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# The Distribution and Significance of IDH Mutations in Gliomas

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Additional information is available at the end of the chapter

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## 1. Introduction

The discovery of somatic isocitrate dehydrogenase (IDH) mutations in gliomas is an example of the powerful impact of the next-generation sequencing on the comprehension of both tumor biology and human diseases.

IDHs catalyze the oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate with production of NADH/NADPH. Thus, they are key enzymes in the Krebs cycle. For this family of metabolic genes, no previous role in human cancer has been described. However, in a recent genome-wide study, recurrent somatic mutations in the IDH1 gene have been identified in patients affected by Glioblastoma Multiforme (GBM) [1]. In successive studies, IDH mutations have also been found in low-grade gliomas, as well as in acute myeloid leukemia.

The aim of this chapter is to review the findings on the epidemiology and significance of IDH mutations in human gliomas, from the discovery to the current knowledge about their molecular pathogenesis.

Special attention will be paid to the powerful diagnostic and prognostic relevance of the IDH mutations in the clinical practice of neuro-oncology.

## 2. Function of isocitrate dehydrogenases

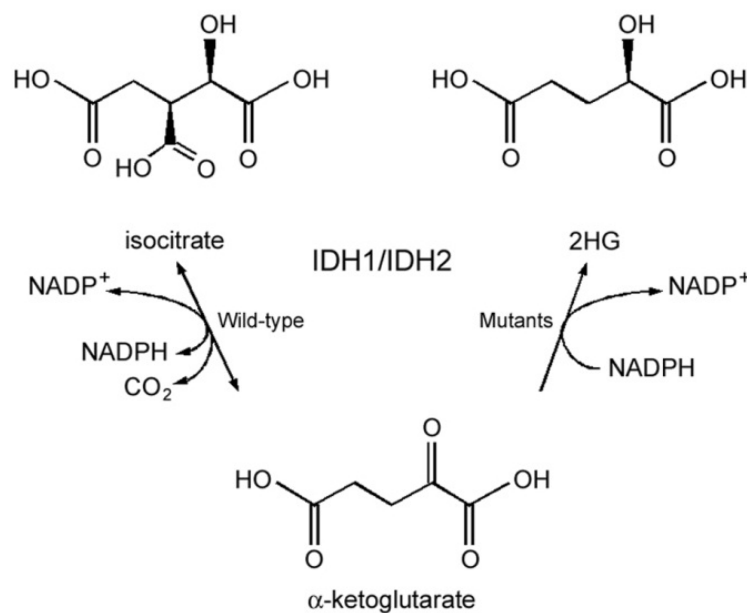
### 2.1. Isocitrate dehydrogenase enzymatic activity and structure

IDHs are enzymes catalyzing the oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG) in the Krebs cycle. During this process, NAD<sup>+</sup> or NADP<sup>+</sup> are respectively reduced to

NADH or NADPH, depending on the isoform that catalyzes the reaction. A scheme of this reaction is shown in Figure 1. In mammalian tissues, three different isoforms have been described: cytosolic NADP(+)-specific IDH (IDH1), mitochondrial NADP(+)-specific IDH (IDH2), and mitochondrial NAD(+)-specific IDH (IDH3).

The IDH1 and IDH2 isoforms are structurally related with 70% of sequence identity [2]. Both function as homodimers, are NADP+ dependent [3] and show a moderate expression in a variety of tissues, including brain [4]. IDH1 is active in cytosol and peroxisomes [5] while IDH2 has a mitochondrial localization [6].

The IDH3 isoform is NAD<sup>+</sup> dependent, functions as heterodimer and is structurally unrelated to IDH1 and IDH2 [7,8]. It is composed of three subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ) organized in a multi-tetrameric structure (2 $\alpha$ , 1 $\beta$  and 1 $\gamma$ ).  $\alpha$ -subunit has a catalytic function whereas  $\beta$ - and  $\gamma$ -subunits have a regulatory function [9,10]. IDH3 is localized in the mitochondria [11].



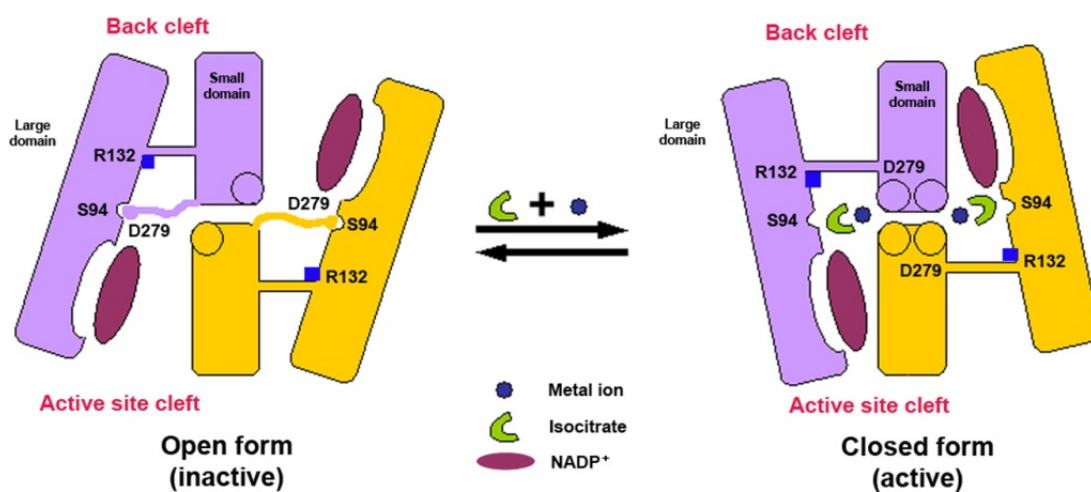
**Figure 1.** Enzymatic activity of wild-type and mutant IDH isoforms.

The activity of IDH1 and IDH2 in normal cells is regulated by the availability of substrate and cofactors. A key feature of this kinetic regulatory mechanism is the directionality of the enzymatic activity. Reactions catalyzed by IDH1 and IDH2 are reversible but not the similar reaction catalyzed by IDH3.

The crystal structure of mammalian IDH1 and IDH2 enzymes is well-known [2,12]. The structure of the wild-type IDH1 homodimer is reported in Figure 2.

In the IDH1 homodimer, each homolog comprises a large domain, a clasp domain and a small domain. Each homodimer contains two asymmetric and identical active sites, each composed of a cleft formed by the large and small domain of the other IDH1 homolog. The active sites are exposed to solvent and are accessible to the substrate and cofactors. The clasp holds the

two subunits together to form the active site. IDH1 shifts between an inactive open, a transitional semi-open and a catalytically active closed conformation. It dimerizes with two active sites in the inactive open conformation, which is maintained by intramolecular interactions between Ser94 and Asp279 residues, blocking access to the active site. Asp279 resides in the position where the isocitrate normally forms hydrogen bonds with Ser94. During the two-step catalysis of the oxidative decarboxylation of isocitrate to  $\alpha$ -KG, IDH1 adopts the active closed conformation where the steric hindrance by Asp279 to magnesium ( $Mg^{2+}$ )-isocitrate complex binding is relieved and the latter binds between the large and small domains of the enzyme [2]. The reaction proceeds with production of  $\alpha$ -KG and NADPH, followed by either the reoccupation of the active site by another  $Mg^{2+}$ -isocitrate complex or by restoring Ser94-Asp279 interactions in the inactive open conformation.



**Figure 2.** Structure of the wild-type IDH1 homodimer.

## 2.2. Function of isocitrate dehydrogenases in cellular metabolism

IDHs play prominent but distinctive roles in a variety of cellular metabolic functions [13]. The main functions of IDH1 are the lipid synthesis and the cellular glucose sensing [14] while IDH2 participates in the control of both the mitochondrial redox balance and cellular oxidative damage [15]. IDH3 plays an integral role in cellular energy metabolism.

In addition, IDHs contribute to the cellular protection generating the reducing equivalent NADPH and, on the other hand, they regulate the function of a variety of  $\alpha$ -KG-dependent processes [16-18]. Several cellular enzymes use  $\alpha$ -KG, partially produced by IDH reactions with isocitrate, as co-substrate.  $\alpha$ -KG is required for the optimal function of 5-methylcytosine (5mC) hydroxylases and histone methyltransferases that are crucial in the regulation of epigenetic processes [18-22].

Likewise,  $\alpha$ -KG plays an important role in the degradation of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ), through a prolyl hydroxylase domain-containing protein (PHD)-mediated pathway [23], and in the glia-specific glutamine and glutamate metabolism [24].

### 2.3. Function of isocitrate dehydrogenases in response to oxidative stress

In mammalian cells, the activity of IDHs increases in response to a variety of oxidative insults, with concomitant decrease of IDH3,  $\alpha$ -KG dehydrogenase and succinate dehydrogenase functions [13]. NADPH plays a major role in the cellular protection against oxidative damage due to free oxygen radicals, and from both gamma and ultraviolet radiations [16,17,25,26]. It is also essential for the regeneration of glutathione (GSH) that neutralizes free radicals and reactive oxygen species (ROS), and for the activity of the thioredoxin system. Although the pentose phosphate pathway is the main source of NADPH, IDH1 and IDH2 enzymes are as much important.

Further evidences do exist for their role in cell protection from different insults. IDH1 and IDH2 deficiency leads to increased lipid peroxidation, oxidative DNA damage, intracellular peroxide generation and decreased survival of a fibroblast cell line after exposure to oxidative agents [16].

## 3. Epidemiology of IDH mutations in human malignancies and genetic disorders

### 3.1. IDH mutations in GBMs

Mutations in the IDH1 gene were identified in a recent genome-wide analysis where 20661 protein coding genes were systematically sequenced on 22 patients affected by WHO (World Health Organization) grade IV GBM. Among the several candidate genes not previously associated with GBM, the IDH1 gene on chromosome 2q33.3 was the most interesting one [1]. Somatic and recurrent IDH1 mutations affecting the highly conserved arginine (R) residue at codon 132 in the enzyme's active site were found with a frequency of 12%. They occurred in a large fraction of younger patients and, especially, in secondary GBMs. They were also associated with a significant increase in patient overall survival (OS).

In agreement with the current WHO classification of the central nervous system (CNS) tumors [27], GBMs are considered to be secondary (sGBMs) or primary (pGBM) tumors according to the histologically verified presence or not of a previous low-grade glioma.

