (French et al., 2005). TP53 (NM_000546.4, GI:187830767) status was determined in 169 samples. Primers and cycling conditions to determine the TP53 mutation status are stated in the Supp. Table S2.

Statistical analysis

Comparison between frequencies of different groups was assessed using the Fisher exact test. The significance of IDH1 mutation type was assessed by univariate and multivariate analysis using Cox Regression. Differences between Kaplan-Meyer survival curves were calculated by the Log-rank (Mantel-Cox) test. Survival time was defined as the period from date of surgery to date of death. If date of death was not available, date of last follow-up was used.

Results and discussion

Dataset

We have screened for mutations in IDH1 in a large cohort of 496 glioma samples. In this dataset, a total of 246 mutations in the IDH1 gene were identified (49.6%). The IDH1 mutation frequency for all grades II and III gliomas was high and ranged from 49% in anaplastic oligoastrocytic tumors to 79% in low grade oligodendrogliomas (Table 1). The frequency of genetic changes in our sample cohort is similar to reported by others (Balss, et al., 2008; Ichimura, et al., 2009; Yan, et al., 2009; Hartmann, et al., 2009; Sonoda, et al., 2009). The frequency of IDH1 mutations in grade II gliomas (76%, n=110/144) was higher than in grade III tumors (58%, n=102/177, P<0.001) (see also (Sanson, et al., 2009)). More specifically, low grade oligodendrogliomas (79%, n=34/43) and oligoastrocytomas (79%, n=22/28) have a higher IDH1 mutation frequency then their corresponding grade III tumors (60%, n=64/106 and 49%, n=19/39 respectively P<0.05). A similar difference in frequency can also be extracted from at least two external sample cohorts (Hartmann, et al., 2009; Watanabe, et al., 2009a).

The observation of a higher mutation incidence in lower grade tumors has thusfar been described for only a few genes (see e.g. (Knowles 2007)). This differential frequency indicates that low grade tumors can accumulate distinct genetic changes and thus represent a different disease entity. Moreover, such differential frequency indicates that the IDH1 mutation status can, at least to some extent, serve as a marker for low grade gliomas (see also (Jones et al., 2008; Korshunov et al., 2009)).

Table 1. The frequency of IDH1 (NM_005896.2, GI:28178824) mutations, LOH on 1p and 19q and mutations in TP53 (NM_000564.4, 187830767) in different histological.

	n	p.R132H	p.R132X	IDH1 wt	1p/19 LOH	no loss	nd	TP53 mut	TP53 wt	nd
ODII	43	34	0	9	21	9	13	0	6	37
ODIII	106	63	1	42	57	31	18	7	18	81
OAII	28	21	1	6	6	10	12	1	3	24
OAIII	39	15	4	20	3	23	13	8	2	29
AII	73	48	6	19	2	53	18	29	18	26
AIII	32	15	4	13	1	18	13	12	6	14
GBM	175	31	3	141	10	73	92	27	32	116
total	496	227	19	250	100	217	0	84	85	0

ODII/ODIII: grade II/III oligodendroglioma, OAII/OAIII: grade II/III oligoastrocytoma, AII/AIII: grade II/III astrocytoma, GBM: glioblastoma. nd: not determined.

Non-p.R132H mutations in IDH1 segregate in distinct glioma subtypes

The majority of mutations (92.3%) in the IDH1 gene were heterozygous c.395G>A missense mutations that result in an arginine to histidine substitution on position 132 (p.R132H). However, 19/246 mutations (7.7%) were non-p.R132H mutations: Single nucleotide changes that result in different amino acid substitutions albeit on the same position. Similar to reported, c.394C>T (p.R132C) was the second most common mutation type in our sample cohort and was identified in nine gliomas. Other non-p.R132H mutations were p.R132S (n=5), p.R132G (n=3), p.R132L (n=1) and p.R132P (n=1).

When focusing on non-p.R132H IDH1 mutations in our dataset, we observed that in classical oligodendrogliomas (combined grades II and III), only one out of the total of 98 IDH1 mutations (1.0%) was non-p.R132H (Figure 1). A significantly larger proportion of non-p.R132H mutations was present in oligoastrocytic tumors (5/41, P<0.01) and in astrocytic tumors (10/73, P<0.001). Significance remained within the different tumor grades (Figure 1). These results indicate that, although non-p.R132H mutations are rare, they are not uniformly distributed across the different histological subtypes of glioma.

We aimed to confirm the differential distribution of non-p.R132H mutations using objective molecular markers. We first screened for combined LOH on 1p and 19q; chromosomal losses that are frequently observed in oligodendrogliomas and relatively rare

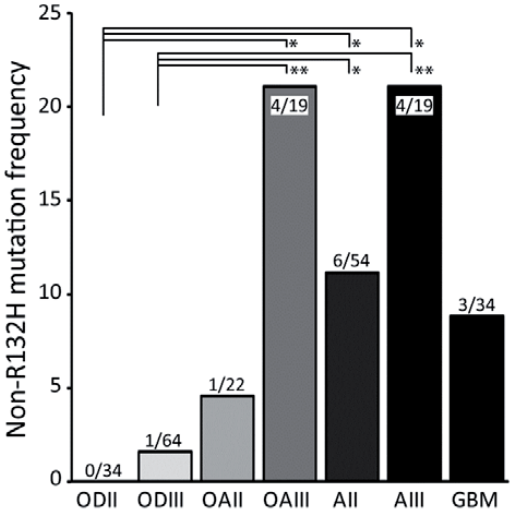

Figure 1. Distribution of non-p.R132H mutations in IDH1 in different histological subtypes of glioma. Plotted is the relative frequency of non-p.R132H mutations in IDH1 mutated histological subtypes of glioma. Absolute numbers for each subtype is stated in/above individual bars. For example, IDH1 mutations are observed in 54 grade II astrocytomas. Of these, six are non-p.R132H. As can be seen, non-p.R132H mutations are sporadically observed in oligodendrogliomas and more frequent in oligoastrocytomas and astrocytomas. *: P<0.05, **: P<0.01, Fishers' exact test.

in astrocytic tumors (Ohgaki and Kleihues 2009; Bromberg and van den Bent 2009)). These losses are caused by an unbalanced translocation between chromosomes 1 and 19 [t(1;19)(q10,p10)] (Griffin et al., 2006; Jenkins et al., 2006). In our entire dataset, 1p/19q LOH was determined in 317 (63.9%) samples. Of the tumors with combined 1p/19q LOH (n=100), a mutation in the IDH1 gene was detected in 75 samples (75%). In these 75 samples, only one non-p.R132H mutation was identified (1.3%, Figure 2). Conversely, in tumors that have retained 1p and/or 19q (n=217), the proportion of non-p.R132H mutations was significantly higher, 13/106 (12.3%, P<0.01). Non-p.R132H mutations therefore segregate in tumors that do not show combined 1p/19q LOH.

We then correlated the type of IDH1 mutation to the TP53 mutation status. TP53 mutations are frequently observed in astrocytomas and secondary glioblastomas but relatively rare in oligodendrogliomas and primary glioblastomas (Collins 2004; Ohgaki and Kleihues 2009). In our entire dataset, TP53 mutation status was determined in 169 samples (34.1 %). Of the tumors with a mutation in TP53 (n=84), a mutation in the IDH1 gene was detected in 65 samples (77.4%). Non-p.R132H mutations were identified in 12 of these 65 samples (17.9%, Figure 2). Conversely, in tumors with wt TP53 (n=85), the proportion of non-p.R132H mutations was significantly lower (3/54) (5.6%, P<0.05). Non-p.R132H mutations therefore accumulate in tumors with a mutation in the TP53 gene.

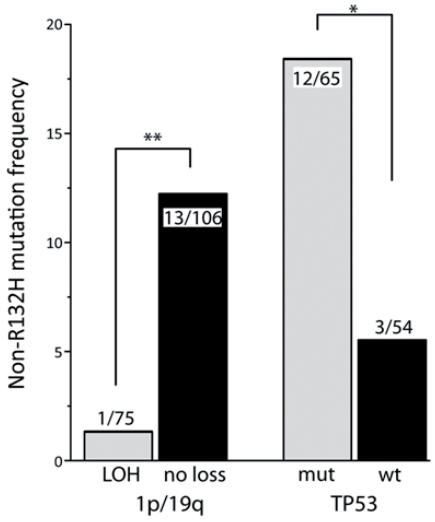

Figure 2. The frequency of non-p.R132H mutations in IDH1 is correlated to distinct genetic changes in gliomas. Non-p.R132H mutations are more prevalent in gliomas that do not show combined LOH on 1p and 19q (left) and in gliomas with a TP53 mutation (right). Absolute numbers for non-p.R132H mutations/total number of IDH1 mutations are stated in/above the individual bars. *: P<0.05, **: P<0.01, Fishers' exact test.

Finally, we determined whether non-p.R132H mutations segregate in intrinsic, gene expression based-, molecular subtypes of glioma. In a recent study, we identified seven intrinsic glioma subtypes based on gene expression profiles of 276 gliomas of all histological subtypes and grades (Gravendeel, et al., 2009). As reported, IDH1 mutations were most frequently observed in clusters 9 and 17 (27/40 and 22/32 respectively). When focusing on the non-p.R132H mutations (n=8) in this cohort, they were relatively rare in cluster 9 (n=1/27, 3.7%) and more frequent in cluster 17 (5/22, 22.7%, P=0.05). Other non-R132 samples were identified in clusters that have relatively few IDH1 mutations; one in cluster 0 and one in cluster 18 (Gravendeel, et al., 2009). Non-p.R132H mutations therefore appear not to be uniformly distributed across intrinsic molecular subtypes of glioma.

Validation of non-p.R132H segregation

The segregation of non-p.R132H mutations into distinct molecular subtypes of gliomas was validated on a recently described independent cohort of glioma samples (Ichimura, et al., 2009). In this study, a total of 119 mutations in IDH1 were identified across various histological subtypes, nine of which were non-p.R132H. Non-p.R132H mutations are clearly more prevalent in IDH1 mutated astrocytomas (7/45) compared to IDH1 mutated oligodendrogliomas (0/35, $P<0.05$). Furthermore, a significantly higher proportion of non-p.R132H mutations was observed in tumors with both IDH1 and TP53 mutation (8/65) compared to tumors with wt TP53 (1/52, $P<0.05$). Finally, all non-p.R132H mutations were identified in tumors that have retained 1p and 19q (9/74 vs. 0/45, $P<0.05$). In summary, non-p.R132H mutations are more prevalent in IDH1 mutated astrocytomas, gliomas with TP53 mutation and gliomas that have retained 1p and 19q in this external dataset. These data therefore confirm our hypothesis that non-p.R132H mutations in IDH1 are more prevalent in distinct subtypes of gliomas.