In a small group of GBM patients without IDH1 mutations, somatic recurrent mutations were identified in the IDH2 gene, even though at lower frequency. They affected codon R172, homologous to R132 of the IDH1 gene [28].

In contrast to sGBMs, IDH1 or IDH2 mutations were only found in a minority of pGBMs (<5-10%) [1,28,29]. The recurrence of IDH1 and IDH2 mutations among GBM patients was confirmed by following studies on larger series in Caucasian patients [30-44] as well as in Japanese, Indian, Korean, Brazilian and Chinese patients [45-49].

In pediatric GBMs, somatic IDH mutations were only rarely identified (approximately 16%) [28,29,50-52]. Pediatric malignant gliomas in patients  $\geq 14$  years showed a higher frequency

(about 35%) of IDH mutations in comparison with children malignant gliomas [52]. This finding suggests that the biology of histologically similar high-grade gliomas in younger pediatric patients differs from adults.

Stabilized GBM cell lines (both neurospheres and adherent cells) did not show IDH1 or IDH2 mutations [53]. This finding is not surprising considering that the majority of GBM cell lines that develop in *in vitro* culture originate from pGBMs.

The frequency of IDH mutations in high-grade gliomas is summarized in Table 1.

Tumor type, (WHO grade)	Total cases	IDH1 and IDH2 mutations detected	%, range	Reference
Pilocytic astrocytoma (I)	221	5	2.3 (0-5.9)	28*,30,32-34,39,42,43,59
Diffuse astrocytoma (II)	608	455	75 (30.7-100)	28*,30,32-34,36,38-43,45,46,56
Anaplastic astrocytoma (III)	566	330	58 (44.4-87.5)	28*,30,32-36,38-42,45,46
Oligodendroglioma (II)	597	366	61 (42.9-100)	28*,30,32-34,36,38-42,46
Anaplastic oligodendroglioma (III)	561	346	62 (46-100)	28*,30,32-36,38-42,45,46,57
Oligoastrocytoma (II)	207	149	72 (49-100)	28*,30,32,33,36,38,41,42,46
Anaplastic oligoastrocytoma (III)	297	202	66 (63-100)	28*,30,32,33,36,38,42,45,46
Primary glioblastoma (IV)	2362	170	7.2 (1.8-19.4)	28*,31-37,38-43,45-49
Secondary glioblastoma (IV)	335	250	75 (15.4-84.6)	28*,31-36,38-42,45,46,48
Giant cell glioblastoma (IV)	18	4	22 (20-25)	28*
Pediatric glioblastoma (IV)	69	2	3 (0-7.6)	30,50-52
Gliomatosis cerebri	15	5	33	58

\* Combined results from Balss and Hartmann studies due to duplication of some cases. Abbreviations: WHO, World Health Organization; IDH, isocitrate dehydrogenase.

**Table 1.** Table 1. Frequency of IDH1 and IDH2 mutations in high- and low-grade gliomas.

### 3.2. IDH mutations in low-grade gliomas

After the discovery of IDH mutations in sGBMs, a great interest arose in verifying whether these mutations were also present among low-grade gliomas. As expected, IDH1 or IDH2 mutations recur in >65-80% of WHO grade II and III astrocytomas [28,30,32-36,38-43,45,46,54-56], and even more (approximately 70-85%) in WHO grade II and III oligoastrocytomas and oligodendrogliomas [28-30,32-36,38-43,45,46,5-59].

Mutations in the IDH1 gene have also been found in some cases of gliomatosis cerebri [58].

In contrast to diffuse gliomas, IDH mutations are very rare or absent in a variety of WHO grade I and II CNS tumors, such as pilocytic astrocytomas, subependymal giant cell tumors, gangliogliomas, ependymomas and pleiomorphic xanthoastrocytomas

[28-30,32-34,39,42,43,59-62]. The frequency of IDH1 and IDH2 mutations in low-grade gliomas is reported in Table 1.

Relatively rare neuronal and glioneuronal tumors as central neurocytomas, dysembryoplastic neuroepithelial tumors and rosette-forming glioneuronal tumors, as well as embryonal tumors as medulloblastomas, do not show IDH mutations [28,32,33,42,59-64].

Mutations in the IDH genes were not detected among non-glioma brain tumors, with the exception of adult (but not children) primitive neuroectodermal tumors (sPNETs) [29,42,65,66], and maybe two cases of atypical meningiomas [67,68].

### 3.3. IDH mutations in other malignancies

IDH1 and IDH2 mutations are relatively glioma specific. However, they have been reported at lower frequency in some other mesenchymal tumors, especially haematopoietic malignancies and chondroid neoplasms [69-74]. IDH mutations were identified in approximately 8% of patients with acute myeloid leukemia (AML), in some myelodysplastic syndromes and myeloproliferative neoplasms [70-72]. Among AML patients, they were mainly found in tumors without cytogenetic abnormalities, and typically affect the IDH2 gene, at codons R140 or R172 [70-74]. Unlike gliomas, rare instances of AML patients with mutations in both IDH1 and IDH2 genes have been reported [75].

IDH1 mutations have also been described as a frequent event (56%) in endochondromas, as well as in central or differentiated chondrosarcomas [69], but not in peripheral chondrosarcomas or osteochondromas [76]. The majority of IDH mutations in chondroid neoplasms are represented by the p.R132C substitution [69].

Mutations in the IDH1 and IDH2 genes are extremely rare in most of other human malignancies [35,47]. Rare IDH1 mutations, mostly characterized by the p.R132C substitution, have been described in prostatic adenocarcinomas, thyroid carcinomas, melanomas and B-acute lymphoblastic leukemia [45,77-79]. No IDH mutations were detected in brain metastases of colorectal cancers [80].

### 3.4. IDH mutations in hereditary diseases

L-2- and D-2-hydroxyglutaric aciduria (L-2-HGA and D-2-HGA, respectively) are rare neurometabolic disorders with mendelian inheritance caused by mutations in the L-2-hydroxyglutarate dehydrogenase (L2HGDH) or D-2-hydroxyglutarate dehydrogenase (type I D2HGDH) genes. Both are characterized by elevated levels of 2-hydroxyglutarate (2-HG) in body fluids including urine, plasma and cerebrospinal fluid [81,82]. L-2-HGA is the most common and severe of the two, and mainly affects the CNS. In contrast, symptoms associated to D-2-HG may be mild to nearly absent. Interestingly, heterozygous mutations at codon R140 of the IDH2 gene were found in a subset of D2HGDH (type II D2HGDH) patients with normal D2HGDH enzymatic activity, no D2HGDH gene mutations but with increased D2HGDH levels in body fluids [83,84].

Although IDH mutations result in D-2-HG accumulation, L-2-HGA but not D-2-HGA patients have unexpectedly been reported to have a higher risk of malignant brain tumors [85-87].

Somatic mosaic IDH1 and IDH2 mutations have also been found associated in humans to multiple endochondromatosis, Ollier disease and Maffucci syndrome [88,89]. Patients with Ollier disease/Maffucci syndrome develop multiple central cartilaginous tumors with an uncertain inheritance (possibly dominant inheritance with reduced penetrance); interestingly, some of them develop gliomas and AML. A model in which IDH1 and IDH2 mutations are early post-zygotic events in individuals affected by these syndromes has been proposed, suggesting their implication in tumorigenesis [88].

## 4. Molecular genetics of IDH mutations in gliomas

### 4.1. Spectrum of IDH mutations

Five genes encoding human isocitrate dehydrogenases have been identified. IDH1 on 2q33.3 encodes for the IDH1 isoform [90], IDH2 on 15q26.1 for the IDH2 isoform [91], IDH3A on 15q25.1 for the  $\alpha$ -subunit of the IDH3 isoform [92], IDH3B on 20p13 for the  $\beta$ -subunit [93] and IDH3G on Xq28 for the  $\gamma$ -subunit [94].

All the identified mutations in gliomas affect either the IDH1 or the IDH2 gene. IDH3A, IDH3B and IDH3G genes have not been implicated to date in gliomas [95].

All IDH1 and IDH2 mutations are somatic, heterozygous and missense changes. They typically affect codon R132 in the IDH1 gene and its homologous R172 in the IDH2 gene, both within the enzyme's substrate binding site. In contrast to ALM, in gliomas mutations in the IDH1 and IDH2 genes are always mutually exclusive.

Over 90% of the reported mutations affect the IDH1 gene and, among them, different types of mutations have been described. The p.R132H substitution accounts for about 92.4%, followed by p.R132C for 3.2%, p.R132G for 2.1%, p.R132G for 1.6% and p.R132L for <1% [28,30-39,41-48,56,57,59]. The rare p.R132V nucleotide substitution, originally proposed as somatic mutation, was later labeled as single nucleotide polymorphism (SNP) [29,78].

Interestingly, >90% of IDH1 mutations at codon R132 leads to a histidine change, suggesting a significant selective advantage in favor of this particular point mutation.

A single report has recently identified a predicted new mutation at codon R100 (p.R100Q), located in the enzyme's active site, in two WHO grade III oligodendrogliomas and in one WHO grade II astrocytoma [54]. Two other very rare IDH1 mutations (p.R49C and p.G97D) have only been described in a single pediatric GBM patient [50].

Mutations in the IDH2 gene are less common in gliomas, accounting for 3-5% or less of all the identified mutations [30,31,34,39,42-45,56,57,59]. The p.R172K mutation accounts for about 60%, followed by p.R172M for 26.7%, p.R172W for 11.1%, p.R172S for 2.2% [30,31,34,39,42-45, 56,57,59]. Rare nonsense IDH2 mutations have been described in a single study, with uncertain significance [39].



The type and frequency of the identified mutations at codons IDH1 R132 and IDH2 R172 are summarized in Table 2. All are reported at the Catalogue of Somatic Mutations in Cancer (COSMIC database) (<http://www.sanger.ac.uk/cosmic/>).

Gene	Nucleotide change	Amino acid change	Total mutated cases	%	Reference
IDH1			1791		
	c.G395A	p.R132H	1655	92.4	28,30-39,41-48,56,59
	c.C394T	p.R132C	58	3.2	28,30-39,41-48,56,59
	c.C394G	p.R132G	38	2.1	28,30-39,41-48,56,59
	c.C394A	p.R132S	29	1.6	28,30-39,41-48,56,59
	c.G395T	p.R132L	11	0.6	28,30-39,41-48,56,59
IDH2			45		
	c.G515A	p.R172K	27	60	30,31,34,39,42-45,56,57,59
	c.G515T	p.R172M	12	26.7	30,31,34,39,42-45, 56,57,59
	c.A514T	p.R172W	5	11.1	30,31,34,39,42-45,56,57,59
	c.G516T	p.R172S	1	2.2	30,31,34,39,42-45,56,57,59

The reported nucleotide and amino acid numbering for the IDH1 and IDH2 genes is relative to the transcription start site (+1) corresponding to the A of the ATG on the respective GenBank reference sequences NM\_005896 and NM\_002168. Abbreviations: IDH, isocitrate dehydrogenase.

**Table 2.** Type and frequency of IDH1 R132 and IDH2 R172 mutations in gliomas.

#### 4.2. Association with clinical and histological features

The occurrence of IDH mutations correlates with some clinical and histopathological features of gliomas. The strongest significant association is between IDH mutations and patient's age at diagnosis in most glioma tumor subtypes [28,30,31,36,42,46,49]. Generally, the mean age of patients with IDH mutations is significantly lower in comparison with patients without IDH mutations. In contrast, in pediatric high-grade gliomas, patients with IDH1 mutations are older than children with wild-type tumors [28].

In GBMs, the average time from the first clinical symptom to the histological diagnosis is significantly longer in patients with IDH mutations than in wild-type patients, consistently with a slower growth and less aggressive tumor [31].

In adult patients, IDH mutations are significantly and inversely associated with the histological malignancy grade [28,36]. Interestingly, they show a non-random distribution within the glioma subtypes [30]. The p.R132C mutation in the IDH1 gene is strongly associated with the astrocytic phenotype while IDH2 mutations mainly affect oligodendroglial tumors [30,41]. When astrocytomas develop in patients affected by Li-Fraumeni syndrome, they always show the p.R132C mutation [55], suggesting that precursor cells, which for definition already carry a germline tumor protein *p53* (TP53) mutation, specifically acquire this relatively uncommon IDH1 mutation.

In GBMs, the occurrence of an oligodendroglial component is significantly more frequent in tumors with IDH mutations, as well as large ischemic and pseudopalisading necroses are typical hallmarks of wild-type tumors [31].

Finally, a preferential distribution of IDH mutations within different regions of the brain has been reported in two recent large-scale studies. WHO grade II-III gliomas and pGBMs with IDH1 mutations are mainly located in the frontal lobe (73.5%) rather than in the temporal lobe (41.7%) [49,96]. This distribution, similar to that of 1p/19q co-deletion, provides further evidence for the distinctiveness of gliomas from different brain lobes. The absence of IDH mutations also identify, among WHO grade II gliomas, a novel tumor subtype characterized by a predominant insular location, greater tumor size, infiltrative aspects on magnetic resonance imaging (MRI) and dismal prognosis [56].

### 4.3. Association with genetic and epigenetic alterations

IDH mutations show significant associations with some of the typical genetic and epigenetic changes of gliomas. IDH mutations are tightly associated with the 1p/19q co-deletion and the methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation status [33,36,37,42,57,97,98].

Interestingly, they are inversely correlated with the specific genetic alterations of pGBMs, such as the epidermal growth factor receptor (EGFR) amplification, the cyclin-dependent kinase inhibitor 2A or 2B (CDKN2A/CDKN2B) deletion and the phosphatase and tensin homolog (PTEN) mutations [28,32]. The mutual exclusion with EGFR amplification coincides with the rarity of IDH mutations in pGBMs [33,36,37,42,57,98].

Furthermore, astrocytic tumors and GBMs with IDH mutations typically show a higher rate of TP53 mutations in comparison with wild-type tumors [28,32,33,46,98].

The strong association between IDH mutations and the 1p/19q co-deletion in oligodendrogliomas corresponds to approximately 100% concordance in the occurrence of both genetic alterations, as confirmed by three independent large-scale studies [28,98,99]. In oligodendrocytic tumors, IDH mutations also correlate with somatic mutations in the homolog of the *Drosophila capicua* (CIC) gene on chromosome 19q13.2, suggesting a putative role of the latter in the pathogenesis of this tumor subtype [100,101]. Interestingly, a significant association was also found with the epithelial membrane protein 3 (EMP3) promoter hypermethylation status [personal data].

IDH mutations and the KIAA1549- v-raf murine sarcoma viral oncogene homolog B1 (BRAF) fusion gene are considered two mutually exclusive genetic events in diffuse and pilocytic astrocytomas, respectively. However, in a series of 185 adult diffuse gliomas, IDH mutations coexist with the KIAA1549-BRAF fusion gene, mainly in oligodendroglial tumors [102].

### 4.4. Timing and relationships with gliomagenesis

The majority of tumors with IDH mutations also harbor either TP53 mutations or 1p/19q co-deletion [28,29,32]. The concurrence of IDH and TP53 mutations is typically observed in WHO

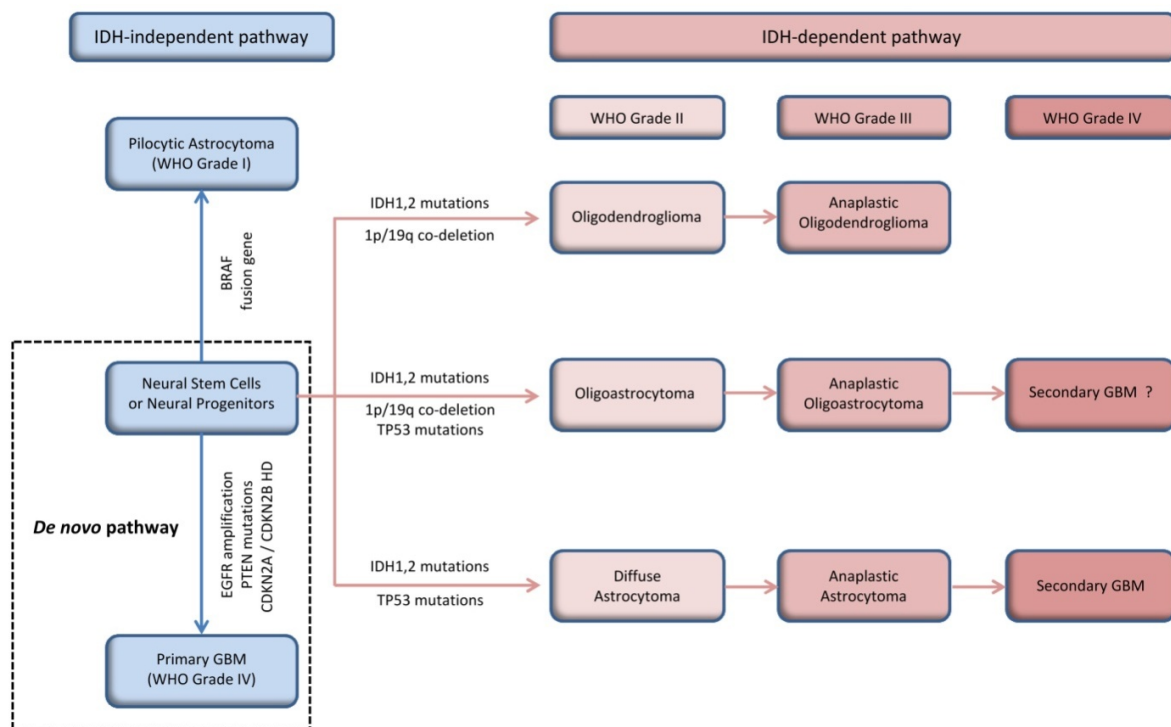
grade II and III astrocytic tumors, as well as in sGBMs. The concomitance of IDH mutations with 1p/19q co-deletion is mainly found in WHO grade II and III oligodendrogliomas. WHO grade II and III oligoastrocytic tumors mostly show IDH mutations in association with TP53 mutations and 1p/19q co-deletion.

The temporal sequence of the genetic and epigenetic events during gliomagenesis has recently been determined [33]. A series of paired initial and recurrent tumors from 51 patients was screened for the occurrence of both IDH1 and TP53 mutations, and 1p/19q co-deletion. In this study, diffuse astrocytomas and oligodendrogliomas with IDH1 mutations at first surgery developed, respectively, either TP53 mutations or 1p/19q co-deletion, at recurrence. In this series, no tumor developed IDH1 mutations after the acquisition of either TP53 mutations or 1p/19q co-deletion [33]. This finding validates the chronological order proposed for the genetic events during gliomagenesis with IDH1 mutations as the earliest genetic event, interesting the common glial precursor cell population, even before EGFR amplification [36,57]. The successive acquisition of TP53 mutations or 1p/19q co-deletion may lead, respectively, to the astrocytic or oligodendroglial differentiation. This hypothesis is further supported by the significant association of IDH mutations with 1p/19q co-deletion in oligodendrogliomas and with TP53 mutations in astrocytomas [57,99].

In contrast, pilocytic astrocytomas showing the KIAA1549-BRAF fusion gene should derive from different progenitors.

Interestingly, IDH mutations do occur in sGBMs, but not in pGBMs. This difference must be referred to the different origin of the two tumor subtypes. sGBM develops from a previous astrocytoma, whereas pGBM is a *de novo* tumor. They differ for the genetic configuration, age, and growth speed [103], but not for location and phenotype; at the most, they can differ for the spreading modalities [104]. It is not known how *de novo* tumors arise, whereas it is believed that secondary ones originate through anaplasia, *i.e.* through dedifferentiation of tumor cells which follows mutation accumulation [27]. Generally, it is known that GBMs originate either from Neural Stem Cells (NSCs) or from astrocytes [105] and this could correspond to the distinction between pGBMs and sGBMs. Obviously, it is likely that the two GBM subtypes must originate *ab initio* from the same Cancer Stem Cells (CSCs). The development of GBM in the emisphere, far away from the sub-ventricular zone (SVZ), could be in contrast with its origin from NSCs of the same region, but this can be got over if we refer to the concept of both asymmetric division and migration of progenitors [106]. A path has been traced from mitotically active precursors to the developed tumors [107], which recognizes in transiently dividing progenitors and in somatic stem cells, the elements where mutations accumulate; they also express EGFR, present in normal progenitors of the SVZ [108]. These cells are the possible source of pGBMs, whereas for sGBMs it is mandatory to refer to a previous astrocytoma [109,110].