IDH1 mutation and patient survival

In gliomas, the presence of an IDH1 mutation has been associated with prolonged survival within gliomas of similar malignancy grade (Parsons, et al., 2008; Sanson, et al., 2009). In our dataset, the IDH1 mutation status was also positively correlated to prolonged survival in the entire dataset (3.3 vs. 0.84 years, $P<0.0001$) and in glioblastomas (2.0 vs. 0.72 years, $P<0.001$). Survival of our sample cohort was similar to population based studies (see e.g. (Ohgaki and Kleihues 2005) and www.cbtrus.org). To determine whether the IDH1 mutation type affects patient prognosis, we performed survival analysis on p.R132H vs. non-p.R132H mutated samples. Univariate analysis indicates that the type of IDH1 mutation does not affect patient survival ($P=0.74$). The IDH1 mutation type also does not affect patient survival within histological and/or molecular subtypes of glioma. For example, in the molecular glioma subtype that has a relatively large proportion of

non-p.R132H mutations (Cluster 17) the median survival between p.R132H (n=17) and non-p.R132H (n=5) mutated samples is similar (3.2 and 3.7 years respectively). It should be noted that the low numbers of non-p.R132H mutations within defined subtypes may obscure relatively small differences. However, multivariate analysis (in which known prognostic factors tumor type, grade and age at diagnosis were included), also indicates that the type of IDH1 mutation does not affect patient survival (P=0.44). Therefore, the IDH1 mutation type does not largely affect patient survival within our sample cohort.

Non-p.R132H mutations in IDH1 have been reported to accumulate at higher frequencies in distinct histological subtypes of gliomas (Hartmann, et al., 2009) and other tumor types (Watanabe, et al., 2009b; Kang et al., 2009; Mardis, et al., 2009). However, histological diagnosis of gliomas is subject to significant interobserver variability (Kros, et al., 2007). This study therefore provides evidence for the preferential accumulation of distinct types of IDH1 mutations not only in histological subtypes of glioma but also using molecular markers such as TP53, 1p/19q LOH and intrinsic molecular subtypes. We demonstrate that non-p.R132H mutations are relatively frequent in astrocytic tumors, tumors with mutation in TP53 and gliomas that do not show combined LOH on 1p and 19q. Although our results indicate that non-p.R132H mutations occur preferentially in tumors with distinct molecular features, it remains to be investigated whether these changes are causal for- or associated with- this increased frequency. The sequence in mutation events may also influence the type of IDH1 mutation acquired.

There are two possible explanations for the differential distribution of non-p.R132H mutations in glioma subtypes. First, it is possible that (epi)genetic differences between tumor types (e.g. differences in DNA repair due to e.g. MGMT (MIM# 156569) promoter methylation) result in the preferential accumulation of distinct mutations (see e.g. (Greenman et al., 2007)). For example, MGMT promoter methylation is associated with transition type mutations in TP53 in colorectal cancer and in gliomas (Esteller et al., 2001; Nakamura et al., 2001). Indeed, a higher percentage of oligodendroglial tumors show MGMT promoter hypermethylation compared to astrocytic tumors (Wick et al., 2009; Jeuken et al., 2007; Yang et al., 2009). Differences in the MGMT promoter methylation status between glioma subtypes may therefore explain the differential distribution of non-p.R132H mutations in IDH1. However, the transition/transversion ratio in IDH1 (i.e. p.R132H,C/p.R132S,G,L,P) between GBMs, astrocytic tumors (AII/III), oligoastrocytic tumors (OAII/III) or oligodendrocytic tumors (ODII/III) is similar (2/32, 5/68, 2/39 and 1/97 respectively). We also failed to observe a difference in transition/transversion ratio in the TP53 gene in the respective histological subgroups (14/12, 26/13, 8/1 and 5/2). The transition/transversion ratio in the TP53 gene between R132H and non-R132H mutated samples is also similar (39/15 and 8/3 respectively). Any possible difference in DNA repair between tumor types therefore is not reflected by a difference in the type of mutations acquired.

A second tentative possibility is that different mutations have different functional properties. For example, non-p.R132H mutations may show some residual activity towards isocitrate either in cis (activity of the mutated enzyme itself) or in trans (by influencing the activity of the remaining wt enzyme (Zhao, et al., 2009)). Alternatively, a recent study described that mutated IDH1 is able to convert α-ketoglutarate into 2¬hydroxyglutarate (Dang et al., 2009). Interestingly, different IDH1 mutation types showed a differential activity towards α-ketoglutarate. Whether this differential activity can explain the differential distribution of specific types of IDH1 mutations requires further detailed analysis.

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Chapter 6

IDH1 R132H Decreases Proliferation of Glioma Cell Lines In Vitro and In Vivo

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Abstract

Objective: A high percentage of grade II and III gliomas have mutations in the gene encoding isocitrate dehydrogenase (IDH1). This mutation is always a heterozygous point mutation that affects the amino acid arginine at position 132 and results in loss of its native enzymatic activity and gain of alternative enzymatic activity (producing D-2-hydroxyglutarate). The objective of this study was to investigate the cellular effects of R132H mutations in IDH1.

Methods: Functional consequences of mutations were examined among others using fluorescenceactivated cell sorting, kinome and expression arrays, biochemical assays, and intracranial injections on 3 different (glioma) cell lines with stable overexpression of $IDH1^{R132H}$.

Results: $IDH1^{R132H}$ overexpression in established glioma cell lines in vitro resulted in a marked decrease in proliferation, decreased Akt phosphorylation, altered morphology, and a more contact-dependent cell migration. The reduced proliferation is related to accumulation of D-2-hydroxyglutarate that is produced by $IDH1^{R132H}$. Mice injected with $IDH1^{R132H}$ U87 cells have prolonged survival compared to mice injected with $IDH1^{wt}$ or green fluorescent protein–expressing U87 cells.

Interpretation: Our results demonstrate that $IDH1^{R132H}$ dominantly reduces aggressiveness of established glioma cell lines in vitro and in vivo. In addition, the $IDH1^{R132H}$-$IDH1^{wt}$ heterodimer has higher enzymatic activity than the $IDH1^{R132H}$-$IDH1^{R132H}$ homodimer. Our observations in model systems of glioma might lead to a better understanding of the biology of IDH1 mutant gliomas, which are typically low grade and often slow growing.

Introduction

Gliomas are histologically classified into astrocytic, oligodendroglial, and oligoastrocytic tumors. They are further subdivided into low grade (grade II), ana-plastic (grade III), or glioblastoma multiforme (grade IV), depending on the presence or absence of malignant features. Mutations in the gene encoding isocitrate dehydrogenase 1 (IDH1)are very common in all grade II and III gliomas, often occurring at frequencies >70%.[1,2] The IDH1 mutation frequency is inversely correlated to tumor grade,[3,4] with the lowest frequency observed in glioblastoma multiformes.[5] IDH1 mutations are always heterozygous missense mutations affecting the arginine at position 132. In >90% of the IDH1 mutations in gliomas, this arginine is replaced by a histidine (R132H mutation).[1,6]

IDH1 mutations are an early event in tumorigenesis; they arise even before TP53 mutations or loss of 1p and 19q.[7] Patients with IDH1 mutations are generally younger, and mutations are associated with prolonged survival.[3,4,8–11] Mutations in the homologous IDH2 gene were also identified, although these occur at much lower frequencies in gliomas (0–6%).[1,12–15] The only other tumor type in which regular IDH1 and IDH2 mutations are detected is acute myeloid leukemia (AML).[16]

IDH1 catalyses the oxidative decarboxylation of isocitrate into a-ketoglutarate (aKG), thereby using nicotinamide adenine dinucleotide phosphate (NADP) as an electron acceptor.[17] The mutated IDH1 enzyme (R132H, IDH1^{R132H}) shows a strongly decreased enzymatic activity toward isocitrate, leading to lower aKG production, thereby increasing hypoxia inducible factor 1a levels.[15,18,19] In addition, IDH1^{R132H} has an altered enzymatic activity and uses aKG as a substrate to produce D-2-hydroxyglutarate (D-2HG).[20–22] Although it is clear that IDH1^{R132H} has altered enzymatic activity, little is known about how the altered enzymatic activity contributes to tumor formation.

In this study, we have therefore studied the cellular IDH1^{R132H} and functional effects of in vitro and in vivo. Our results show that IDH1^{R132H} expression decreases proliferation rates and alters the morphology and migration pattern of these cells. Although the reduced aggressiveness/proliferation found in our study was identified in established cell lines that are different from the in vivo growing tumors, our results may help to understand the biology of IDH1 mutated gliomas, which are associated with improved outcome and are predominantly low grade.

Materials and Methods

IDH1 mutation analysis was performed using forward and reverse primers as described.[3] and IDH1^{R132H} IDH1^{wt} were cloned into pEGFP-C2bio, which contains a biotin tag before the green fluorescent protein (GFP) tag. U87, U138, or HEK stable cell lines overex, IDH1^{R132H}-GFP pressing IDH1$^{wt\text{-}GFP}$, or GFP were selected for using Geneticin (Gibco, Invitrogen, Breda, the Netherlands). Morphology analysis, scratch assay, WST proliferation assay,[23] fluorescence-activated cell sorting (FACS) cell cycle analysis, and Ki67 staining and apoptosis assay were performed as described in the Supporting Information Methods. Cells plated in a 6-well plate were labeled with 1mM bromodeoxyuridine (BrdU) (BD Biosciences, Breda, the Netherlands) for 2 hours. Cells were fixed and stained using the BrdU In-Situ Detection kit (BD Biosciences) according to the manufacturer's instructions. Visualization of BrdU-labeled cells was performed using biotin-conjugated anti-BrdU (1:10) and allophycocyanin-conjugated streptavidin (1:400). Visualization of intracranially injected U87 cells was performed using a mouse antivimentin antibody (DAKO, Heverlee, Belgium) and Alexa568-conjugated antimouse antibody (Invitrogen) at 1:300 and 1:500 dilution, respectively. Sections were counterstained using 4,6-diamidino-2-phenylindole (DAKO).

EGFP-C2-bio-IDH1^{wt} or EGFP-C2-bio-IDH1^{R132H} in combination with BirA were transiently expressed by HEK cells, and purified using Streptavidin Dynabeads M-280 (Invitrogen) and a Magna-Sep (Invitrogen). Enzyme measurements of isolated recombinant enzymes, of the stable cell lines and in primary tumors, were performed as described (see also Supporting Information Methods).

Intracranial injections were performed with the U87 stable cell lines. C.B-17/Icr-Hantmhsd-Prkdcs SCID mice (Harlan) were stereotactically injected with 1 x 10^5 tumor cells according to a protocol previously described.[24] Tumor growth was measured 3x/wk in the IVIS 200 system (Xenogen, Alameda, CA).

L-2-HG and D-2-HG were measured as described.[25] Both L-2-HG and D-2-HG had no effect on the pH of the medium in the concentrations used in this study.

Stable U87 and U138 cell lines were used for mRNA expression analysis on U133 Plus2 arrays (Affymetrix, Santa Clara, CA). Data were normalized using both the MAS 5.0 (Affymetrix) and Robust Multi-array Average (RMA)[26] algorithms. Only genes were considered that (1) had a >20% dif IDH1^{R132H}-GFP ferent expression level in -expressing cells $_{IDH1}$wt-GFP compared to -expressing cells (in both U87 and U138), (2) had the same direction of difference between IDH1^{R132H}-GFP-expressing and GFP-expressing cells, and (3) had expression of the gene in both of the 2 compared groups (MAS 5.0 > 30 and RMA > 7.0).

For kinome analysis stable U87 cell lines were used. Pepchip kinomics arrays (Pepscan, Lelystad, the Netherlands) were hybridized according to the manufacturer's pro-

tocol and as described in Parikh et al[27] and Sikkema et al[28]). Pathway analysis was performed using the Ingenuity Pathways Analysis tool (Ingenuity Systems, Mountain View, CA). To compare groups, 2-sided Student t tests were used; otherwise, the statistical test used is stated in the text. For more detailed information see also Supporting Information Methods.

Results

Proliferation

Three independent cell lines (U87, U138, and HEK) were created that stably overexpress IDH1$^{R132H\text{-}GFP}$, IDH1$^{wt\text{-}GFP}$, or GFP. In all 3 cell lines, we observed a IDH1$^{R132H\text{-}GFP}$ decreased proliferation rate of the expressing cell lines compared to control (IDH1$^{wt\text{-}GFP}$expressing or GFP-expressing) cell lines. The decreased proliferation was observed by WST assays (Fig 1 and Supporting Information Fig 1) and confirmed by FACS cell cycle analysis (U87). For example, FACS analysis on exponentially growing cells showed that the percentages of cells in S-phase, G2, or mitosis were lower in IDH1$^{R132H\text{-}GFP}$IDH1$^{wt\text{-}GFP}$-expressing cells than in expressing or GFP-expressing cells (18.3% vs 22.5% and 22.4%, respectively, $p < 0.05$, Mann-Whitney U test). IDH1^{R132HGFP}-expressing cells also had concurrent fewer IDH1$^{wt\text{-}GFP}$

Ki67-positive cells compared to -expressing cells (33.6% and 45.5%) and had a reduced number of BrdU-positive (proliferating) cells in IDH1^{R132HGFP} compared to IDH1wt-GFP cells (17.5% vs 19.2%) as determined by FACS analysis. The decreased proliferation was not due to differences in apoptosis. Our data therefore demonstrate that established cell lines expressing IDH1^{R132H} show lower proliferation rates due to a reduced cell cycle activity.

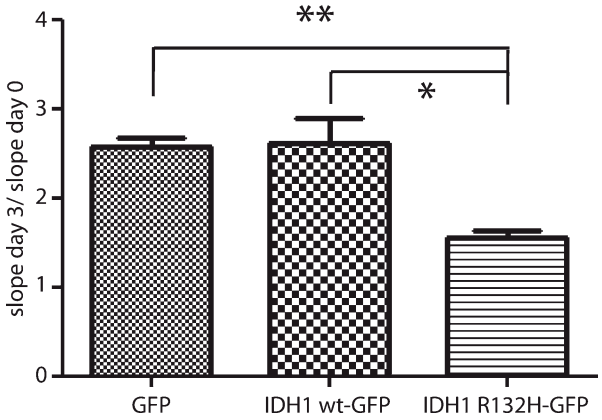

FIGURE 1: Glioma cells expressing mutant IDH1 show reduced proliferation in vitro. WST-1 proliferation data of stable U87 cells expressing gamma-fetoprotein (GFP), IDH1wt-GFP, or IDH1$^{R132H\text{-}GFP}$ are shown. Error bars represent the standard deviation. *$p < 0.05$, **$p < 0.01$. wt = wild type.

To determine whether the reduced proliferation rate of IDH1$^{R132H-GFP}$-expressing cells is also observed in vivo, we performed intracranial injection in mice with our 3 U87 cell lines. Mice injected with IDH1$^{R132H-GFP}$-expressing cells had significantly better survival (mean 40.3 6 6.9 days) than both the control groups injected with cells expressing either IDH1wt-GFP (mean 29 ± 5.8 days, p = 0.0012) or GFP (mean 28.6 ± 5.2 days, p = 0.00058) (Fig 2). The difference in survival was obvious, and histological analysis of 1 of the mice injected with IDH1$^{R132H-GFP}$-expressing cells revealed complete absence of tumor at 75 days, whereas the initial IVIS measurements did detect tumor burden (see Fig 2 and Supporting Information Fig 2). Immunohistochemistry showed a reduced GFP staining in some of the IDH1$^{R132H-GFP}$-induced but none of the IDH1^{wt-GFP}-induced tumors, indicating that IDH1$^{R132H-GFP}$ expression is, at least partially, lost in the absence of selection pressure (Supporting Information Fig 3). Mutant tumor cells were present as indicated by elevated D-2-HG levels (see below). The reduced proliferation of IDH1$^{R132H-GFP}$-expressing cells in vitro therefore is also apparent in vivo following intracranial injection of IDH1$^{R132H-GFP}$-expressing U87 cells.

To further investigate the cellular consequences of the IDH1^{R132H} mutation, we performed a cell migration assay. Cells expressing IDH1$^{R132H-GFP}$ showed a different and more

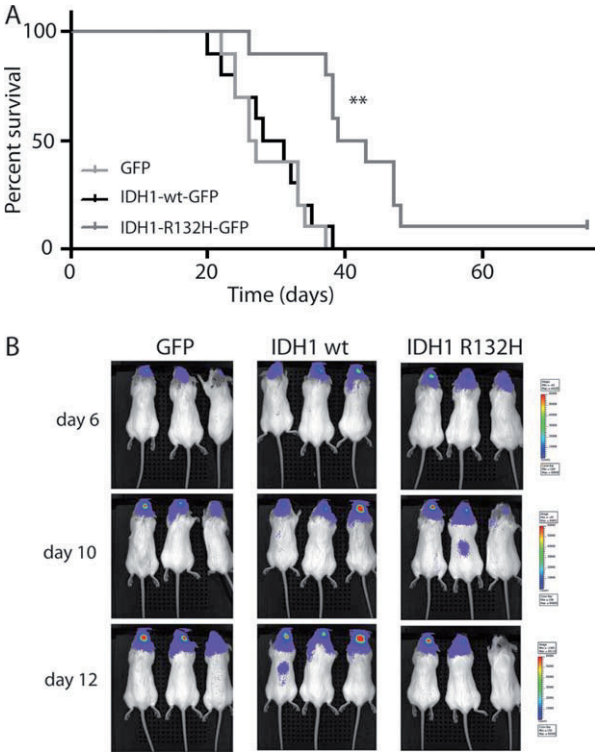

FIGURE 2: Glioma cells expressing mutant IDH1 show reduced proliferation in vivo. (A) Survival curve of 10 mice injected with U87-IDH1wt-GFP (black line), 10 mice injected with U87-IDH1$^{R132H-GFP}$ (dark gray line), and 10 mice injected with U87-GFP (light gray line). The mice injected with U87-IDH1$^{R132H-GFP}$ have a significantly longer survival ($p < 0.01$); 1 mouse survived >75 days. (B) IVIS imaging on day 6, 10, and 12 of mice injected with U87 cells expressing GFP (left), IDH1wt-GFP (middle), and IDH1R132H-GFP (right). Notice in the right panel that the initial tumor burden in the rightmost mouse decreases. GFP = gamma-fetoprotein; wt = wild type. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

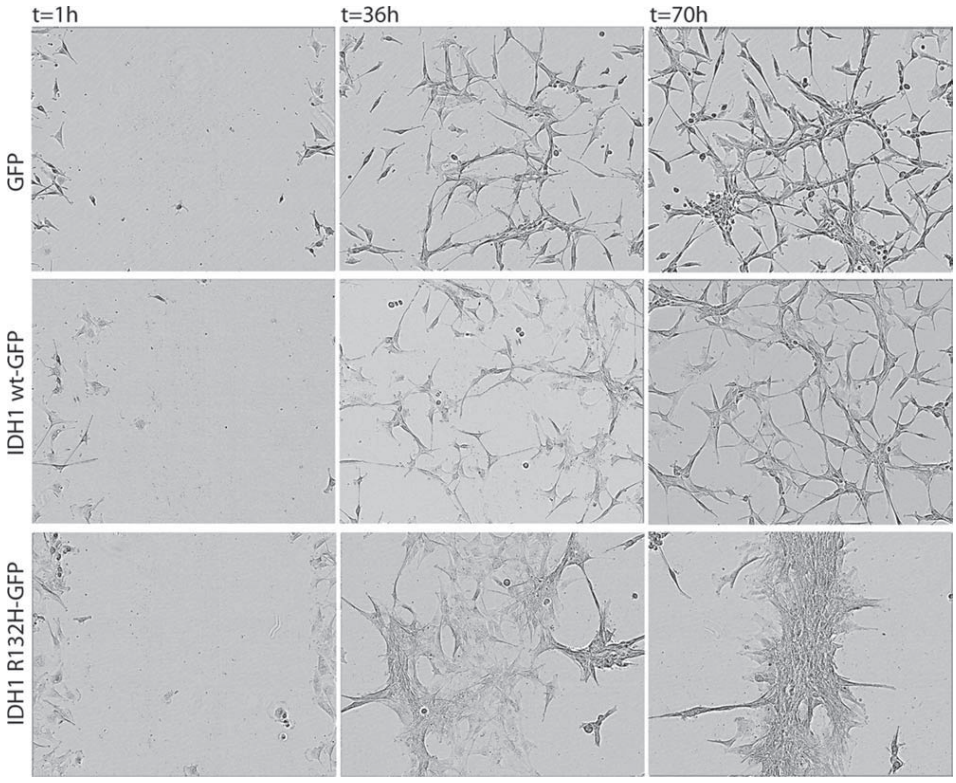

FIGURE 3: Glioma cells expressing mutant IDH1 have an altered migration pattern. Scratch assay results of U87 cells overexpressing gamma-fetoprotein (GFP), $IDH1^{wt\text{-}GFP}$, or $IDH1^{R132H\text{-}GFP}$ are shown. The U87 cells overexpressing $IDH1^{R132H\text{-}GFP}$ show a reducedmotility and cluster tightly together on the scratched surface area (see also Supporting Information Videos 1–3). wt = wild type.

clusterlike (adherent to other cells) migration pattern, ultimately resulting in a tightly squeezed line of cells in the middle of the scratched region (Fig 3 and Supporting Information Videos 1–3). When counting individual cells (n = 20) in the scratched region, the overall motility was significantly less in U87 cells expressing $IDH1^{R132H\text{-}GFP}$ than in U87 cells expressing $IDH1^{wt\text{-}GFP}$ or GFP (18.0 ± 2.1µm/h vs 26.3 ± 3.3 and 27.5 ± 3.9 respectively, both $p < 0.0001$). $IDH1^{R132H\text{-}GFP}$-expressing U87 cells also show an altered migration in vivo following intracranial injection; clusters of vimentin-positive infiltrative foci were approximately twice as frequent in the tumors expressing $IDH1^{R132H\text{-}GFP}$ as in those expressing $IDH1^{wt\text{-}GFP}$ in vivo (Fig 4). Such an increase in infiltrative foci in U87 cells has also been observed by others.[29] The $IDH1^{R132H}$ mutation therefore is not only associated with a reduced proliferation rate but also associated with an altered cellular migration pattern in an established glioma cell line. Whether these 2 are correlated remains to be investigated.

Altered Enzymatic Activity of IDH1$^{R132H\text{-}GFP}$ Is Increased in the Presence of IDH1$^{wt\text{-}GFP}$

Thus far, all reported enzymatic experiments have been performed using extracts of cells overexpressing wild-type or mutant IDH1.[15,18] Similar to the findings reported by these groups, our cell lines stably expressing IDH1^{R132HGFP} also show reduced enzymatic activity toward isocitrate and increased activity toward αKG compared to IDH1$^{wt\text{-}GFP}$ (Fig 5 and Supporting Information Figs 4 and 5). Patient-derived tumors with IDH1 mutations (n = 5, all R132H) also showed reduced enzymatic activity toward isocitrate (p = 0.037) (see Supporting Information Figs 5 and 6).