A genetic model for the origin and progression of the different glioma subtypes is shown in Figure 3.



Abbreviations: IDH, isocitrate dehydrogenase; BRAF, v-raf murine sarcoma viral oncogene homolog B1; CDKN2A/B, cyclin-dependent kinase inhibitors 2A and 2B; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog; TP53, tumor protein *p53*; HD, homozygous deletion; GBM, glioblastoma multiforme; WHO, World Health Organization.

**Figure 3.** Model for the development of astrocytic and oligodendrocytic tumors. Two different pathways have been proposed, according to the IDH mutation status [111]. In the IDH-dependent pathway, WHO grade II astrocytomas and oligodendrogliomas arise by first acquisition of IDH mutations and successive development of either TP53 mutations or 1p/19q co-deletion. They further progress to WHO grade III astrocytomas or oligodendrogliomas and, ultimately, become secondary GBMs. WHO grade II and III oligoastrocytomas show genetic changes common to both astrocytomas or oligodendrogliomas. In the IDH-independent pathway, primary GBMs de novo develop irrespective of IDH mutations, by acquisition of the following genetic alterations: EGFR amplification, PTEN mutations and CDKN2A/CDKN2B homozygous deletion. Pilocytic astrocytomas arise by acquisition of the KIAA1549-BRAF fusion gene (modified from Ref.111).

#### 4.5. Association with the Proneuronal glioma subtype

Gene expression profiling studies of high-grade gliomas have permitted a subclassification of tumors according to a molecular signature [112]. In a recent study on 115 WHO grade II and III astrocytomas, three distinct subgroups termed Proneuronal, Proliferative and Mesenchymal have been identified, in agreement with similarities in expression profiling of survival-related genes [112]. The Proneuronal group is characterized by a better prognosis and by expression of genes associated with normal brain and neurogenesis. The Proliferative group has a markedly better prognosis and shows expression of genes associated with both proliferation and angiogenesis, whereas the Mesenchymal group has a poorer prognosis and expresses genes associated to mesenchymal origin. Most WHO grade III gliomas, as well as

75% of low-grade gliomas, were classified as Proneuronal. Interestingly, IDH mutations were significantly associated to the latter subgroup.

Recently, the Cancer Genome Atlas (TCGA) Research Network has been hired to generate the comprehensive catalogue of genomic abnormalities occurring during tumorigenesis in the majority of human cancers [113]. Among them, GBM was chosen as the pilot disease for this project. Based on gene expression profiling of 206 GBMs, the following four clinically relevant subtypes were identified: Classical, Proneuronal, Neuronal and Mesenchymal. Aberrations and gene expression of EGFR, neurofibromatosis type I (NF1) and platelet-derived growth factor receptor A (PDGFRA)/IDH1 each defines the Classical, Mesenchymal and Proneuronal subtype [113]. Notably, most sGBMs were classified as Proneuronal. The successive up-dated TCGA report confirmed the association with this favorable GBM subtype [114].

An independent study by array-based comparative genomic hybridization (CGH) analysis identified distinct genomic and expression profiles between pGBMs and sGBMs [44]. Few pGBMs with IDH1 mutations have similar expression profiles as sGBMs with IDH1 mutations [44].

Similar findings were described in low-grade gliomas [115]. In a series of 101 diffuse astrocytic gliomas, IDH mutation status discriminated molecularly and clinically distinct low-grade glioma subsets, where tumors with IDH mutations show TP53 mutations, PDGFRA overexpression, and better survival. In contrast, tumors without IDH mutations show EGFR amplification, PTEN loss, and unfavorable disease outcome. Furthermore, global expression profiling revealed three robust molecular subclasses within lower grade diffuse astrocytic gliomas, two of which mainly characterized by IDH mutations and TP53 mutations and one prevalently wild-type. The former can be distinguished from each other on the basis of TP53 mutations, DNA copy number abnormalities, and links to distinct stages of neurogenesis in the SVZ [115].

#### **4.6. Association with the G-CIMP hypermethylated phenotype**

In a genome-wide DNA methylation analysis in human gliomas distinct methylation profiles have been associated with gene expression subtypes [114]. Interestingly, a specific glioma CpG island methylator phenotype (G-CIMP) was found, able to define a distinct molecular subclass of tumors. G-CIMP is analogous to the CpG island methylator phenotype (CIMP) previously described in a number of human malignancies, including AML and colorectal carcinoma [116]. Similarly to these, G-CIMP is characterized by a high number of hypermethylated *loci*. The G-CIMP prevails among low-grade gliomas, shows distinct copy number alterations and correlates with better survival and younger age [114].

Notably, it is strongly associated with IDH mutations and the Proneuronal subtype [114,117,118]. The significant association between IDH mutations and MGMT promoter hypermethylation found by us [42,97] and by others [33,36,37,57] in low-grade gliomas is in line with this finding.

Remarkably, IDH mutations alone are sufficient to establish the G-CIMP, by remodelling the methylome and the transcriptome. Introduction of mutant IDH1 into primary human astro-

cytes alters specific histone marks, induces extensive DNA hypermethylation and reshapes the methylome [119].

As for gliomas, IDH mutations have also been found associated with peculiar DNA hypermethylation pattern in AML [116]. In the latter, hypermethylation may be alternatively caused by inhibition of the oncogene family member 2 (TET2). TET2 encodes an  $\alpha$ -KG-dependent dioxygenase that catalyzes the formation of 5-hydroxymethylcytosine (5hmC), with subsequent DNA demethylation [120,121].

## 5. Molecular pathogenesis of IDH mutations

### 5.1. Biochemistry of mutant isocitrate dehydrogenases

All the identified IDH mutations in gliomas are amino acid substitutions at codon R132 in the IDH1 and codon R172 in the IDH2 gene. Both residues are highly conserved and involved in the formation of the active site of the two enzymes.

During the two-step catalysis of the oxidative decarboxylation of isocitrate to  $\alpha$ -KG, IDH adopts an active closed conformation where the  $Mg^{2+}$ -isocitrate substrate complex binds between large and small domains of the enzyme [2]. The active site is composed of two groups of key residues. The first consists of two essential catalytic residues in IDH1, tyrosine (Y) 140 and lysine (K) 212, that participate in the acid-base catalysis during decarboxylation of isocitrate and are evolutionarily highly conserved [122]. The second consists of a triad of arginines (R100, R109 and R132 in IDH1 and R140, R149 and R172 in IDH2) in the  $Mg^{2+}$ -isocitrate complex recognition site that forms a salt-bridge with the substrate [77].

Modelling studies on human cytosolic IDH1 structure suggest that substitutions at R132 would impair interactions of the enzyme with the substrate [23]. Among all the amino acid residues involved in the binding with isocitrate, R132 only forms three hydrogen bonds with the  $\alpha$ - and  $\beta$ -carboxyl of isocitrate, while all the others no more than one or two. Therefore, substitutions at R132 may weaken the hydrogen bond of IDH1 to substrate. Since R132 contacts Asp297 in the transitional semi-open conformation of IDH1, it may be important in the transition from open to closed conformation [19]. In this regard, R172, as well as R140 in IDH2, is equivalent to R132. These chemical factors may explain why mutations in the IDH1 and IDH2 genes exclusively affect these amino acids. The position, rather than the nature of the amino acid substitution, seems to affect the IDH activity, by conferring a selective advantage to tumor cells with IDH mutations [77].

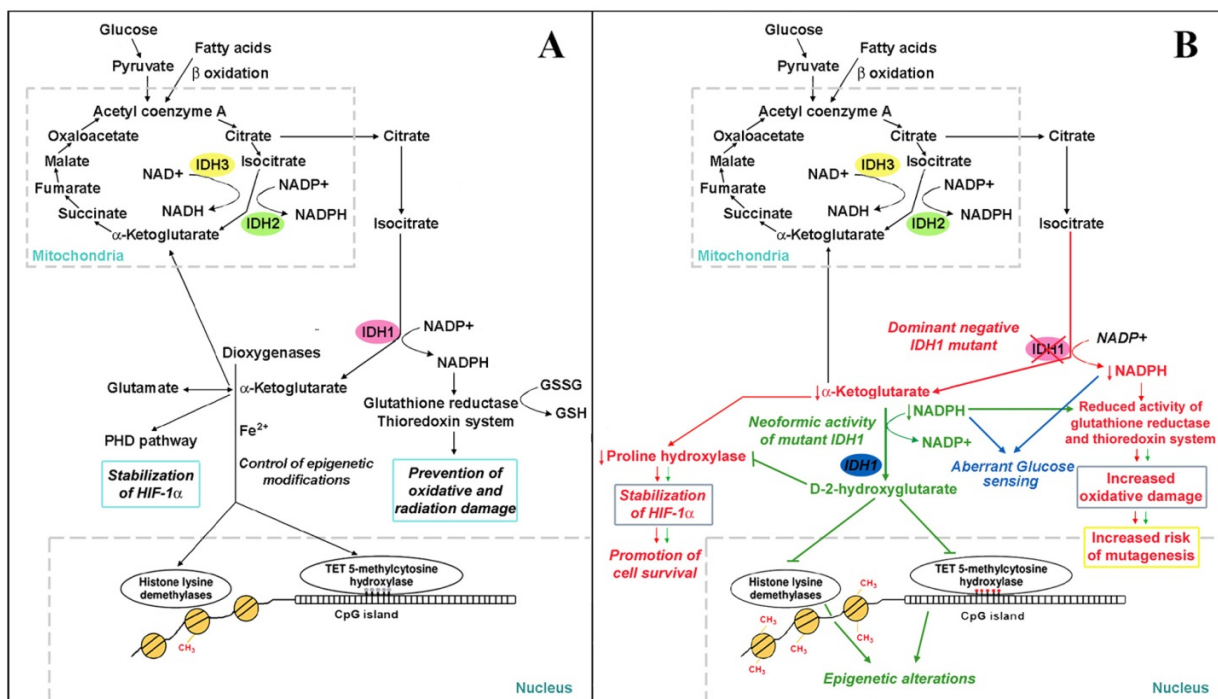
To date, all the identified mutations have been tested, and all of them impair the normal IDH enzymatic activity [23,32]. The enzymatic activity of IDH mutant proteins is significantly reduced, irrespective of the nature of the amino acid substitution [35,111]. Any amino acid substitutions in one of the two homologue arginines R100 or R132 in IDH1 and R140 and R172 in IDH2 lead to the same gain of function in both proteins. Interestingly, the third conserved arginine (R109 in IDH1 and R149 in IDH2) of the triad has never been reported to be mutated [54].