Subsequently we sought to examine whether the altered enzymatic activity of IDH1R132H could be recapitulated in a purified protein system with our constructs. For these experiments, we generated constructs in which a biotag was added to the N-terminal end of the protein. Biotagged constructs could then be purified following biotinylation and streptavidin-conjugated magnetic beads. As reported using extracts of cell lines, IDH1bio-wt-GFP had enzymatic activity toward isocitrate and not toward aKG and vice versa for IDH1bio-R132H-GFP. The activity was dose dependent for both isocitrate and enzyme concentration (not shown). A 1:1 mixture of IDH1wt-GFP and IDH1$^{R132H\text{-}GFP}$ showed intermediate activity toward isocitrate (see Fig 5). Surprisingly, in this purified protein system,

the IDH1$^{bio\text{-}R132H\text{-}GFP}$ activity was more active toward aKG in the presence of IDH1bio-wt-GFP enzyme. More αKG is converted in these mixture experiments, although they contained only half of the IDH1$^{bio\text{-}R132H\text{-}GFP}$ of the nonmixture experiments (all experiments contained equimolar amounts of enzyme). These results indicate that the presence of IDH1$^{bio\text{-}wt\text{-}GFP}$ increases the enzymatic activity of IDH1$^{bio\text{-}R132H\text{-}GFP}$ toward αKG.

2-Hydroxyglutarate Reduces Proliferation

Our next experiments were aimed at determining whether the reduced proliferation rate of IDH1$^{R132H\text{-}GFP}$-expressing cells is a consequence of the D-2-HG produced by the mutant enzyme. In our U87 cell lines, D-2-HG concentrations are strongly increased, both in cell pellets (68µmol/mg protein) and in the medium (55µmol/l) (Supporting Information

Fig 7). L-2-HG levels were not affected by IDH1$^{R132H\text{-}GFP}$ expression. Similar increases were observed in cell extracts of intracranially injected tumors (26.9µmol/l vs 1.35µmol/l, p = 0.014, see also Supporting Information Fig 7).

The concentrations of D-2-HG measured in pellets and media were used to spike this metabolite into the medium of U87 cells expressing GFP. Both D-2-HG and L-2-HG reduced the proliferation of these cells in a dose-dependent way as measured by FACS cell cycle analysis (Fig 6). The percentage of cells in G2-SM phase was reduced from 20.1% to 15.2% and 7.7% (p = 0.018 and p = 0.037, respectively) by the addition of 3

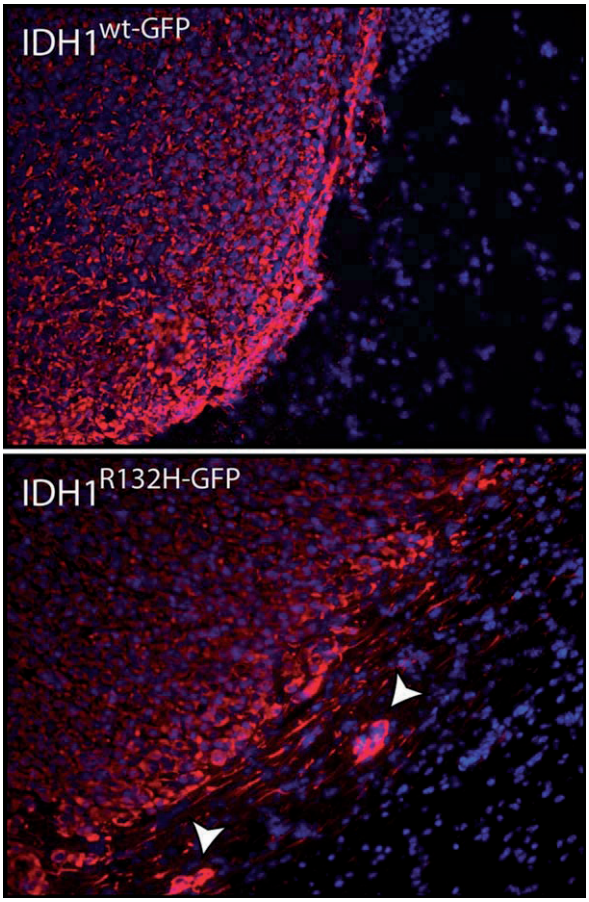

FIGURE 4: Glioma cells expressing mutant IDH1 have an altered migration pattern in vivo. Intracranially injected U87 cell tumors expressing $IDH1^{R132H\text{-}GFP}$ (bottom panel) are characterized by the presence of infiltrative foci (arrowheads). Such foci were observed much less frequently in tumors expressing $IDH1^{wt\text{-}GFP}$ (top panel). Red = vimentin; blue = 4,6-diamidino-2-phenylindole; wt = wild type; GFP = gammafetoprotein. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

and 6mmol/l D-2-HG. This was confirmed by WST analysis using 30mmol/l D-2-HG (46% decrease in growth compared to U87 without D-2-HG, $p < 0.001$). A reduction in cellular proliferation was also observed when performing a BrdU proliferation assay; the number of BrdU-positive U87 cells was reduced from 26.6% to 23.4% in the presence of 1.2mM D-2-HG. Similar percentages of BrdU-positive U87 cells have been observed by others.30 These results indicate that the reduced proliferation rate observed in U87 cells in vitro is at least partly mediated by the accumulation of D-2-HG.

The decrease in proliferation was not accompanied by an increase in apoptosis as determined by FACS analysis; the number of annexin V-positive cells in U87 cells was not increased in the presence of D-2-HG (5.5%, 5.2%, and 5.9% for 0.12, 0.6, and 1.2mM D-2-HG, respectively) compared to control U87 cells (6.9%). These results are in line with our observation that IDH1R132H-GFP-expressing cells do not show an increase in apoptosis (see above) despite having elevated levels of D-2-HG.

Downstream Pathway Analysis

It is unknown which oncogenic pathways are affected by IDH1^{R132H} and D-2-HG. We therefore performed expression profiling to further analyze the downstream pathways. Only 7 genes (Table) were identified that show an IDH1R132H-GFP-associated differential expression of >20% in both U87 and U138 cells. Ingenuity pathway analysis indicated that these genes are not significantly linked to any canonical pathway, but can be linked to a single network involved in tumor morphology, cellular development, and cellular growth and proliferation (Supporting Information Fig 8). However, the number of differentially expressed genes is too few to significantly address downstream pathway activation.

Kinome profiling on our 3 U87 cell lines was then performed to further examine the pathways affected by IDH1$^{R132H\text{-}GFP}$. Seven kinases were significantly differentially activated between IDH1$^{R132H\text{-}GFP}$-expressing cells and both IDH1$^{wt\text{-}GFP}$-expressing and GFP-expressing cells ($p < 0.05$) (see Table). One of the most differentially activated kinases was AKT1, showing less activity in IDH1R132H-GFP-expressing cells. Immunocytochemistry using phospho-AKT specific antibodies confirmed the reduced AKT1 activity in IDH1$^{R132H\text{-}GFP}$-expressing cells (Supporting Information Fig 9). As AKT1 activation is positively correlated with cell proliferation, kinome profiling confirms the reduced proliferation observed in IDH1R132H-GFP-expressing U87 cells.

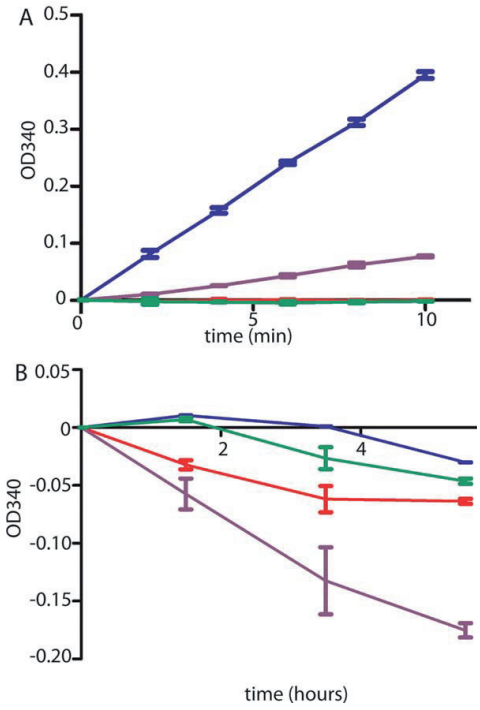

FIGURE 5: Wild-type (wt)–mutant IDH1 dimers are enzymatically more active toward a-ketoglutarate than mutant IDH1. (A) Enzymatic activity of purified enzyme as measured by nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) production (expressed by OD340) in the presence of isocitrate and NADP; IDH1$^{wt\text{-}GFP}$ (*blue line*) and IDH1$^{R132H\text{-}GFP}$ (*red line*) or a 1:1 mixture of both (*purple line*). Gamma-fetoprotein (GFP) (*green line*) is used as a control. (B) Enzymatic activity as measured by NADPH use in the presence of a-ketoglutarate of IDH1$^{wt\text{-}GFP}$ (*blue line*), IDH1$^{R132H\text{-}GFP}$ (*red line*), a 1:1 mixture of IDH1$^{wt\text{-}GFP}$ and IDH1$^{R132H\text{-}GFP}$ (*purple line*), and GFP (*green line*). Error bars represent the standard error of the mean.

Discussion

To our knowledge, we are the first to describe the cellular effects of IDH1R132H-GFP expression in established cell lines. Our data strongly indicate that IDH1^{R132HGFP} reduces the proliferation rate of various cell lines, as is demonstrated by WST assays, FACS cell cycle analysis, and Ki67 staining. These results were supported morphologically (visualized by a reduced population of elongated, actively dividing, cells) and by kinome profiling (as indicated by a reduced AKT activation). The reduced proliferation in vitro was confirmed in vivo following intracranial injection of IDH1$^{R132H-GFP}$-expressing cells.

It is possible that the reduced proliferation rate in established glioma cell lines explains why only 1 tumor cell line expressing IDH1^{R132H} has been reported,[31,32] although xenografts of glioblastomas with IDH1 mutations have been described.5 In fact, we examined 13 primary cell cultures on serum propagated from IDH1 mutated gliomas. No primary tumor cell culture bearing the IDH1 mutation could be established from any of these gliomas. A reduced presence of the IDH1 mutation was already visible in the first passage of these cells as a reduced peak on sequence chromatograms (R.B. and M.L., unpublished observations). However, a correlation between difficulties of culturing IDH1 mutated gliomas and reduced proliferation in established cell lines should be treated with caution, as many other factors might contribute to poor growth of (lower grade) gliomas in vitro.

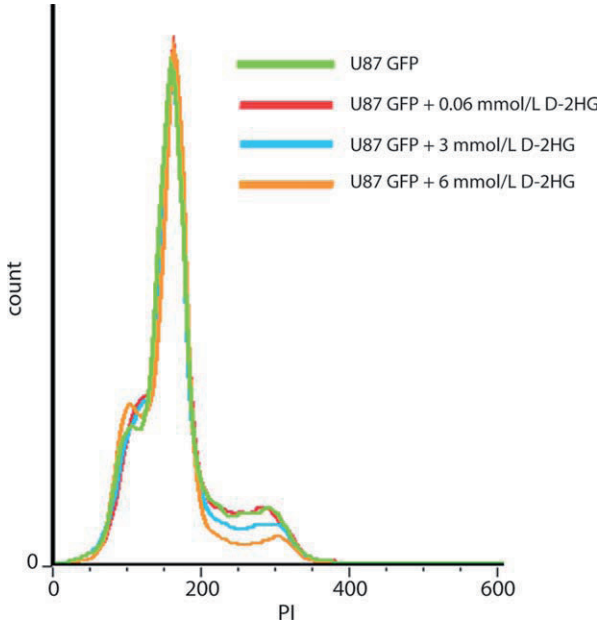

FIGURE 6: D-2-hydroxyglutarate (D-2HG) reduces proliferation of glioma cells. Dose-dependent effects of D-2-hydroxyglutarate on the cell cycle of U87 cells expressing gammafetoprotein (GFP) as measured by fluorescence-activated cell sorting are shown. PI = propidium iodide.

TABLE I: Differentially Expressed Genes and Activated Kinases in Mutant IDH1-Expressing Cell Lines

Gene	Expression in Mutant	Fold Change
BTG3	Overexpressed	1.672
CCNG1	Overexpressed	1.514
CD99	Overexpressed	1.727
SERPINE1	Overexpressed	2.865
HNRPDL	Underexpressed	0.714
PPP1CB	Underexpressed	0.703
TMED7	Underexpressed	0.540
Kinase	**Activation in Mutant**	**Fold Change**
Activin A receptor, type 1	Higher	2.15
MARK	Higher	1.62
AKT1	Lower	0.82
MAPK4	Lower	0.66
Ribosomal protein S6 alpha 4	Lower	0.64
SHP2	Lower	<<
TAO kinase 2	Lower	0.66

Gene = Differentially expressed genes in $IDH1^{R132H\text{-}GFP}$-expressing cell lines (U87 and U138) compared to $IDH1^{wt\text{-}GFP}$ expressing cell lines. Kinase = Differentially activated kinases in IDH1R132H-GFP-expressing U87 cell lines compared to $IDH1^{wt\text{-}GFP}$-expressing and GFP-expressing U87 cell lines. < = no expression detectable anymore. wt = wild type; GFP = gamma-fetoprotein.

It is interesting that the effects of $IDH1^{R132H}$ were so apparent although the mutation was introduced into highly malignant glioma cell lines. This demonstrates the dominant nature of mutant IDH1 in established cell lines. If mutant IDH1 is also causal for reduced proliferation in gliomas, it might explain why $IDH1^{R132H}$ is inversely correlated with tumor grade3,4 and why $IDH1^{R132H}$ is an independent favorable prognostic marker in glioma patients1,4 However, it is possible that the effect of $IDH1^{R132H}$ expression observed in this study is associated with its expression in highly malignant cell lines that have a distinct set of genetic changes (see, eg, Clark et al[33]). Therefore, the effect of $IDH1^{R132H}$ expression on nonmalignant cell types, low-grade gliomas, and/or cancer stem cells remains to be determined.

Our results appear contradictory, with a presumed oncogenic role of IDH1R132H and D-2-HG as an oncometabolite.[20,22,34] However, our results may be explained if IDH1 mutation only has a favorable role in tumor initiation, but is unfavorable for tumor progression. Indeed, $IDH1^{R132H}$ is the first hit,7 does not accumulate secondary to tumor formation, and anecdotally is lost in part of the tumor after malignant progression.35

However, our results may also be explained if IDH1R132H does not contribute to tumor formation, or if effects observed in this study are specific to the cell lines used. A causal role has not yet been established. Alternative functions of $IDH1^{R132H}$ can also explain a possible role in oncogenesis.

A similar discussion can be held on D-2-HG as an oncometabolite. IDH1 or IDH2 mutations result in an increase in D-2-HG in serum and tumors of AML21,22 and glioma20 patients (see also Supporting Information Fig 7), whereas we demonstrate that D-2-HG reduces the proliferation rate of glioma cells. These results appear contradictory to a presumed role of D-2-HG as an oncometabolite, but it is possible that D-2-HG only plays a role in tumor initiation. However, accumulation of D-2-HG is also not associated with brain tumor formation (whereas L-2-HG is),36 which might suggest D-2-HG has no tumor-initiating capacities and is solely an inevitable byproduct. If so, mutated IDH1 or IDH2 has oncogenic properties not associated with D-2-HG. It has been reported that the IDH1 and IDH2 mutation spectra differ across the various diseases (glioma, AML and D-2-HG aciduria).6,13,36,37 It is tempting to speculate that these oncogenic properties vary across the different IDH1 and IDH2 mutations.

To further elucidate a possible function of mutant IDH1, we have recently performed methylation profiling (Infinium Human methylation 27 arrays [Illumina, San Diego, CA]) on a panel of 68 anaplastic oligodendrogliomas and oligoastrocytomas (P.J.F., manuscript in preparation). In this study, the CpG island methylator phenotype (CIMP) was highly correlated with the presence of IDH1 mutation. These data are in line with the recently suggested hypothesis that IDH1 induces promoter methylation.[38] However, not all IDH1 mutated tumors are CIMP positive, nor is the effector gene TET2 differentially expressed between IDH1wt-GFP-expressing and $IDH1^{R132H\text{-}GFP}$-expressing U87 and U138 cells. It therefore remains to be determined whether IDH1 mutations also result in promoter methylation in glioma.

Our purified enzyme system allowed us to examine the properties of wild-type–mutant enzyme mixtures. Interestingly, our results show that the 1:1 mixture of $IDH1^{bio\text{-}wt\text{-}GFP}$-expressing and $IDH1^{bio\text{-}R132H\text{-}GFP}$-expressing cells has the highest reductive enzymatic activity.

The altered enzymatic activity therefore is stimulated by the presence of wild-type enzyme. To our knowledge, this is the first paper to examine the altered enzymatic activity of $IDH1^{R132H\text{-}GFP}$ in a purified protein system. Others have studied and reported on IDH1 mutated enzymes from cell lysates that also contain endogenous IDH1[22] or have not tested the enzymatic activity toward aKG of the combination of IDH1 mutant and wild-type enzyme.[19,21] The increased alternative enzyme activity could be explained by the fact that IDH1 functions as a dimer. In this model, the aKG produced by wild-type IDH1 in wild-type–mutant heterodimers improves substrate availability for mutant IDH1. Alternatively, heterodimers confer a more active conformational state of mutant IDH1.

IDH1 can only function as a dimer,[1] and therefore it is possible that wt-IDH1 enzymes in wt-mutant IDH1 heterodimers may induce a different conformational state than IDH1 mutant homodimers. Such an altered state can result in increased activity of the mutant enzyme. The increased alternative enzyme activity of the wild-type–mutant combination is in line with the observation that virtually all IDH1 mutations are heterozygous.

In conclusion, $IDH1^{R132H\text{-}GFP}$-expressing cell lines show a reduced proliferation rate in vitro and better survival in vivo. Our results obtained in model systems of glioma may help explain the biology of IDH1 mutated gliomas, which are typically low grade and slow growing.

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Chapter 7

Absence of Common Somatic Alterations in Genes on 1p and 19q in Oligodendrogliomas

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Abstract

A common and histologically well defined subtype of glioma are the oligodendroglial brain tumors. Approximately 70% of all oligodendrogliomas have a combined loss of the entire 1p and 19q chromosomal arms. This remarkably high frequency suggests that the remaining arms harbor yet to be identified tumor suppressor genes. Identification of these causal genetic changes in oligodendrogliomas is important because they form direct targets for treatment. In this study we therefore performed targeted resequencing of all exons, microRNAs, splice sites and promoter regions residing on 1p and 19q on 7 oligodendrogliomas and 4 matched controls. Only one missense mutation was identified in a single sample in the ARHGEF16 gene. This mutation lies within-and disrupts the conserved PDZ binding domain. No similar ARHGEF16 mutations or deletions were found in a larger set of oligodendrogliomas. The absence of common somatic changes within genes located on 1p and 19q in three out of four samples indicates that no additional ''second hit'' is required to drive oncogenic transformation on either chromosomal arm.

Introduction

A common and histologically well defined subtype of glioma are the oligodendroglial brain tumors. Oligodendrogliomas differ from the other glioma subtypes in clinical behavior with respect to overall prognosis (median survival 3 years versus less than one year) and a relatively better and longer lived response to chemotherapy and radiotherapy [1–3]. Oligodendrogliomas have clearly distinct gene expression profiles [4–6] and are also cytogenetically distinct: approximately 70% of all oligodendrogliomas have a combined loss of the entire short arm of chromosome 1 (1p) and loss of the entire long arm of chromosome 19 (19q) [1,3–5,7]. Loss of these chromosomal arms in oligodendrogliomas is highly correlated with chemosensitivity; approximately 80–90% of oligodendroglial tumors with LOH (loss of heterozygosity) on 1p and 19q respond to chemotherapy [1,2,8]. Conversely only 25– 30% of tumors that have retained the short arm of chromosome 1p are sensitive to chemotherapy. In summary, oligdendrogliomas are a clinically, histologically, cytogenetically and molecularly distinct and well defined subgroup of glioma.

In spite of these clearly distinct clinical, histological and molecular features, little is known on the genetic changes that drive these tumors. Thusfar, IDH1/IDH2 (70%) and, to a much lesser extent, TP53 (15–25%) and PIK3CA (10–15%) are the only genes that are mutated at significant frequency in this tumor type [9–15]. The remarkably high frequency of LOH of 1p and 19q suggests the remaining arms harbor yet to be identified tumor suppressor genes (Knudson two-hit hypothesis [16]). Identification of the causal genetic changes is important because they form direct targets for treatment: Tumor growth depends on these acquiredsomatic-changes both in oncogenes (‘‘oncogene addiction’’ [17]) and in tumor suppressor genes [18,19]. In this study we therefore aimed to identify genetic changes in all exons, microRNAs, splice sites and promoter regions on 1p or 19q using array capture and Next Generation Sequencing. Experiments were performed on 7 oligodendrogliomas and 4 had matched control DNA samples.

Materials and Methods

Glioma samples were collected from the Erasmus MC tumor archive. Samples were collected immediately after surgical resection, snap frozen, and stored at 280uC. The use of patient material was approved of by the Institutional Review Board of the Erasmus MC, Rotterdam, the Netherlands (nr MEC 221.520/2002/262; date of approval July 22, 2003, and MEC-2005-057, date of approval February 14, 2005). For this use, patients gave written informed consent according to institutional and national guidelines.

All oligodendrogliomas used (n = 7) had proven loss of 1p and 19q as assessed by SNP 6.0 or 250 k NspI arrays (both Affymetrix, Santa Clara, USA) [7] and highly similar RNA expression profiles (i.e. belong to the same molecular subgroup) [6]. Control DNA was available in 4/7 cases. The candidate variations of 4 samples were used for validation experiments. By using 4 samples we have a 76.0% chance of identifying each mutation with a frequency of 30%. DNA was amplified using a Repli-G midi kit (Qiagen, Venlo, the Netherlands) to ensure sufficient DNA amounts. Patient characteristics are listed in table 1.

Capture arrays (Nimblegen, Roche NimbleGen, Inc., Waldkraiburg, Germany) were designed to enrich for all exons, miRNAs, splice sites (defined as 10 bp up-or downstream of a coding exon) and promoter regions (defined as 100 bp upstream of a transcript) of transcripts present in Refseq, Ensembl or Vega based on the NCBI36/hg18 build. Two capture arrays were designed covering around 5 million bp of sequence each. We were able to design capture probes for 96.0% and 94.4% of all regions on chr 1 and chr 19 respectively, remaining sequences contained non unique sequences (.5 fold presence in the human genome). Amplified DNA samples were fragmented by sonication, end repaired and ligated to paired end adaptors. Samples were size selected (300 bp), enriched for 1p and 19q by array capture, PCRamplified and 76 bp paired end sequenced using the Illumina GA2x sequencer. The Illumine Casava pipeline was used for base calling and quality control.