Recently, IDH1 mutations at codons R100 (in adult glioma) and G97 (in pediatric GBM and colon cancer cell lines), as well as at the predicted Y139, have been found to be associated with D-2-HG production [124]. In contrast, IDH1 SNPs V71I and V178I, as well as several single report non-synonymous substitutions, have no effect on cellular D-2-HG levels and retain the wild-type ability for isocitrate-dependent NADPH production [123].

## 5.2. Dominant negative enzymatic activity

The mechanism by which IDH1 and IDH2 mutations mediate oncogenesis in gliomas is not completely clarified [20,24,124]. However, two hypotheses have been proposed (Figure 4) [125-128]. Both IDH1 and IDH2 mutant enzymes show a loss of function in the forward reaction leading to a reduced production of  $\alpha$ -KG and NADPH [23] and a gain of function in the reverse reaction leading to an increased production of D-2-HG [19]. Both loss- and gain-of function reactions may have significant implications for cellular metabolism in glioma tumor cells.

In agreement with the loss of function hypothesis, IDH mutations may dominantly inhibit the wild-type copy of the homodimer. By *in vitro* studies, it was found that the IDH1 wild-type/mutant heterodimer exhibits only 4% of the enzymatic activity of the IDH1 wild-type homodimer, with a reduced production of  $\alpha$ -KG and NADPH [23]. Nevertheless, this dominant negative inhibition by the mutant proteins may not be observed for all the known IDH mutant types [129]. Definite evidence that dominant negative activity occurs *in vivo* is lacking [73, 128].



**Figure 4.** Functions of normal (A) and mutant (B) isocitrate dehydrogenase (IDH) enzymes (modified from Ref. 126).

### 5.3. Neomorphic enzymatic activity

The only consequence universally recognized for all types of IDH mutations is the acquisition of a neomorphic enzymatic activity to reduce  $\alpha$ -KG to the new metabolite D-2-HG [19]. This finding is in favor of the oncogenetic function of the IDH mutant proteins. Furthermore, the mutation pattern with hot-spot missense nucleotide substitutions in a heterozygous status is typical of activated oncogenes, for example v-ki-ras2 kirsten rat sarcoma viral oncogene homolog (KRAS) and BRAF [128]. Thus, IDH1 and IDH2 genes may function as oncogenes rather than tumor suppressor genes, and IDH1 and IDH2 mutations as oncogenic driver rather than passenger mutations [77]. Moreover, the 2q34 region, in which IDH1 maps, is one of the most stable chromosomal regions in gliomas, with rare deletions described.

The specific orientation by which the hydride transfer takes place in the IDH1 active site results in the production of the R(-) enantiomer of 2-HG, that is equivalent to the D-isomer [77]. Interestingly, the IDH mutant proteins only produce D-2-HG but not its enantiomer L-2-HG [19]. This unique feature may be useful to understand the mechanism by which IDH mutations contribute to gliomagenesis [127].

The occurrence of IDH mutations results in an increased (approximately 100-fold) amount of D-2-HG [19,73]. D-2-HG is structurally similar to  $\alpha$ -KG and then it functions as a competitive inhibitor of multiple  $\alpha$ -KG-dependent enzymes with important roles in cancer. They are mainly dioxygenases, which use  $\alpha$ -KG as co-substrate to catalyze a variety of reactions, including the repair of alkylated DNA, the response to hypoxia and the biosynthesis of collagen or L-carnitine [130]. Three relevant classes of dioxygenases are PHDs, histone demethylases and the TET family of 5mC hydroxylases [131].

### 5.4. Hypoxia signaling pathway

PHD promotes the degradation of the HIF-1 $\alpha$ . The latter is a transcription factor of crucial importance in the cellular response to hypoxia. It activates the transcription of genes involved in apoptosis, cell survival, and angiogenesis, most notably vascular endothelial growth factor (VEGF) [23]. A variety of human cancers show up-regulation of HIF-1 $\alpha$ , probably associated with the VEGF-mediated angiogenesis. D-2-HG produced by the IDH mutant enzymes may compete with  $\alpha$ -KG and inhibit the PHD-mediated degradation of HIF-1 $\alpha$ . Increased HIF-1 $\alpha$  levels may induce VEGF expression and consequently promote angiogenesis and enhancement of tumor growth [23]. In favor of this hypothesis, induction of R132H mutant IDH1 up-regulates HIF-1 $\alpha$  inducible genes including VEGF in U87MG glioma cells [23]. However, the up-regulation of HIF-1 $\alpha$  in glioma tissues with IDH1 mutations has not been replicated by others [132]. Moreover, WHO grade II and III gliomas are not so vascularized and do not show angiogenesis, as it would expect for tumors that activate this hypoxia signaling pathway.

### 5.5. Genome-wide epigenetic deregulation

IDH mutations may promote tumorigenesis by genome-wide epigenetic deregulation through the inhibition of demethylation of 5mC and histones. A member of the  $\alpha$ -KG-dependent dioxygenases is the TET family of 5mC hydroxylases [18,131]. TET2 is a putative tumor



suppressor gene at chromosome 4q24 that plays an important role in the regulation of DNA methylation by conversion of 5mC into 5hmC. The great majority of DNA methylation sites are 5mC at CpG dinucleotides and 5hmC is regarded as an intermediate product of DNA demethylation. D-2-HG produced by the IDH mutant proteins may inhibit TET2-mediated demethylation, leading to a global hypermethylation [131].

Interestingly, a subset of AML patients with TET mutations, which are mutually exclusive with IDH mutations, show the same hypermethylation phenotype of patients with IDH mutations, suggesting that IDH-mutant tumors may be mediated by TET2 inhibition [120]. This finding also suggests that IDH and TET2 mutations may be functionally redundant.

The tight association between the occurrence of IDH mutations and the before mentioned G-CIMP in gliomas, as well as with the CIMP in AML, is a strong evidence in favour of this hypothesis. Not least is the direct evidence that IDH mutations alone establish G-CIMP, as recently reported [119]. Moreover, overexpression of R132H mutant IDH1 or R172K mutant IDH2 in 293T cells leads to increased D-2-HG and to a significant up-regulation of global 5mC levels [120].

An alternative epigenetic mechanism involved in the regulation of gene expression is represented by histone lysine methyltransferases that mediate the methylation of histone proteins [131]. Increased D-2-HG levels may result in alterations of the methylation status of lysine residues in histones, such as H3K9, H3K27 and H3K79 [132]. D-2-HG-producing IDH mutants can prevent the histone demethylation required for lineage-specific progenitor cells to differentiate into completely differentiated cells [133]. The introduction of either mutant IDH or cell-permeable 2-HG is associated with repression of the inducible expression of lineage-specific differentiation genes and with a block in cell differentiation. Notably, the aberrant histone methylation may precede and occur independently of DNA methylation [133].

Overall, by these two mechanisms IDH mutations may induce DNA hypermethylation at a number of target genes and globally deregulate gene expression.

### 5.6. Response to oxidative insults

An alternative hypothesis is related to the IDH function in the cellular protection by generation of the reducing equivalent NADPH. The latter plays an important role in the protection of cell from oxidative damage and radiation-induced stress by contributing to GSH reductase and thioredoxin systems.

Human brain is highly susceptible to oxidative stress because of high oxygen consumption, relative lack of antioxidant enzymes and large amount of iron, a well-known pro-oxidant [134]. The main source of DNA damage in brain is represented by ROS produced during normal cellular metabolism [135]. GSH is the most important antioxidant against the oxidative stress caused by ROS. NADPH is required for the reduction of glutathione disulfide (GSSG) to GSH by glutathione reductase and for the maintenance of the antioxidant status of GSH in the cell. The cytosolic IDH1 contributes to the NADPH pool in the cell, even though two other cytosolic enzymes, glucose 6-phosphate dehydrogenase (G6PD) and the malic enzyme, also generate NADPH [16].

Under oxidative stress conditions, the activity of NAD<sup>+</sup>-dependent IDHs increases. IDH mutations reduce the ability of IDH to produce NADPH from NADP<sup>+</sup> [19,23,32,111], but also deplete NADPH by consuming it as cofactor to convert  $\alpha$ -KG in D-2-HG with NADP<sup>+</sup> production [19]. Cells with reduced NADP-dependent IDH activity have increased oxidative DNA damage, higher GSSG to total GSH ratio and reduced survival on exposure to oxidative stress [16].

Oxidative DNA damage may promote the occurrence of other genetic changes, for example TP53 mutations or t(1;19) translocation favoring, respectively, the differentiation of the astrocytic or oligodendrocytic lineage. Furthermore, DNA damage may lead to DNA double-strand breaks [136] and ROS accumulation has been shown to induce unbalanced translocation in leukemia cells [137]. This finding may explain the high prevalence of t(1;19) translocation (corresponding to the total 1p/19q co-deletion) in oligodendrogliomas with IDH mutations.

This hypothetical model may also explain why D-2-HGA patients do not develop gliomas, because mutations in the D2HGDH gene in these patients would not affect the redox system *via* GSH [127].

### 5.7. Aberrant glucose sensing

A possible selective advantage for tumor cells could derive from the role of IDH1 in the glucose sensing. IDH1 participates in a glucose-sensing pathway in pancreatic islets [138] by signaling the presence of high glucose to downstream members of this pathway by raising the NADPH levels. IDH1 and IDH2 mutant proteins consume NADPH to convert  $\alpha$ -KG to D-2-HG decreasing the cytosolic NADPH level. This may be wrongly considered as signal of a low nutrient status in the glucose-sensing pathway. Cells may compensate this status by increasing cellular nutrient consumption or by blocking cellular differentiation. The former is known as a typical tumor hallmark and it may give tumor cells a selective growth advantage. Indeed, both glioma and AML tumor cells are relative undifferentiated [73]. The block of differentiation, finally, may benefit tumor cells by self-renewal.

## 6. Assessment of IDH mutation status

Currently, different methods are available to determine the IDH mutation status. They analyze either the nucleotide sequence of the gene or the altered structure of the protein.

### 6.1. Gene sequencing

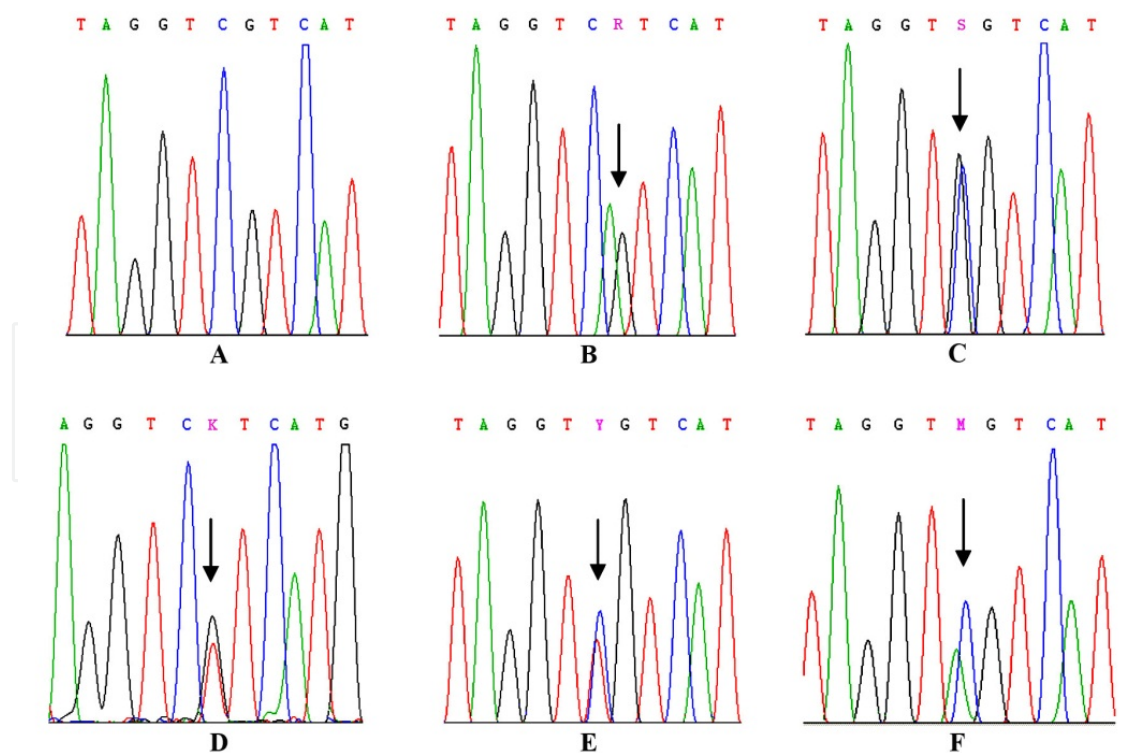
Practical guidelines are available for a reliable detection of IDH mutations with molecular genetics techniques. In this regard, crucial aspects are the availability of tumor tissue, the tumor cell content and the quality of the respective genomic DNA (gDNA). The amount of tumor tissue available for the genetic analysis is often limited, especially for stereotactic biopsies. It also depends on the modality of tissue dissection, by manual macrodissection or laser capture microdissection (LCM).

The content in tumor cells is a critical point because contaminating cells from adjacent normal brain tissue, lymphocyte infiltrates, microglial and endothelial cells may dilute the mutant allele below the detection threshold level leading to false negative results. Consequently, each tumor sample should be as pure as possible and should reflect the highest percentage of tumor cells. This can be particularly problematic in tumor biopsy specimens. Prior to gDNA extraction, the identification and selection of tumor areas as proliferating, by haematoxylin and eosin (H&E) staining and microscopic examination, is therefore mandatory.

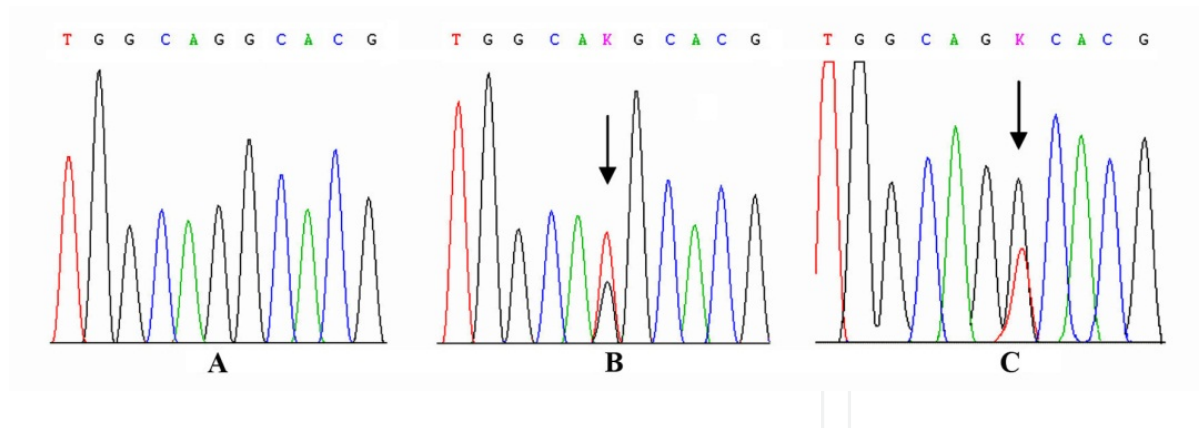
Among DNA-based methods, conventional Sanger sequencing is the most frequently used. It is a relatively inexpensive method for laboratories with access to an automated sequencer and it represents to date the “gold standard” for the detection of IDH mutations. It allows to identify all the possible sequence variations in the amplified region with a sensitivity of approximately 20% of the mutant allele in a wild-type background. Typically, Sanger sequencing is carried out on formalin or RCL2 fixed paraffin embedded tumor tissue, rather than on fresh frozen tissue, because the latter is frequently unavailable. The effects on gDNA quality of the different variables affecting tissue fixation are not to be neglected.

Bi-directional Sanger sequencing on at least two replicates is strongly recommended [139].

Representative electropherograms of IDH1 and IDH2 mutations detected in our previous study [42] are shown in Figures 5,6.



**Figure 5.** Electropherograms of representative mutations in the IDH1 gene. A – IDH1 wild-type; B – c.G395A (p.R132H); C – c.C394G (p.R132G); D – c.G395T (p.R132L); E – c.C394T (p.R132C); F – c.C394A (p.R132S).



**Figure 6.** Electropherograms of representative mutations in the IDH2 gene. A – IDH2 wild-type; B – c.G515T (p.R172M); C – c.G516T (p.R172S).

Alternatively to Sanger sequencing, several studies have successively applied pyrosequencing technique [40,140]. The tightly clustered nature of the IDH mutations makes them ideal candidates for pyrosequencing. This technique allows a quantitative analysis and high throughput, with a sensitivity of 5-7% of the mutant allele in a wild-type background.

## 6.2. Alternative molecular techniques

Alternative methods to assess the IDH mutation status exist. They include derived cleaved amplified polymorphic sequence (dCAPS) [141], PCR-based restriction length polymorphism assays [142], cold PCR high resolution melting (HRM) [143], post-PCR fluorescence melting curve analysis (FMCA) [39] and SNaPshot assays [144]. The two latter methods are characterized by a sensitivity of approximately 2 and 5%, respectively.

Among the above mentioned techniques, melting curve analysis is currently approved for clinical use in the detection of BRAF and KRAS point mutations [62].

Anyway, the choice of the detection method both depends on the researcher's expertise and the laboratory facilities.

## 6.3. Immunohistochemistry

Two monoclonal antibodies (Mab), H09 (referred in some papers as anti-mIDH1<sup>R132H</sup>) and IMab-1, have been developed to recognize the mutant-specific epitope of the IDH1 R132H mutant protein [145,146]. These antibodies can be used for both immunohistochemical analyses of tumor tissue and Western blotting analyses of tumor cell lysates. However, the latter procedure is not used in the clinical practice. Currently, the clone H09 antibody is the only commercially available (Dianova, Hamburg, Germany), with satisfactory results in the routine immunohistochemistry of formalin or RCL2 fixed paraffin embedded tissues [29,34,38,42,43].

The sensitivity and specificity of the clone H09 to detect positive tumor cells has been widely demonstrated in several studies and approaches 100% [38,42,145]. In comparison with IMab-1, clone H09 shows superior staining results [139].

Recently, a new Mab (SMab-1) directed to the p.R132S mutation of the IDH1 gene has been developed [148]. SMab-1 seems to show a specificity similar to that of clone H09 in both immunohistochemistry and immunocytochemistry; further studies are required to validate its use. The monoclonal antibody SMab-1 should be soon commercially available.

While the current clone H09 is highly specific for the IDH1 p.R132H mutation, it does not detect the other rare substitutions in the IDH1 or IDH2 genes [38]. For this reason, Sanger sequencing of the relevant exons of the IDH1 and IDH2 genes is always recommended to exclude the occurrence of other types of mutations in immunonegative cases [42,59,62,56,148].

The immunohistochemical analysis of the IDH1 p.R132H mutation can be manually performed or by automated immunostaining instruments. The Euro-CNS research committee has recently proposed practical guidelines to standardize the diagnostic IDH test with clone H09 by both procedures [139]. The “Vienna protocol”, established at the Institute of Neurology, Medical University of Vienna (Austria), is recommended for manual immunostaining. In contrast, the “Heidelberg protocol”, established at the Department of Neuropathology, Institute of Pathology, Ruprecht-Karls-Universität Heidelberg (Germany), is recommended for automatic immunostaining on Ventana BenchMark immunostainers (Ventana Medical Systems, Tucson, AZ, USA) [139].