The CLC Bio Genomics Workbench (Aarhus, Denmark) was used to align sequence reads against the reference genome. All the experiments were successful except for one of the two capture arrays of sample 229, which was omitted from the analysis. We defined reads subject for mutational analysis as being covered at least 7 times in the tumor sample and at least 8 times in the matching control sample. This coverage was set deliberately low (often $30 fold coverage is used) and was aimed to include as many evaluable targeted regions as possible.

Single Nucleotide Variants (SNVs) and Deletion or Insertion Variants (DIVs) were detected by the CLC Bio Genomic Workbench and filtered using the following criteria (coverage of at least 7 (tumor) or 8 (ctr), variant frequency at least 70% (tumor) or 30% (controls). The difference in variant frequency between tumors and normals is because

only homozygous changes are expected in the tumor (because of the 1p19q LOH). To allow for tumor heterogeneity (presence of non-neoplastic tissue) and stochastic effects (allele specific sequencing) these percentages were set lower than 100% and 50%.

Results

The mean total number of matched bp sequenced per sample was 2.89 billion (1.17 billion in sample 229). Of these, 73.5% was on our targeted regions (range 70.4–84.8%, one outlier at 41.3%) confirming capture efficiency. The coverage of our targeted regions was at least 7 in 96.5% of our target regions (range 87.6– 98.9) (see also figure 1). Coverage of genes suggested to be involved in (oligodendro-) glioma genesis CAMTA1 [20], EMP3 [21], CHD5 [22], DIRAS3 [23] and PLA2G4C [24] is listed in table 2.

We first calculated the tumor percentage of all samples. The tumor content can be estimated by the observed B allele frequency of SNPs using tumor samples only. For example, in case of 50% tumor, the observed B allele frequency would amount to 66.7% (in case of LOH in the tumor). In our sample cohort, the observed B allele frequency was 91.2–97.3% (except for sample 11 with an observed frequency of 85.0%), corresponding to 90.3–97.2% tumor (82.4% for sample 11). A tumor content $82.4% indicates that a mutant allele frequency of 70% (corresponding to a tumor percentage of 82.4%) is a suitable value as detection cutoff for the mutant allele frequency.

Table 1. Patient characteristics of all tumor samples.

Sample	Gender	Diagnosis	Age	KPS	Surgery	RT	CT	Alive	Surv (years)
8	F	OD III	44	100	PR	yes	no	Dead	9.82
11	M	OD III	38		CR	yes	no	Dead	8.92
13	M	OD III	33	90	PR			Dead	8.59
21	M	OD III	31	100	PR	yes	no	Dead	6.81
23	F	OD III	44	90	CR	yes	no	Dead	8.12
229	M	OA III	35	90	CR	yes	Adj PCV	Alive	6.8
538	F	OD II	44	80	SB			Alive	3.27

OD = oligodendroglioma, OA = oligoastrocytoma, KPS = Karnofsky performance score, PCV = procarbazine, lomustine, vincristine.

F = female, M= male, OD = oligodendroglioma, OA = oligoastrocytoma, grades II or III. Age = age at diagnosis. KPS = Karnofsky performance score, PCV =

procarbazine, lomustine, vincristine. Surgery types: PR = partial resection, CR = complete resection, SB = stereotactic biopsy. RT = radiotherapy, CT = Chemotherapy.

We then prioritized all changes identified based on their associated function into: tier 1 (coding exons, splice sites and miRNAs), tier 2 (promoters and UTRs), tier 3 (intronic regions) and tier 4 (SNPs and personal SNPs). In tiers 1, 2, 3 and 4 we identified 431, 1380, 16293 and 72466 SNVs and DIVs respectively. A high number of all variants present in tier 4 were not present in dbSNP130, and are likely to reflect personal SNPs and sequencing artefacts (see also table 3). We then performed direct sequencing on all tier 1 candidates and all candidates within promoter regions (part of tier 2). 505 of the 514 sequence reactions were successful; only 2/9 unsuccessful candidates were predicted to result in a change in the primary protein sequence (both missense mutations).

Of the 514 candidate variants 77% (n = 394) were not confirmed on tumor DNA using direct sequencing (false positive). Such variants likely represent amplification artefacts (due to e.g. whole genome amplification or the post capture PCR amplification) and/or sequencing artefacts (e.g. sequencing errors). A further 21% (n = 110) could be confirmed in the tumor samples, but the variant was also present in the matched control DNA. These variants may represent selective allele amplification and sequencing. In summary, of the 514 candidates subject to direct sequencing, one variant was validated. This variant is a missense mutation (c.2125 G.A) (figure 2a) and affects the last amino acid (p.V709M) of ARHGEF16 (RefSeq: NM_014448.3) in sample 8. It should be noted that the absence of trace wt sequence in the chromatogram confirms the high tumor percentage in this sample. The base is highly conserved (GERP conservation score 3.35 [25]. This amino acid is located within a PDZ-binding domain (ETDV, a protein-protein interaction domain). However, it remains to be determined whether the identified mutation affects its RhoA guanine exchange function and oncogenic transformation potential [26].

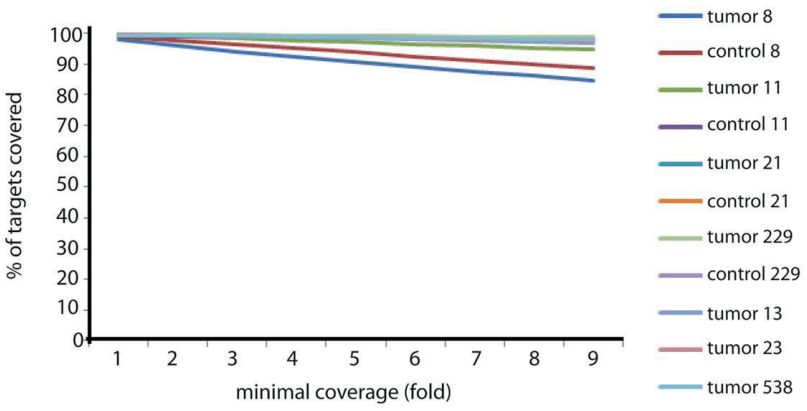

Figure 1. Coverage plot of all samples. Depicted is the percentage of targeted bases (y-axis) that is covered at least n times (x-axis) per sample.

None of the other 6 samples contained changes in the coding sequence of ARHGEF16. In addition, we failed to identify mutations in the last exon of ARHGEF16 in an additional 32 samples from the same molecular cluster [6] using direct sequencing. No small homozygous deletions were identified on SNP 6.0 and 250 k Nsp arrays from 23 oligodendrogliomas [7]. The ARHGEF16 promoter does show hypermethylation on Infinium Methylation arrays (Illumina, San Diego, USA), on 68 anaplastic oligodendrogliomas and oligoastrocytomas; PF, manuscript in prep) and is correlated with loss of 1p and 19q ($p = 0.035$, Fisher exact test). Data are listed in table 4. In addition, tumors with hypermethylated ARHGEF16 promoter have a better survival (5.62 years versus 1.31 years; p,0.0001) (figure 2b). Promoter methylation of ARHGEF16 may therefore be involved in the formation of gliomas with loss of 1p and 19q.

Table 2. Per base coverage of known candidate genes in oligodendrogliomas located on 1p or 19q.

Sample		CAMTA	CHD5	DIRAS3	PLA2G4C	EMP3
8	cov	200.1	264.4	107.2	32.3	57.0
	min	0	5	26	2	15
	max	896	1198	186	87	131
	% covered	96.2	99.6	100	93.1	100
11	cov	58.6	20.2	127.0	79.0	90.2
	min	0	0	12	7	33
	max	275	107	231	214	183
	% covered	93.5	82.1	100	100	100
13	cov	184.6	135.8	98.5	183.8	343.1
	min	0	0	14	24	126
	max	562	677	178	447	625
	% covered	98.2	94.0	100	100	100
21	cov	163.8	77.7	208.1	202.8	281.3
	min	0	2	24	50	95
	max	527	329	360	449	599
	% covered	96.8	98.9	100	100	100
23	cov	178.8	69.0	245.8	189.4	202.3
	min	0	0	35	21	70
	max	531	353	468	487	374
	% covered	95.9	89.9	100	100	100

Min/Max: lowest/highest coverage, % covered: the percentage of bases sequenced at least 7 times (the cutoff used for our analysis).

Table 3. Candidate genetic variations after filtering in all samples with controls.

SNVs			tier 1			tier 2		tier 3	tier 4	
sample	array	chr	exons	miRNA	spl site	prom	UTRs	introns	SNVs dbSNP	personal SNVs
8	1	1	13	0	4	9	96	97	4312	1577
	2	1	33	0	3	3	218	38	1020	841
	2	19	19	0	0	6	37	23	1350	1120
11	1	1	80	0	3	4	110	68	3342	852
	2	1	14	0	2	5	110	204	1787	3400
	2	19	108	0	1	3	59	369	2703	4146
21	1	1	17	0	2	4	92	891	4450	1095
	2	1	13	0	0	7	94	3166	2110	7892
	2	19	25	0	0	4	63	3887	3090	9028
229	2	1	20	0	3	9	144	3505	146	3887
	2	19	44	0	2	5	135	3543	287	13213
DIVs			tier 1			tier 2		tier 3	tier 4	
sample	array	chr	exons	miRNA	spl site	prom	UTRs	introns	DIVs dbSNP	personal DIVs
8	1	1	0	0	10	11	60	79	179	0
	2	1	0	0	2	1	6	3	18	0
	2	19	1	0	0	1	9	7	54	0
11	1	1	0	0	6	6	27	51	118	1
	2	1	0	0	0	0	1	0	1	0
	2	19	0	0	0	0	0	0	0	0
21	1	1	0	0	0	0	0	0	0	0
	2	1	0	0	0	0	0	1	0	0
	2	19	0	0	0	0	0	0	0	0
229	2	1	2	0	0	5	24	134	181	28
	2	19	3	0	1	0	12	227	177	61

UTR = untranslated region, SNV = single nucleotide variation, DIV = deletion/insertion variation, chr = chromosome, array = capture array, spl site = splice site, prom = promoter.

Discussion

We have systematically sequenced all exons, miRNAs, splice sites and promoter regions on 1p and 19q. Of the 514 candidate variants in coding exons, miRNAs, splice sites and promoter regions, only one was validated: a missense mutation in ARHGEF16 affecting the PDZ-binding domain. ARHGEF16 lies on 1p36 a region that is commonly deleted in gliomas [20,22,27]. However, no other genetic changes were detected in the ARHGEF16 gene in a panel of 32 additional oligodendrogliomas, though the promoter is frequently hypermethylated. Future experiments should determine whether this specific mutation contributes to the pathogenesis of the disease.

In our sample cohort, only one somatic mutation in a single sample was identified among the ,10^{7} bases of sequence evaluated. The overall mutation rate in oligodendrogliomas therefore is at least an order of magnitude lower than reported for many other cancer types including glioblastomas [12,28,29]. Recent reports however, have highlighted tumor types that also have a very low somatic mutation rate [30,31].