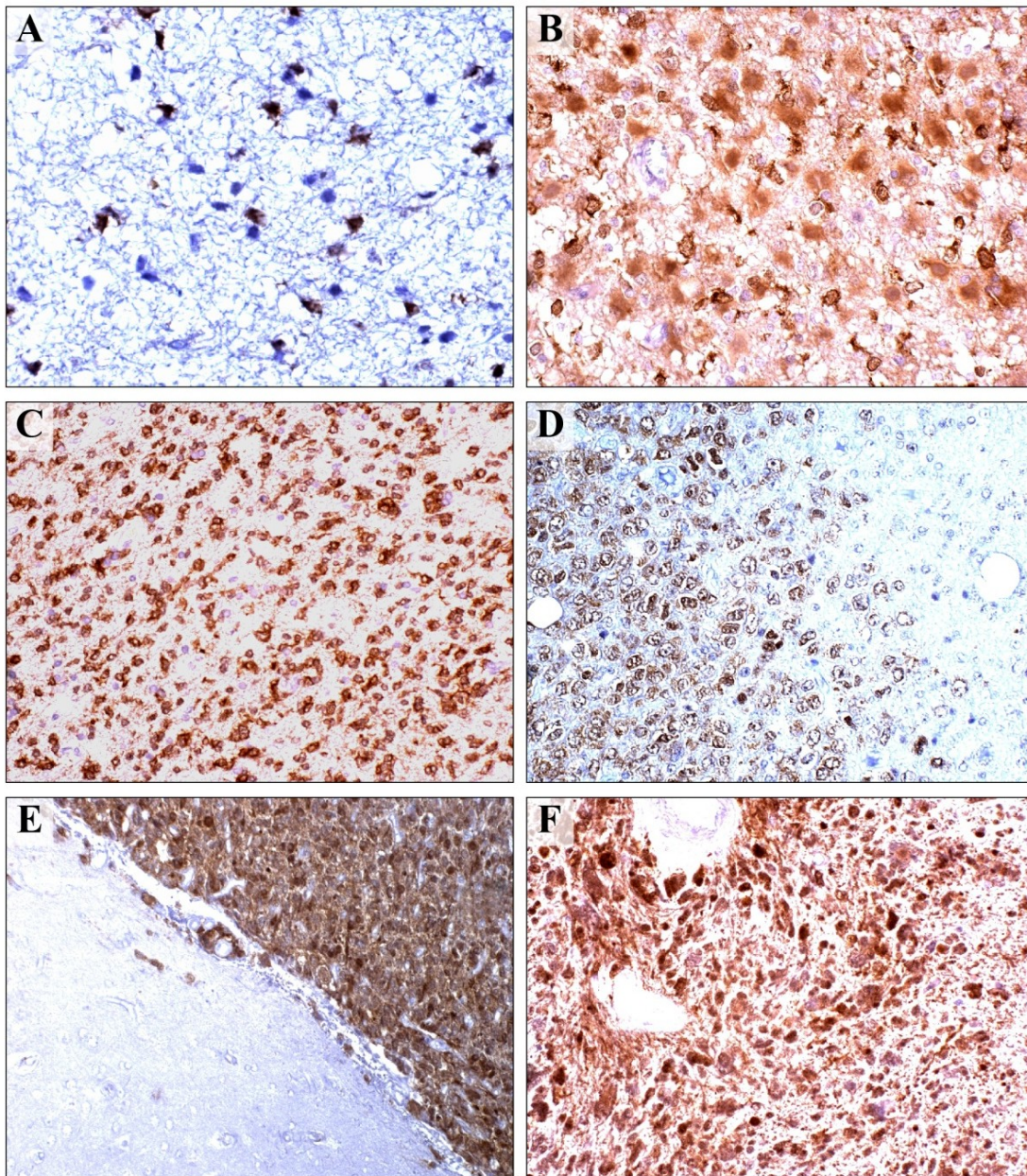
#### 6.4. Immunoreactivity of the IDH1 R132H mutant protein

In our experience, as well as in the experience of others, the anti-mIDH1<sup>R132H</sup> immunoreactivity is cytoplasmic and perinuclear. In a variable percentage of cases, the staining is diffuse in the glia-fibrillary network. In some instances, an additional slightly weaker nuclear staining can be observed, although IDH1 is physiologically located in cytoplasm and peroxisomes. Normal cells, endothelial cells and lymphocytes are immunonegative.

In positive diffuse astrocytomas, all the cells show immunoreactivity (Figure 7A). The gemistocytic cells of astrocytomas are weakly positive with a reduced reaction in the center of the cells (Figure 7B). In this case, the doubt that it is not a specific reaction cannot be excluded.

In oligodendrogliomas, the staining is intense and perinuclear (Figure 7C). The minigemistocytes show a more compact staining of the cytoplasm. In oligodendroglial tumors with a sharp boundary with the normal tissue, the protein expression abruptly ceases at the tumor border; only isolated and rare positive cells are scattered in the normal tissue. In infiltrating tumors, a gradient of positive cells at the border with normal tissue is clearly visible (Figure 7D). In these tumors, tumor cells positive for the clone H09 are mixed with and distinguishable from normal oligodendrocytes (Figure 8E). The latter can be identified for their Cyclin D1 positive expression, being the two stainings complementary. In gliomas, only cycling cells such as tumor oligodendrocytes and reactive and tumor astrocytes express Cyclin D1 while all the other cells are immunonegative. In contrast, normal oligodendrocytes of the cortex and white matter, and microglial cells exhibit a Cyclin D1 positive nuclear staining [149,150]. Applied to oligoastrocytomas, the analysis of Cyclin D1 could prevent the identification of tumor cells as normal oligodendrocytes. In tumors with high cell density, oligodendrocytes appear compacted with a round cytoplasm, whereas in infiltrated areas they acquire an elongated form with polar processes, similar to that observed by silver impregnation (Figure 8B).

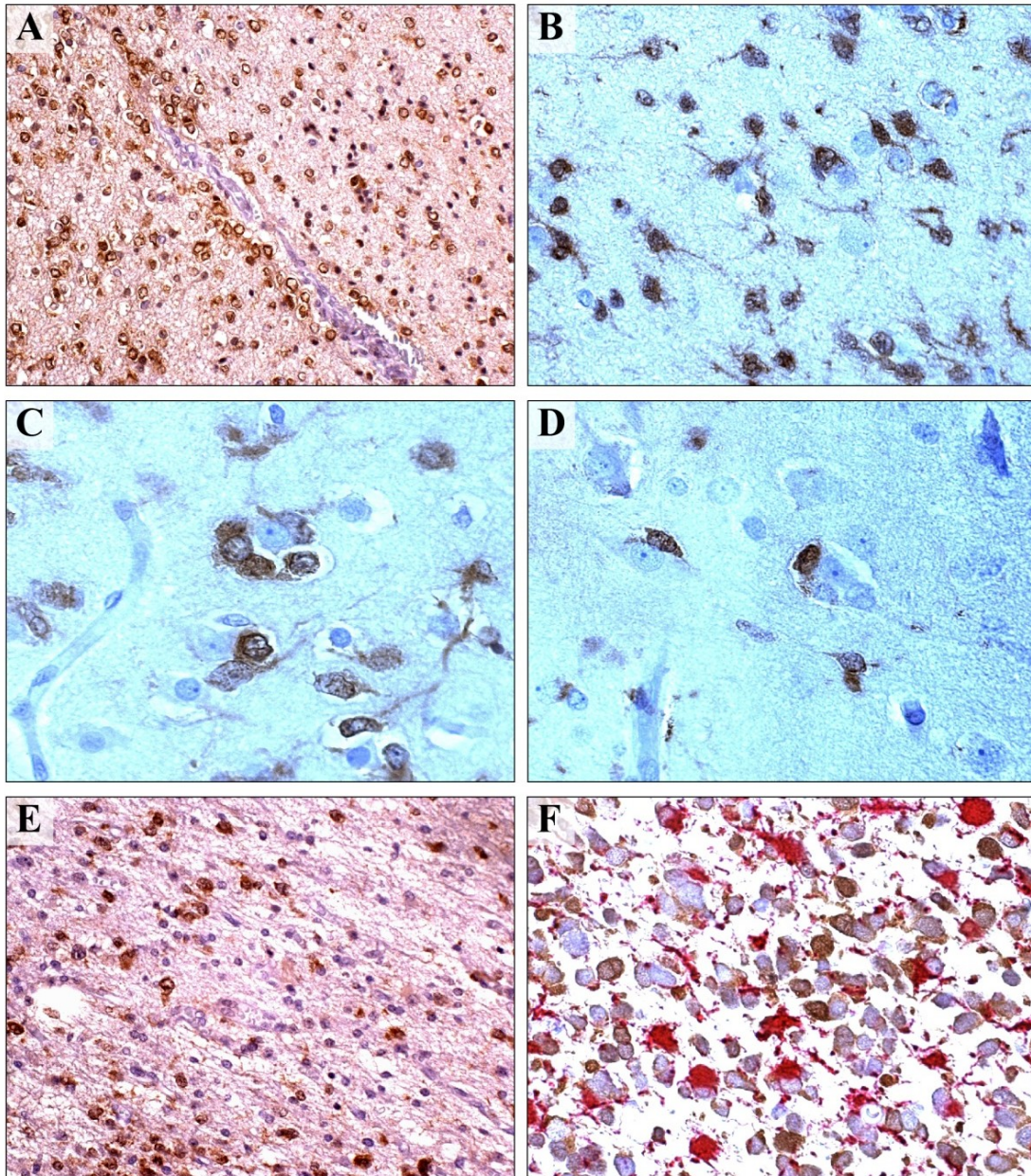
The majority of perivascular oligodendrocytes (Figure 8A) and perineuronal satellites are positive with elegant images (Figure 8C), with some exceptions (Figure 8D). Some of them are immunonegative for clone H09 but immunopositive for Cyclin D1.



**Figure 7.** Anti-mIDH1<sup>R132H</sup> immunohistochemistry. A – Diffuse astrocytoma, immunoreactive cells; B – Gemistocytic astrocytoma, immunoreactive cells; C – Oligodendroglioma, diffuse immunoreactivity of the cells; D – Oligodendroglioma, gradient of immunoreactivity toward the cortex; E – sGBM, sharp border; F – sGBM, immunoreactive cells. All DAB, x200.

In GBMs, positive cells may be polymorphous and the tumor borders could be either sharp or with a gradient of positive cells (Figures 7E,F). Cells crowded around vessels are strongly positive. Giant cells could be either positive or negative.

Pilocytic astrocytomas rarely show immunoreactivity. Reactive astrocytes could be easily recognized for their H09-negative and GFAP-positive immunostaining (Figure 8F).



**Figure 8.** Anti-mIDH1<sup>R132H</sup> immunohistochemistry. A – Oligodendroglioma, pericapillary cell crowding. DAB, x200; B – Oligodendroglioma, various cell forms in the tumor periphery. DAB, x400; C – Oligodendroglioma, positive perineuronal satellites. DAB, x400; D – Oligodendroglioma, positive and negative perineuronal satellites. DAB, x400; E – Oligodendroglioma, positive and negative cells in an infiltrated area. DAB, x200; F – Double immunohistochemistry showing mIDH1<sup>R132H</sup>-positive tumor oligodendrocytes and GFAP-positive reactive astrocytes. DAB and Alkaline Phosphatase Red, respectively, x400.

## 7. Diagnostic, prognostic, predictive and therapeutic considerations

### 7.1. Diagnostic relevance of IDH mutation assessment

The anti-mIDH1<sup>R132H</sup> immunohistochemical evaluation is of great utility in the diagnosis of human brain tumors. As already reported, it allows differential diagnosis between gliomas and non-neoplastic CNS lesions (astrocytosis or therapy-induced changes) [151,152], between gliomas and non-glial CNS tumors, and within glioma subtypes [88,90,153].

In our experience, it is useful in the further following diagnostic situations.

1. In small stereotactic biopsies with hypercellular white matter, to recognize tumor infiltration, because single tumor cells can be detected among normal cells in the infiltrating nervous tissue. This is especially relevant in oligodendrogliomas, where it allows to identify the pattern of the tumor infiltration. A clear-cut distinction can be made between tumors with a sharp border with the normal tissue and the infiltrating tumors, where a gradient of tumor cells can be observed toward the normal tissue. Perineuronal and pericapillary satellitoses are immunopositive for the clone H09, with the exception of some cells that may remain unstained. The latter should represent normal perineuronal satellites joined by tumor cells. However, this finding leaves unresolved the question whether the increased number of satellites corresponds to infiltrating cells or whether they derive by transformation of normal satellites. In infiltrating oligodendrogliomas, Cyclin D1 positive normal oligodendrocytes are recognizable by H09-positive tumor oligodendrocytes. This finding may be useful when an oligoastrocytoma must be distinguished from an astrocytoma infiltrating the white matter, contributing to the resolution of the diagnostic ambiguity of these tumors.
2. To discriminate between tumor astrocytes and reactive astrocytes. The latter point is sometimes of paramount importance, especially when the diagnosis must be carried out on small samples, for example in stereotactic biopsies where infiltrative cells must be recognized among normal cells. Immunoreactivity for the clone H09 is a strong evidence for the tumor nature of positive cells among normal or reactive cells. However, the absence of immunoreactivity does not exclude the occurrence of a glioma.
3. In the differential diagnosis between diffuse astrocytomas, frequently immunopositive, and pilocytic or pleomorphic astrocytomas, both typically immunonegative.
4. Among GBMs, to differentiate primary from secondary tumors (see 4.4). Interesting is the diagnostic utility of the IDH status, together with the assessment of TP53 mutation status, in the recognizing of secondary gliosarcoma [154].
5. To discriminate gemistocytes that show variable immunopositivity with usually a less stained center, from minigemistocytes of oligodendrogliomas, in contrast, uniformly and intensely stained [38,145].
6. In the diagnostic ambiguity of oligoastrocytomas or of a tumor with astrocytes, where it is important to identify the normal or tumor nature of oligodendrocytes and the tumor or normal reactive nature of astrocytes [155].



## 7.2. Prognostic significance of IDH mutations

The occurrence of IDH mutations predicts significantly longer survival for patients affected by GBMs and by WHO grade III astrocytomas and oligodendrogliomas. This was reported in the original discovery of IDH mutations in GBMs [1] and successively confirmed in a large study on WHO grade II and III astrocytomas, oligodendrogliomas and GBMs [28]. Later, the association of IDH mutations with a better patient overall survival (OS) and progression-free survival (PFS) has been confirmed by several reports [36,37,42,57,156-160].

In a recent and partially retrospective study, including the NOA-04 trial cohort of anaplastic astrocytomas and GBMs, IDH mutations are the most powerful single prognostic factor for improved OS, followed by age, tumor type and MGMT promoter hypermethylation [161]. The most favorable outcome is observed in anaplastic astrocytomas with IDH1 mutations, followed by GBMs with IDH1 mutations, anaplastic astrocytomas without IDH1 mutations and GBMs without IDH1 mutations [162].

Likewise, IDH mutations also confer an independent favorable prognosis in WHO grade III oligodendrogliomas, as reported by the European Organization for Research and Treatment (EORTC) trial 2951 [57].

In GBMs, a significant association of IDH1 mutations with OS and PFS was found in primary tumors [37,49] with limited independent prognostic effect [45,159].

In low-grade gliomas, however, the prognostic role of IDH mutations is still controversial, although patients with IDH mutations tend to show a longer survival [36,98,163]. In independent studies on WHO grade II and III gliomas, IDH mutations are prognostic on OS [30,36,56] and PFS [56]. However, in the largest series of low-grade gliomas so far analyzed, no prognostic role of IDH mutations was found in diffuse astrocytomas and oligodendrogliomas [164], in agreement with two recent reports on low-grade astrocytomas [165,166]. Furthermore, patients with low-grade gliomas without IDH1 mutations have a dismal prognosis [56].

In Japanese glioma patients, IDH mutations were significantly associated with increased OS and PFS in WHO grade III tumors but not in WHO grade II [68,98] or in GBMs [98].

In two recent reports on gliomatosis cerebri IDH mutations are strongly correlated to better OS [167,168].

Anyway, the prognostic significance of IDH mutations may be secondary to their prevalence among younger patients, and age is a well-known prognostic factor in gliomas [27]. An explanation for the better prognosis may potentially be related to the biological effect of the IDH mutations. Indeed, IDH1 R132H mutant enzyme seems to impede both migration and growth of stabilized transfected glioma cell lines [169].

In conclusion, the consistent finding of a more favorable outcome in malignant glioma patients with IDH mutations suggests the evaluation of IDH mutation status for prognostic considerations in the clinical setting [139].

### 7.3. Predictive significance of IDH mutations

Generally, IDH mutations do not show correlations with response to antineoplastic therapy in GBMs [37], anaplastic gliomas [159,161], anaplastic oligodendrogliomas [57] or progressive low-grade gliomas [156]. However, IDH mutations correlate with a higher rate of responses to up-front Temozolomide (TMZ) in a series of 84 low-grade glioma patients, independently from the 1p/19q co-deletion [157]. They also show evidence for differential responsiveness to genotoxic therapy of low-grade glioma patients [163]. The occurrence of IDH mutations is associated with favorable PFS and OS in a cohort of WHO grade II gliomas who received radiotherapy or chemotherapy at diagnosis but not in a cohort of low-grade diffuse gliomas initially treated with surgery alone. In a single report, IDH mutations predict response to TMZ in sGBMs [170].

Interestingly, patients with IDH mutations treated for recurrent gliomas have a longer OS from the time of recurrence when treated with the VEGF (receptor) (VEGF(R))-targeted agents sunitinib malate and bevacizumab, rather than with the EGFR-targeted blocking monoclonal antibody cetuximab. This finding supports the hypothesis that IDH1 mutation may benefit from VEGF(R)- versus EGFR-targeted therapy at the time of recurrence [158].

### 7.4. Therapeutic significance of IDH mutations

As before mentioned, the impact of IDH mutations on both radio- and chemotherapy responses in gliomas has not yet been clarified. Anyway, a higher therapeutic sensitivity is expected for IDH mutant rather than wild-type tumor cells.

No therapy that specifically targets IDH mutations is currently available. However, mutant IDH enzymes are attractive candidate for a target therapy in gliomas and there is increasing interest in the development of IDH-related therapies. The standard goal should be the block of the D-2-HG oncometabolite by inhibition of IDH mutant enzymes. Restoring normal IDH function, replacing depleted  $\alpha$ -KG and/or depleting D-2-HG should be beneficial for glioma patients.

Importantly, in the design of inhibitor molecules for IDH mutant proteins in gliomas, compound selection criteria should include consideration for blood-brain barrier penetration. To date, only few reports of inhibitors against dehydrogenases are available. Among them, inositol monophosphate dehydrogenase (IMPDH) inhibitors only have been introduced into clinical development [171].

A recent intriguing possibility is the opportunity to *in vivo* measure D-2-HG levels on glioma patients. Different methods are available, including direct liquid-chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and non invasive imaging techniques as proton magnetic resonance spectroscopy (MRS). Preliminary data in the use of D-2-HG as pharmacodynamic biomarker seem to be promising. Interestingly, D-2-HG levels detected by MRS in glioma patients correlates with the occurrence of IDH mutations [172-175]. However, in a small series of gliomas, D-2-HG levels in serum do not correlate with IDH mutations [176], in contrast to previous findings in AML [177].

In conclusion, IDH mutations identify in gliomas a biologically distinct tumor identity [109]. Suggestions are in favor of the hypothesis that rare pGBMs with IDH mutations might be sGBMs progressed by clinically undetected preceding gliomas of lower malignancy grade. Likewise, sGBMs without IDH mutations might be pGBMs, unrecognized because of inappropriate histological sampling [111]. Anyway, GBMs with IDH mutations seems to be a distinct entity [111] and stratification of GBMs patients according the IDH mutation status should be mandatory.

## 8. Conclusions

The discovery of recurrent somatic IDH mutations in gliomas is the most significant advance in the field of neuro-oncology in the recent years. This finding emphasizes the putative role of the IDH1 and IDH2 metabolic genes in the molecular pathogenesis of gliomas and, more importantly, the translational relevance of the IDH mutations.

Currently, they are considered a strong prognostic marker for glioma patients, independently from the other well-known prognostic factors. For this reason, the knowledge of the IDH mutation status has a great importance for the diagnosis and prognosis of patients, especially when affected by WHO grade III gliomas and GBMs.

This finding suggests revisions of the current WHO classification of human brain tumors with the addition of the IDH mutation status, as well as the KIAA1549/BRAF fusion gene for pilocytic astrocytomas and the total 1p/19q co-deletion for oligodendrogliomas. Common opinion is in favor of a molecular stratification over the conventional WHO grading for prognostic and therapeutic considerations in both low- and high-grade glioma patients. This may also contribute to solve the ambiguity in the histopathological diagnosis of oligoastrocytoma [155,164].

One of the major question remains the molecular pathogenesis of WHO grade II and III gliomas without IDH mutations, which often do not show alterations in genes typically involved in gliomas, as TP53 mutations or CDKN2A homozygous deletion. Whole-genome or exome sequencing by the next generation sequencing technology may be useful in future to identify the molecular basis of these tumors and to a better comprehension of the molecular pathogenesis of gliomas.

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