One important observation by our study is the fact that in 3 out of the four samples examined no genomic second hit was found in any of the screened regions. Our data therefore indicate that no additional ''second hit'' is required on these chromosomal arms to drive oncogenic transformation. It is possible that a second hit is present on the remaining alleles but has escaped detection (e.g. due to a skewed distribution in sequencing of the non-neoplastic alleles derived from ''contaminating'' normal tissue) or is located in regions not captured or covered by our capture array. Alternatively, promoter methylation and/or haploinsufficiency of one or more genes could drive oncogenesis in oligodendrogliomas with loss of 1p and 19q. In line with this hypothesis is the high frequency of IDH1 mutations in oligodendrogliomas with 1p and 19q LOH (see e.g. [32]) and the observation that IDH1 mutations induce chromatin remodeling and promoter hypermethylation [33,34]. IDH1 mutations are associated with the CpG island hypermethylation phenotype (MvdB and PF, submitted), and promoter hypermethylation in cancer often occurs in the promoter regions of tumor suppressor genes [35,36]. Promoter hypermethylation of tumor suppressor genes in combination with somatic mutations in a limited number of genes therefore may drive oncogenesis in oligodendrogliomas with 1p and 19q LOH.

Table 4. Percentage methylation of two different CpG sites (cg24919884 and cg02737335) within the ARHGEF16 locus.

	ARHGEF16								
Sample	cg24919884	cg02737335	survival	censoring	Sample	cg24919884	cg02737335	survival	censoring
1	0.767	0.69	1.104	1	42	0.874	0.817	1.633	1
10	0.642	0.754	1.071	1	43	0.691	0.794	1.208	1
11	0.777	0.829	4.008	0	44	0.788	0.739	1.063	1
12	0.657	0.285	3.408	0	45	0.879	0.845	7.734	0
13	0.251	0.313	2.548	1	47	0.565	0.663	1.31	1
14	0.828	0.831	0.775	1	48	0.81	0.821	0.523	1
15	0.773	0.771	0.326	1	49	0.876	0.84	5.003	1
16	0.565	0.737	1.663	1	5	0.617	0.813	2.732	1
17	0.867	0.872	3.54	1	50	0.623	0.8	0.195	1
18	0.487	0.609	1.416	1	51	0.866	0.802	5.814	1
19	0.706	0.688	0.819	1	52	0.855	0.834	8.227	0
20	0.681	0.688	1.625	1	53	0.862	0.835	2.997	1
21	0.851	0.823	2.485	1	54	0.439	0.469	1.34	1
22	0.878	0.829	2.627	0	55	0.879	0.824	8.036	1
23	0.835	0.822	0.625	1	56	0.852	0.831	8.233	0
24	0.857	0.849	5.827	0	57	0.704	0.4	0.707	1
25	0.412	0.729	1.625	1	58	0.836	0.778	0.975	1
26	0.615	0.646	1.447	1	59	0.846	0.824	2.022	1
27	0.821	0.854	6.17	0	6	0.503	0.468	1.014	1
28	0.773	0.818	1.236	1	60	0.856	0.85	3.038	1
29	0.635	0.712	0.548	1	62	0.817	0.819	0.997	0
3	0.888	0.861	1.222	1	63	0.882	0.83	6.992	0
30	0.883	0.874	4.26	1	64	0.803	0.758	0.258	1
31	0.439	0.635	1.134	1	65	0.88	0.854	5.019	1

32	0.851	0.811	3.488	0	66	0.819	0.798	3.304	1
33	0.415	0.53	4.849	0	67	0.527	0.752	0.537	1
34	0.858	0.826	3.488	0	68	0.884	0.844	5.622	1
35	0.882	0.857	6.874	0	69	0.847	0.813	15.819	1
36	0.853	0.802	0.348	1	7	0.494	0.692	1.616	1
37	0.866	0.83	6.71	0	70	0.843	0.841	18.715	1
39	0.839	0.809	2.836	1	73	0.465	0.631	3.652	1
4	0.909	0.815	6.31	0	74	0.723	0.625	1.175	1
40	0.413	0.63	7.526	1	8	0.307	0.715	1.345	1
41	0.834	0.721	3.795	1	9	0.835	0.817	2.038	1

Values correspond to the fraction of methylation (scale 0–1).

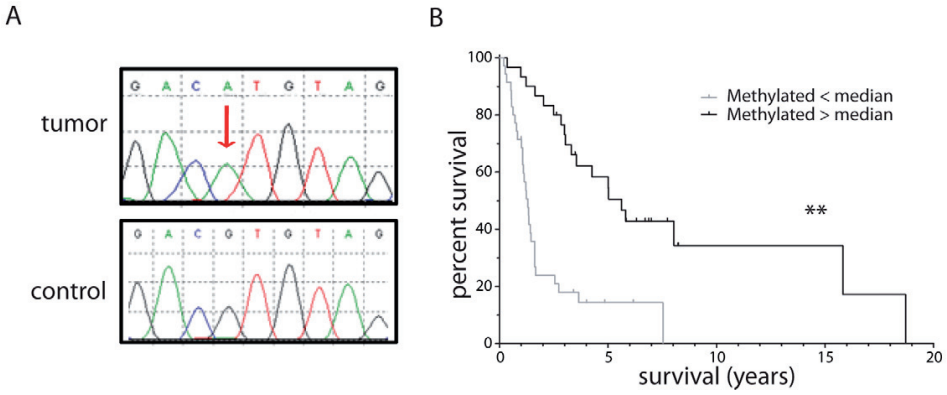

Figure 2. ARHGEF16 (RefSeq: NM_014448.3) mutation and promoter methylation. A; Upper lane: part of the sequence of ARHGEF16 with the missense mutation (2125G-.A) in tumor sample 8. Lower lane: sequence of the same region of ARHGEF16 in the matching control DNA. B; Kaplan Meier survival curve of oligodendrogliomas (n = 39) and oligoastrocytomas (n = 11) with unmethylated ARHGEF16 (, median) (black line) or methylated ARHGEF16 (. median) (grey line). ** = p,0.01.

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Concluding remarks and future directions

In this thesis novel methods to screen for translocations are described and one candidate gene, the *CASPR2* gene, was functionally analyzed and was found to be a tumor suppressor gene in glioma. We found that a subgroup of the recently discovered R132 mutations in *IDH1* (the non-p.R132H mutations) are segregated in distinct molecular subgroups. The functional analysis of the R132H mutation in the *IDH1* gene showed that the expression of this mutated gene in glioma cell lines decreases proliferation *in vitro* and also *in vivo*. Our next generation sequencing mutation screen in oligodendrogliomas with loss of 1p and 19q showed no additional mutations above detection levels in genes on the remaining alleles.

The last decade, mRNA expression- and SNP arrays have produced large amounts of data that have resulted in a much greater insight in glioma genomics and genetics (see also chapter 3 of this thesis). More recently, large scale sequencing efforts have revealed an even greater insight into the biology of gliomas (Parsons, Science 2008, Bettegowda, Science 2011). Caveats of this deep sequencing techniques are that allele specific sequencing, regions poorly covered and the mutation detection algorithm may all result in false negative mutation calls, as was the case in our mutation screen (see chapter 7).

The rapid development of novel sequencing techniques will lead to even more genetic data in the next decade. Therefore, it can be expected that virtually all genetic changes in all glioma subtypes will be identified in the near future.

As the knowledge of all regions of the DNA expands (e.g. think about the discovery of miRNAs), also the value of the changes in what is now called “junk” DNA will be clarified (Pink, RNA 2011). In spite of the importance of whole genome sequencing, it has to be taken into account that genetic alterations are at the beginning of mRNA and protein level changes. Epigenetic alterations (e.g. methylation (Noushmehr, Cancer Cell 2010)), and post transcriptional and translational modifications of proteins can have their effects on cell behavior and in that way can contribute to cancer formation.

Cancer is thought of as a genetic disease and before normal cells become malignant tumor cells they need genetic alterations in genes in several pathways, like pathways involved in growth and apoptosis. Logically, the genetic alterations in these genes and their effects are good targets for treatment. For example, in leukemia this resulted in the use of imatinib, which is a specific tyrosine kinase inhibitor that works on leukemic cells that express the BCR-ABL fusion protein (Buchdunger, Cancer Res 1996).

One of the emerging concepts that came out of genome sequencing efforts is that not all genetic changes in gliomas (or other tumors) are causal for the disease. These so-called "passenger" mutations can arise during cell division and are then found throughout the tumor due to clonal expansion (Greenman, Nature 2007). In fact, the majority of somatic mutations in cancer may be such "passenger" mutations (Greenman, Nature 2007). Distinguishing the causal "driver" mutations from such "passenger" mutations is therefore required and will be a major focus of cancer research in the near future.

One way to distinguish driver from passenger mutations is by frequency analysis: causal genetic changes are thought to occur at a higher incidence than predicted by chance. This strategy is used in the studies described in chapter 3, 4, 5 and 7 of this thesis. Specifically, in chapter 5 we show that frequency analysis within different glioma subtypes (molecular and histological) can also give insight into probable functional differences of the distinct mutation types within a gene. However, few genetic changes are recurrent events (so-called "mountains" in the genomic landscape of tumors (Wood, Science 2007)); only a handful of genes are mutated at frequencies >10% not only in gliomas (TCGA, Nature 2008; Parsons, Science 2008; Greenman, Nature 2007) but also in many other cancer types (Sjoblom, Science 2006; Parsons, Science 2010). These studies show that many more genes are mutated at low frequencies (so-called "hills"). In chapter 4 we show that such infrequent candidates can contribute to tumor formation and/or progression and therefore can give insight into the process of tumorigenesis.

At this moment the functional analysis is too low throughput for all future genetic alterations to be analyzed. The technical revolution that has already taken place in the field of the detection of genetic alterations will have to spread to the functional analysis of these genes. This high throughput functional testing will be the next necessary step in the process to distinguish the driver from the passenger mutations (Lecault, Nature Methods 2011). Fast or automatic techniques for genetic manipulation of cell lines have to be developed as current RNAi knockdown techniques and expression of mutated transcripts are time consuming (Fujita, Methods Mol Biol 2010).

However, each model system has its own limitations, and it is possible that a specific genetic change in one cell type has no effect in another, or that an effect *in vivo* is not observed *in vitro.* As most commonly used glioma cell lines are high grade cell lines and have been cultured for many passages, genes that could be oncogenic in normal cells or low grade cell cultures, could not have an effect or (as we demonstrate in chapter 6) even an inverse effect on these high grade cell cultures.

The best cell culture systems to test the effect of a gene on tumorigenesis is an oligodendroglial or astrocytic cell culture which only has a few oncogenic hits. Only then the effect on tumorigenesis can be established. The additional effect of an oncogene or the loss of a tumor suppressor gene on the proliferation rate of tumor cells could also

be usefully tested in cell lines of the tumor in which the alteration is discovered. This will mean that efficient systematic large scale projects have to be undertaken.

Eventually, the effect of oncogenes and the loss of tumor suppressor genes has to be tested in the normal environment of tumor cells. We know that the surrounding stroma is also involved in tumorigenesis (Jones, Oncogene 2011) as well as in angiogenesis and in the immune reaction against tumor cells. Combined tumor and stroma cell cultures can be useful, but good animal models are necessary to make sure all microenvironmental factors are taken into account.

Many infrequent "hills" could indicate that each tumor has its own unique spectrum of causal genetic changes. Treatments aimed at targeting these individual genetic changes may therefore be difficult. There are several ways to approach this problem. Firstly, it is likely that different genetic changes are part of a select set of molecular pathways (TCGA, Nature 2008). Therefore, pathway inhibition or reactivation can be used to target a broader range of tumors. Because of the fast evolutionary selection that is taking place in tumor growth (Navin, Nature 2011), a cocktail of targeted therapies will probably give the most promising results on tumor control. However, the problem of toxicity to normal cells has to be taken into account (for example see Reardon, Cancer Chemother Pharmacol 2011). Good local administration of the drugs is an important factor in preventing unwanted toxicity, but a trade-off with the number of invasive tumor cells reached by the drugs is unavoidable.

Apart from small molecule inhibition based on genetic changes, we can also use the tumor specific genetic alterations and the proteins they produce to target the tumor cells with specific antibodies or oncolytic viruses. For such targeting, however, only alterations in proteins that are expressed on the cell surface can be used. A recent example of such a specific antibody is the EGFRVIII antibody CH12 (Jiang, J Biol Chem 2011)**.** The big advantage of this tumor specific approach is the theoretically low toxicity to normal cells.

In conclusion, large scale sequencing projects will unravel all genetic alterations in all different subtypes of glioma. The future challenge will be to translate these findings into clinical practice. For this we need to perform systematic functional analysis of these genetic alterations in appropriate cell cultures and animal models. In addition, we require the development of specific treatments that target these changes. Eventually, the knowledge of all these genetic alterations will be used to develop patient tailored targeted therapy, in which all (tumor specific) genetic alterations of a patient will at first be helpful to predict the outcome to the in-use chemotherapeutics and in the future will decide which tumor specific drugs can be administered. As whole genome sequencing

will become more and more affordable in the near future, especially the first will soon become a realistic option.

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Summary

Gliomas are tumors with a dismal prognosis and it is therefore important to improve survival. The first step to accomplish this is to understand gliomagenesis at the molecular level, and to discover all tumor suppressor and oncogenes. In chapter 1, the most important genetic alterations and their molecular pathways in gliomas are discussed. Affected pathways include EGFR/PI(3)K/AKT pathway, the P53 pathway and the RB pathway in glioblastoma. In grade II and III gliomas LOH of 1p19q and the recently detected mutations in the *IDH1* gene are very common. Chapter 2 gives a detailed overview of the IDH1 mutation frequencies, the IDH1 mutation types and the prognostic value of IDH1 mutations in glioma patients. It also hypothesizes about the functional consequences of IDH1 mutations.

In chapter 3 and 4, novel methods to detect genetic changes are described. In chapter 4 we identified transclocations using aberrant exon expression, in chapter 3 we identify translocations using integrated genomic approaches. Translocations identified include LEO1-SLC12A1 and EST AI364529-CASPR2. In chapter 3 RBBP4 was identified as a candidate oncogene and Pragmin as a candidate tumor suppressor gene. In chapter 4 functional analysis of the CASPR2 gene showed that the translocation reduces CASPR2 protein expression, 2 additional samples with loss of (part) of the CASPR2 gene were also identified and it was shown that glioma cells overexpressing CASPR2 had a reduced growth. CASPR2 therefore is likely to function as a tumor supressor gene in glioma.

In chapters 5 and 6 we focus on IDH1 mutations in glioma. In chapter 5 we find that IDH1 non-p.R132H mutations are unevenly distributed between histologic subgroups (less in classic oligodendrogliomas). They occur in gliomas harboring TP53 mutations, but are virtually absent in tumors with LOH of 1p19q. Segregation in distinct subgroups could point to functional differences between IDH1 R132H and IDH1 non-p.R132H mutations. In chapter 6 we functionally characterize IDH1 R132H mutations. Although IDH1 R132H is a candidate oncogene, in glioma cell lines overexpression of IDH1 R132H resulted in a reduced proliferation, both in vitro and in vivo. These cell lines also had a more cluster like and slower migration pattern. These results could give an explanation for the fact that IDH1 mutations are a positive prognostic marker.

In chapter 7 we make use of Next Generation Sequencing to screen in oligodendrogliomas with LOH of 1p19q for genetic alterations (point mutations and small insertions and deletions) on these chromosomal arms. We enriched the DNA of 7 samples for all coding exons, miRNAs, splice sites and promoter regions on 1p and 19q using capture arrays. We could not detect a common geneticly altered gene on 1p or 19q, thereby likely ruling out that the second hit on 1p or 19q is a genetic alteration in captured regions.

In summary, we describe several novel mechanisms to identify causal genetic changes in gliomas (chapter 3, 4 and 7), and characterize 2 of them (IDH1 and CASPR2).

Samenvatting

Gliomen zijn tumoren met een erg slechte prognose en vaak fatale afloop. Het is daarom belangrijk om de vooruitzichten voor patienten te verbeteren. Een eerste stap in dit proces is de tumorgenese proberen te begrijpen op het moleculaire niveau en alle tumorsuppressorgenen en oncogenen in kaart te brengen.

In hoofdstuk 1 worden de belangrijkste genetische veranderingen in gliomen besproken samen met hun moleculaire interacties in netwerken. Betrokken netwerken zijn het EGFR/PI(3)K/AKT netwerk, het P53 netwerk en het RB netwerk. In graad II en III gliomen komen het verlies van 1p19q en de recent ontdekte mutaties in het IDH1 gen vaak voor. Op het resterende 1p of 19q allel zou een tot nu toe onbekend tumorsuppressorgen kunnen zitten dat geheel uitgeschakeld wordt door een tweede genetische verandering. Hoofdstuk 2 geeft een gedetailleerd overzicht van de IDH1 mutatiefrequenties en mutatietypen en de prognostische waarde van IDH1 mutaties in patiënten. Daarnaast komen er meerdere hypothesen aan bod over de functionele consequenties van IDH1 mutaties.

In hoofdstuk 3 en 4 worden nieuwe methoden om genetische veranderingen op te sporen beschreven. In hoofdstuk 4 identificeren we translocaties met behulp van exonen met een afwijkende expressie; in hoofdstuk 3 doen we dit met behulp van exon-expressiedata geïntegreerd met data over allelverlies. Translocaties die op deze manieren gevonden zijn zijn LEO1-SLC12A1 en EST AI364529-CASPR2. Daar komt bij dat in hoofdstuk 3 RBBP4 en Pragmin naar voren komen als kandidaat oncogen en tumorsuppressorgen. In hoofdstuk 4 laat de functionele analyse van het CASPR2 gen zien dat er geen expressie is van CASPR2 eiwit in de tumor met de translocatie, er 2 tumoren zijn waarin er verlies is van (een gedeelte van) het CASPR2 gen en dat overexpressie van CASPR2 in glioom cellen voor een verminderde groei van deze cellen zorgt. Het is daarom aannemelijk dat CASPR2 een tumorsuppressorgen is.

Hoofdstuk 5 en 6 beschrijven de functionele analyse van mutaties in het IDH1 gen. In hoofdstuk 5 laten we zien dat IDH1 non-R132H mutaties onevenredig verdeeld zijn over histologische subgroepen (minder in klassieke oligodendrogliomen). Ze komen voor in gliomen met TP53 mutaties, maar zijn zo goed als afwezig in tumoren met verlies van 1p19q. Deze seggregatie in specifieke subgroepen kan wijzen op functionele verschillen tussen IDH1 R132H and IDH1 non-p.R132H mutaties. In hoofdstuk 6 brengen we IDH1 R132H mutaties functioneel in kaart. Hoewel IDH1 R132H een kandidaat oncogen is, laten glioomcellen met overexpressie van IDH1 R132H een verminderde proliferatie zien, zowel in vitro als in vivo. Deze cellijnen hebben ook een langzamer en meer geclusterd migratiepatroon. Deze resultaten kunnen een verklaring geven voor het feit dat IDH1 mutaties een positieve prognostische waarde hebben.

In hoofdstuk 7 maken we gebruik van Next Generation Sequencing technieken om in oligodendrogliomen met verlies van 1p19q te screenen voor genetische veranderingen (puntmutaties en kleine inserties en deleties) op 1p en 19q. Het DNA van 7 tumoren werd verrijkt voor alle coderende exonen, miRNA's, splice sites en promoter regio's op 1p en 19q met behulp van capture arrays. We konden geen regelmatig aangedaan gen vinden op 1p of 19q. Hiermee sluiten we uit dat de uitschakeling van het tot nu toe onbekende tumorsuppressorgen op 1p/19q plaats vindt door middel van een tweede genetische verandering (ten minste voor de genen in de verrijkte regio's).

Samenvattend beschrijven we in dit proefschrift verscheidene nieuwe methoden om causale genetische veranderingen in gliomen te identificeren (hoofdstuk 3, 4 en 7) en analyseren we 2 van deze genen functioneel (IDH1 en CASPR2).

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Curriculum Vitae

Linda Bralten studied Medicine at the RijksUniversiteit Groningen of which she accomplished the first year cum laude. At the same University she also accomplished the first year of the study Mathematics. She did a master thesis project about Parkinson's disease at Hospital Universidad of the University of Barcelona and external clinical internships at the Tygerberg Hospital in Stellenbosch, South Africa, and at the VU University in Amsterdam. During her PhD project she completed her Neuroscience Research Master at the Erasmus University cum laude. In 2010 Linda won the stophersentumoren.nl stimulation award.

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1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Biomedical English Writing and Communication	2008	4 ECTS
- Research Integrity	2010	2 ECTS
- Introductory Course on Statistics & Survival Analysis for MDs	2010	0.4 ECTS
Specific courses (e.g. Research school, Medical Training)		
- Research Management for PhD students and Postdocs	2010	0.2 ECTS
- Cold Spring Harbor Course on Mechanisms of Neural Differentiation and Brain Tumors	2008	0.75 ECTS
- Molecular Diagnostics III	2008	0.6 ECTS
- Basic and Translational Oncology	2007	0.6 ECTS
- Course Molecular Medicine	2007	0.6 ECTS
- Signalling Transduction	2007	0.6 ECTS
Seminars and workshops		
- Department book/journal club	2007/2009	4 ECTS
- Imaging Workshop for MDs	2010	0.25 ECTS
- Browsing Genes and Genomes with Ensembl	2007	0.4 ECTS
(Inter)national conferences & Presentations		
- 15th Society for Neuro-Oncology Annual Meeting (poster)	2010	0.4 ECTS
- Dutch National Neuro-Oncology Workgroup (oral presentation)	2010	0.4 ECTS
- KWF Tumor Cell Biology Meeting (oral presentation)	2009	0.4 ECTS
- 14th Society for Neuro-Oncology Annual Meeting (poster)	2009	0.4 ECTS
- 100th American Association for Cancer Research Annual Meeting (poster)	2009	0.4 ECTS
- 12th Molecular Medicine Day (oral presentation)	2008	0.4 ECTS
- Dutch National Neuro-Oncology Workgroup (oral presentation)	2007	0.4 ECTS

2. Teaching		
Supervising MLO technicians in training		
- Rosanna van der Dungen	2008/2009	2 ECTS
- Lariesa Lapré	2009/2010	2 ECTS
Organizing skills		
- Board member Promeras (PhD representative organization)	2007/2010	2 ECTS
- Member of the PhD committee	2007/2010	2 ECTS
- EPAR faculty representative	2007/2008	2 ECTS